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

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ABSTRACT

The genus *Ambrosiella* accommodates species of *Ceratocystidaceae* (*Microascales*) that are obligate, mutualistic symbionts of ambrosia beetles, but the genus appears to be polyphyletic and more diverse than previously recognized. In addition to *Ambrosiella xylebori*, *Ambrosiella hartigii*, *Ambrosiella beaveri*, and *Ambrosiella roeperi*, three new species of *Ambrosiella* are described from the ambrosia beetle tribe *Xyleborini*: *Ambrosiella nakashimae* sp. nov. from *Xylosandrus amputatus*, *Ambrosiella batrae* sp. nov. from *Anisandrus sayi*, and *Ambrosiella grosmanniae* sp. nov. from *Xylosandrus germanus*. The genus *Meredithiella* gen. nov. is created for symbionts of the tribe *Corthylini*, based on *Meredithiella norrisii* sp. nov. from *Corthylus punctatissimus*. The genus *Phialophoropsis* is resurrected to accommodate associates of the *Xyloterini*, including *Phialophoropsis trypodendri* from *Trypodendron scabricollis* and *Phialophoropsis ferruginea* comb. nov. from *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 TEF1α DNA sequences suggest that these three fungal genera within the *Ceratocystidaceae* independently adapted to symbiosis with the three respective beetle tribes. In turn, the beetle genera with large, complex mycangia appear to have evolved from other genera in their

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respective tribes that have smaller, less selective mycangia and are associated with *Raffaelea* spp. (*Ophiostomatales*).

Introduction

The six recognized species of *Ambrosiella* Brader ex Arx & Hennebert 1965 (*Sordariomycetes*: *Microascales*: *Ceratocystidaceae*) are obligate, mutualistic symbionts of ambrosia beetles. Ambrosia beetles are an ecological group of more than 3400 species of mycophagous sapwood-boring beetles in the subfamilies Platypodinae and Scolytinae (Coleoptera: Curculionidae). Whereas their bark beetle relatives generally feed on the nutritious inner bark (secondary phloem) of trees, ambrosia beetles tunnel in the nutrient-poor sapwood and depend on mutualistic fungi for their nutrition (Harrington 2005). The beetles generally do not eat wood while boring (Beaver 1989), though the larvae of some ambrosia beetles ingest fungus-colonized wood (xylomycetophagy) (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 (*Ophiostomatales*) (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 are 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 reticular 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.* 2007; Stone *et al.* 2007; Hulcr & Cognato 2010; Cognato *et al.* 2011a). 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 & Rudinsky 1969b).

Ambrosiella initially included ambrosia beetle symbionts with percurrent proliferation of conidiogenous 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 ferruginea* (Math.-Käärik) L.R. Batra 1968 produced conidia from phialides. Harrington *et al.* (2010) limited *Ambrosiella* spp. to the phialidic ambrosia fungi within the *Ceratocystidaceae* and *Raffaelea* spp. to symbionts within the *Ophiostomatales*. All known *Ambrosiella* spp. produce a fruity aroma (Harrington 2009), and these volatiles may play a role in attracting ambrosia beetles within the galleries (Hulcr *et al.* 2011).

As now recognized, most *Ambrosiella* spp. produce large, thick-walled, ovoid, terminal aleurioconidia with inconspicuous collarettes and/or basipetal chains of cylindrical to barrel-shaped phialoconidia via ring-wall building (Minter *et al.* 1983; Nag Raj & Kendrick 1993; Riggs & Mims 2000). The presence of deep-seated phialides in the symbiont of *Trypodendron scabricollis* (tribe *Xyloterini*), *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 synonomy 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. ferruginea* 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 columbianus* (Batra 1967; Nord 1972) and *Corthylus punctatissimus* (Roeper 1995). Besides *An. dispar*, *A. hartigii* has been reported from *Anisandrus sayi* and *Anisandrus obesus* (Hazen & Roeper 1980; Roeper &

French 1981), as well as *Xylosandrus germanus* (Weber & McPherson 1984; Roeper 1996). *A. ferruginea* has been reported from several *Trypodendron* spp., including *T. lineatum*, *Trypodendron domesticum*, *Trypodendron retusum*, *Trypodendron rufitarsis*, and *Trypodendron betulae* (Batra 1967; French & Roeper 1972; Roeper and French, 1981; Roeper 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 from beetles with large mycangia suggested that there was more species diversity within *Ambrosiella* than previously recognized, and each studied ambrosia beetle with large, complex mycangia appeared to be associated with a single, unique species, either within *Ambrosiella* or a closely related genus in the *Ceratocystidaceae*.

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

Materials and methods

Beetle collection and fungal isolation

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

Most fungal isolates were obtained by grinding beetles and dilution plating (Harringon & Fraedrich 2010; Harrington *et al.* 2011) or by placing whole beetles or parts of beetles containing mycangia on plates of SMA (1 % malt extract, Difco; 1.5 % agar, Sigma–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 forceps and a scalpel. An incision was made just posterior to the scutellum to expose the interior of the beetle without damaging the mycangium, which sits directly beneath the mesonotum and is attached to the scutellum. Fine forceps were then used to gently tease out the mycangium and scutellum. The mycangium/scutellum was either transferred to a drop of Cotton blue on a slide and covered with a cover slip for microscopic examination, or the spore mass separated and used for isolation of the fungal symbiont or for DNA extraction with PrepMan® Ultra (Applied Biosystems, Foster City, CA).

DNA extraction and sequencing

Isolates were grown at room temperature on MYEA, and DNA was extracted using one of two methods: either the cultures were grown 2–7 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 fungal material in beetle galleries or beetle mycangia. In some cases, the extracted DNA was concentrated using Amicon® Ultra-0.5 Centrifugal Filter Devices (EMD Millipore, Billerica, CA). Whole beetles preserved in ethanol were ground with a tissue grinder and Prepman® Ultra extraction buffer, and the resulting mix was transferred to a microcentrifuge tube for DNA extraction.

ITS barcoding

Sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA for initial identification of unknown cultures utilized the general fungal primer ITS1-F (Gardes & 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 Ceratо1F (5′ GCGGAGGGATCATTACTGAG 3′) and ITSCer3.7R (5′ GTGAAATGACGCTCGGACAG 3′) for ITS1 and primer pair ITSCer3.1 (5′ CAACGGATCTCTTGGCTCTA 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) for ITS2 (Harrington *et al.* 2014).

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

Phylogenetic analysis

Sequences of the small subunit rDNA (SSU, 18S rDNA) and translation elongation factor 1-alpha (*TEF1α*) were used for phylogenetic placement of the ambrosia beetle symbionts. Taxa selected (Table 1) included a representative of each symbiont and other representatives of the newly recognized genera in the *Ceratocystidaceae*, which were previously treated as *Ceratocystis* spp. or *Thielaviopsis* spp. (de Beer *et al.* 2014). The representative taxa are well characterized, except for the former 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

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

	Culture or specimen identification numbers	Associated ambrosia beetle	GenBank accession no		
			ITS	SSU	*TEF1α*
Ambrosiella					
A. batrae	C3130 (CBS 139735)	*Anisandrus sayi*	KR611322	KR673881	KT290320
A. beaveri	C2749 (CBS 121750)	*Cnestus mutilatus*	KF669875	KR673882	KT318380
A. grosmanniae	C3151 (CBS 137359)	*Xylosandrus germanus*	KR611324	KR673884	KT318382
A. hartigii	C1573 (CBS 404.82)	*Anisandrus dispar*	KF669873	KR673885	KT318383
A. nakashimae	C3445 (CBS 139739)	*Xylosandrus amputatus*	KR611323	KR673883	KT318381
A. roeperi	C2448 (CBS 135864)	*Xylosandrus crassiusculus*	KF669871	KR673886	KT318384
A. xylebori	C3051 (CBS 110.61)	*Xylosandrus compactus*	KF669874	KR673887	KT318385
Ambrosiella sp.	M257	*Eccoptopterus spinosus*	KR611325		
Meredithiella					
M. norrisii	C3152 (CBS 139737)	*Corthylus punctatissimus*	KR611326	KR673888	KT318386
Meredithiella sp.	M260	*Corthylus consimilis*	KR611327		
Phialophoropsis					
P. ferruginea	M243 (BPI 893129)	*Trypodendron lineatum*	KR611328	KR673889	KT318387
Phialophoropsis sp.	C2230 (CBS 460.82)	*Trypodendron domesticum*	KC305146	KR673890	KT318388
Phialophoropsis sp.	CBS 408.68	*Trypodendron retusem*	KC305145		
P. trypodendri	SUTT	*Trypodendron scabricollis*	KR611329		
Ceratocystis and other *Ceratocystidaceae*					
C. adiposa	C999 (CBS 183.86)		=JN604448	KR673891	HM569644
C. fagacearum	C927 (CBS 129242)		=KC305152	KR673892	KT318389
C. fimbriata	C1099 (ICMP 8579)		AY157957	KR673893	HM569615
C. norvegica	C3124 (UAMH 9778)		DQ318194	KR673894	KT318390
Endoconidiophora coerulescens	C301 (CBS 100.198)		KC305116	KR673895	HM569653
Huntiella moniliformis	C1007 (CBS 204.90)		=DQ074739	KR673896	KT318391
Huntiella sp.	C792		KR611330	KR673897	KT318392
H. moniliformopsis	C1934 (DAR 74609)		=NR119507	KR673898	HM569638
Thielaviopsis ethacetica	C1107		=KJ881375	KR673899	HM569632

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; *Pseudallescheria ellipsoidea* for 18S rDNA (U43911) and *Pseudallescheria angusta* for *TEF1α*) and *Gondwanamyces capensis* (18S rDNA, FJ176834). Outgroup taxa were *Plectosphaerella cucumerina* (18S rDNA, AF176951) and *Neurospora crassa* (18S rDNA, X04971).

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

Amplification of *TEF1α* used the forward amplification primer EFCF1a (5′ AGTGCGGTGGTATCGACAAGCG 3′) or EFCF1.5 (5′ GCYGAGCTCGGTAAGGGYTC 3′) and the reverse primer EFCF6 (5′ CATGTCACGGACGGCGAAAC 3′) following the protocol of Oliveira *et al.* (2015). Sequencing was generally performed with the PCR primers as well as the internal primers EFCF2 (5′ TGCTCAACGGGTCTGGCCAT 3′) and EFCF3 (5′ ATGGCCAGACCCGTGAGCA 3′). The aligned sequences were approximately 1200 bp.

A combined SSU and *TEF1α* dataset (TreeBase URL: http://purl.org/phylo/treebase/phylows/study/TB2:S17680) of 2781 characters was used for phylogenetic analysis. Model testing using ModelTest 2.1.7 v20141120 (Darriba *et al.* 2012) on both the combined dataset and the separate datasets for each gene showed the GTR + I + G model to be most appropriate. MrBayes 3.2.1 (Ronquist & 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 (5^{th} 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 14 studied species of ambrosia beetles with

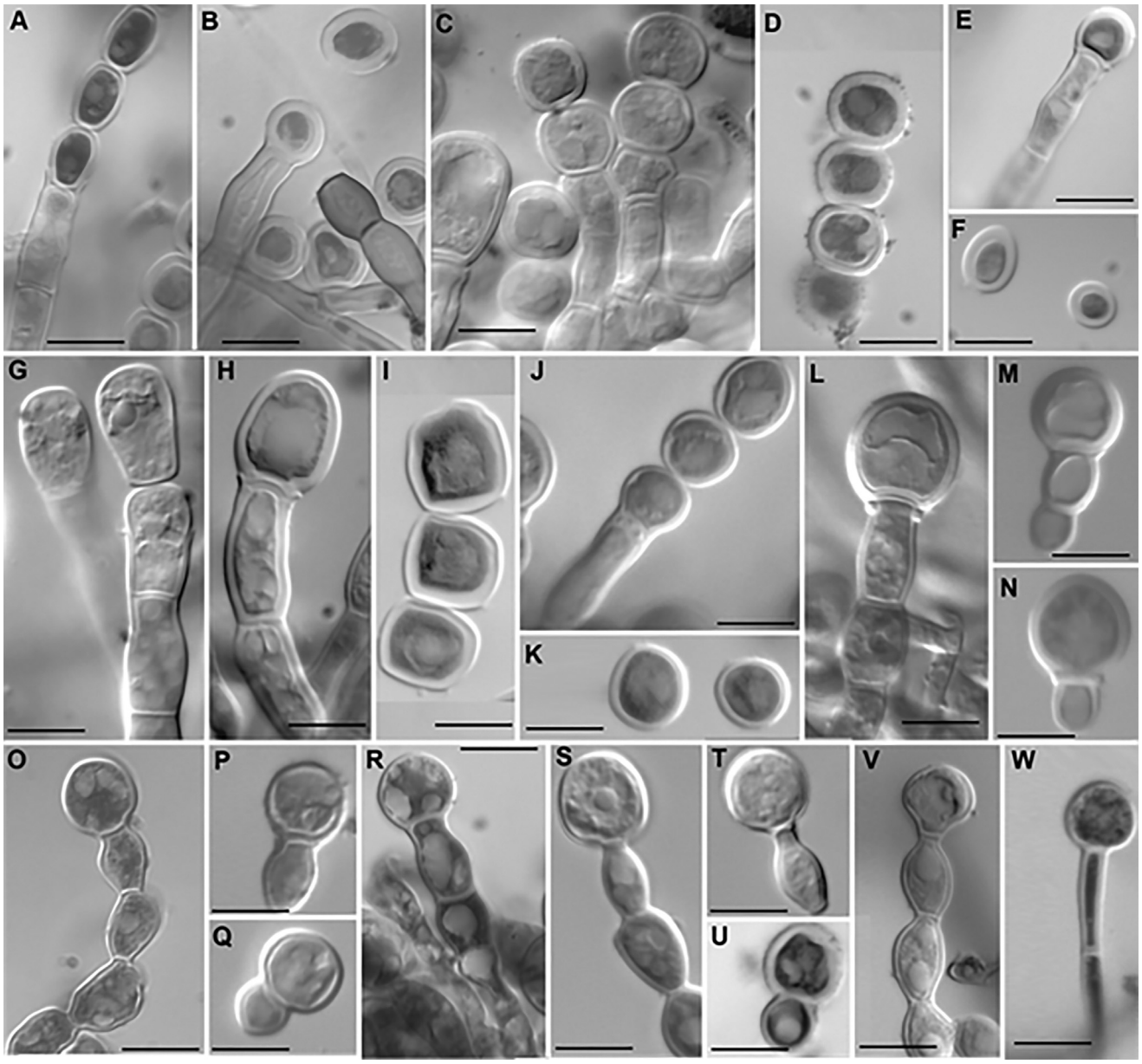

Fig 1 – Conidiophores and conidia of *Ambrosiella* spp. (A, B). *A. beaveri* isolate C2749 (CBS 121750, ex para-type). (A). phialoconidiophore. (B). aleurioconidiophore. (C–F). *A. nakashimae*. (C, D). in gallery, specimen BPI 893134 (holotype). (E, F). Isolate C3445 (CBS 139739, ex-type). (G–I). *A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

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.

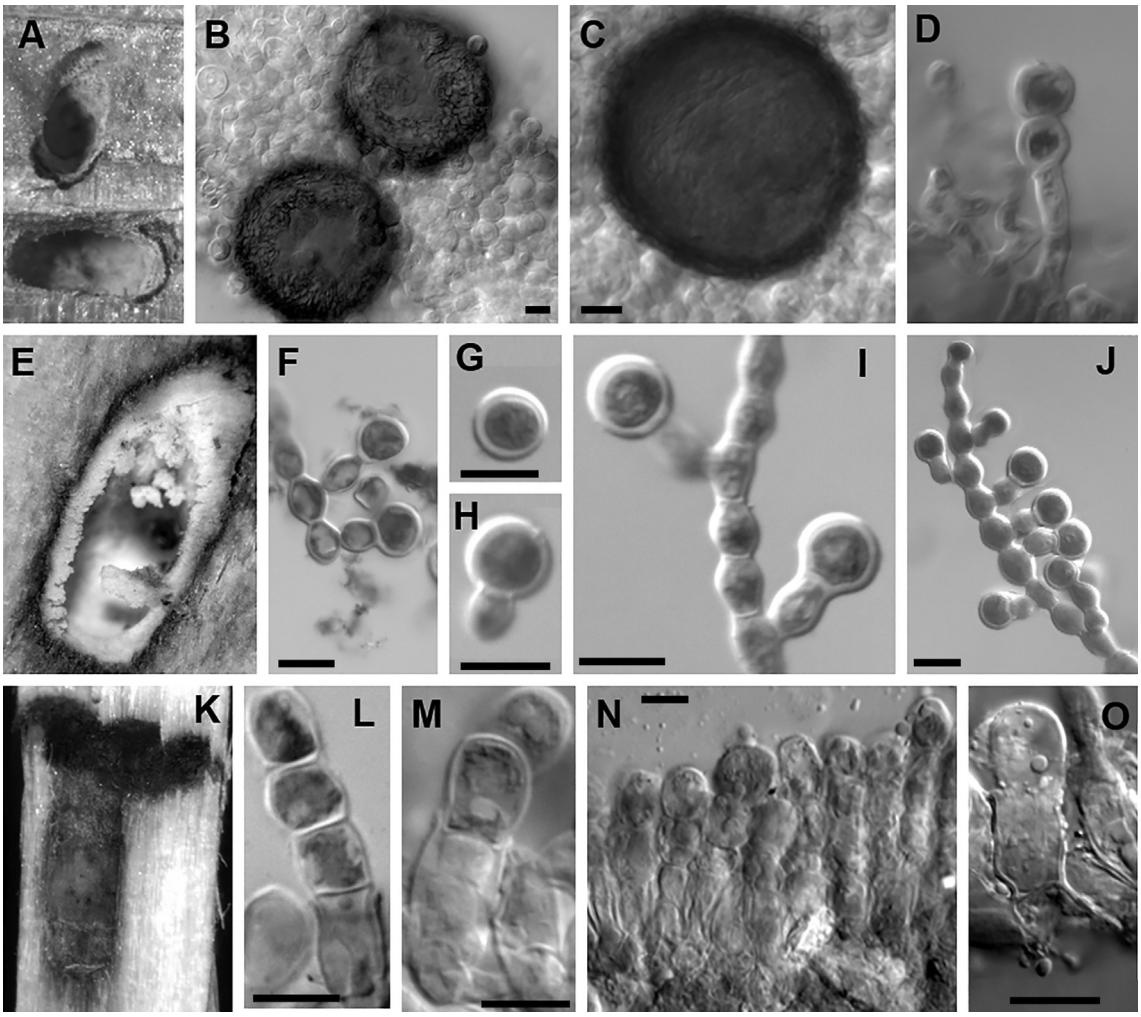

Fig 2 – Growth and sporulation of ambrosia fungi in galleries of ambrosia beetles from three beetle tribes. (A–D). *Ambrosiella nakashimae* in galleries (BPI 893134) of *Xylosandrus amputatus*. (A). growth in tunnels. (B, C). protoperithecia and aleurioconidia. (D). chained aleurioconidia on aleurioconidiophore.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

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

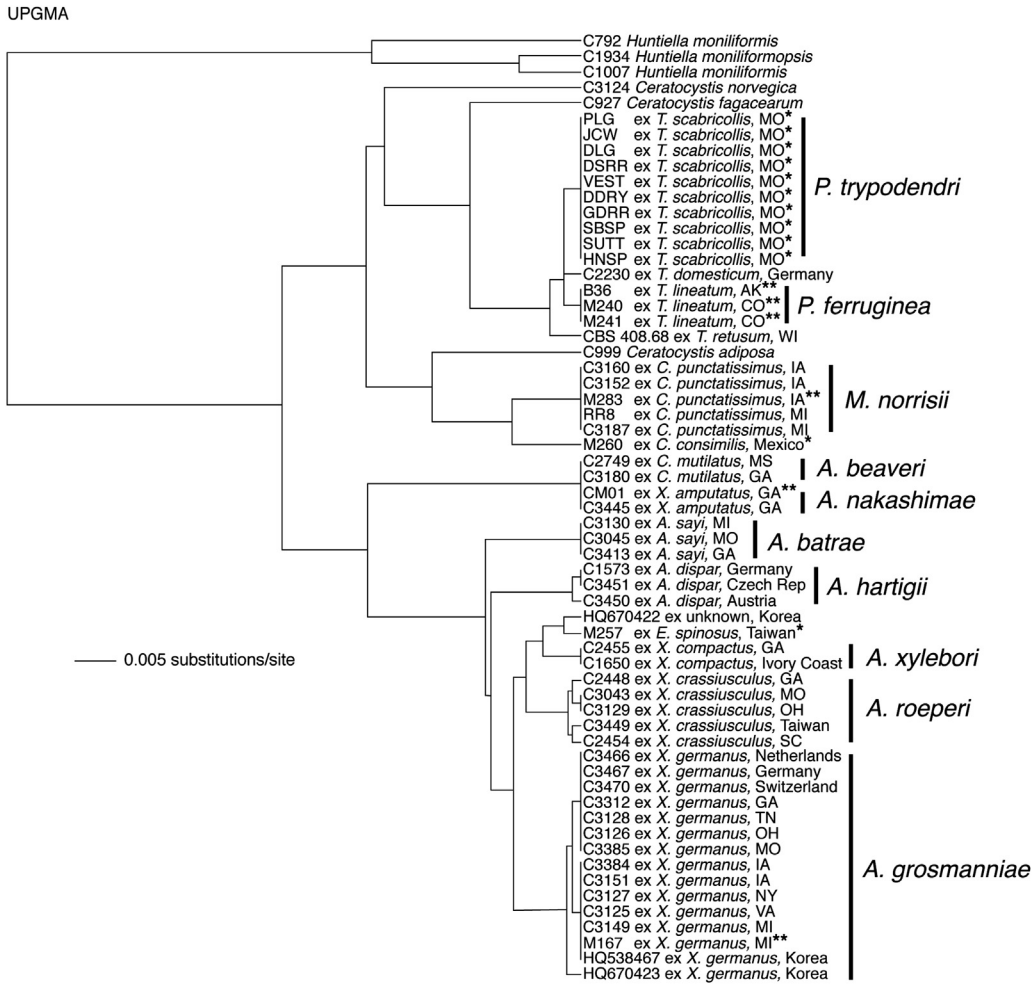

Fig 3 – Unrooted UPGMA distance tree of ITS rDNA sequences of *Ambrosiella* spp. from *Cnestus*, *Xylosandrus*, and *Anisandrus* spp.; *Meredithiella* spp. from *Corthylus* spp.; *Phialophoropsis* spp. from *Trypodendron* spp.; and several representatives of the *Ceratocystidaceae*. Single asterisks 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.

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 *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 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 *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 hartigii* 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 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 1J–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

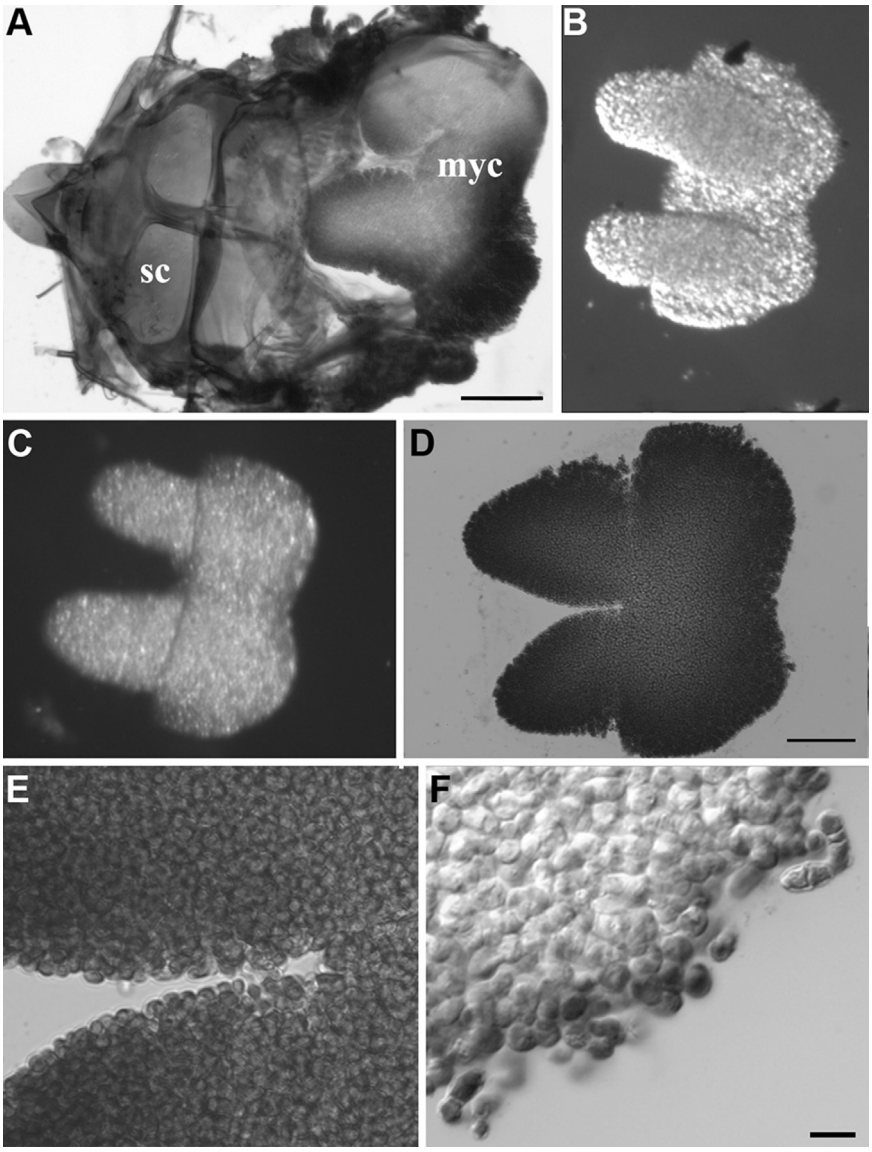

Fig 4 – Mycangium and excised spore mass of *Ambrosiella grossmaniae* from a female *Xylosandrus germanus*. (A). excised mycangium (myc) and attached scutellum (sc). (B–F). mycangial spore mass. (B). ventral aspect. (C). dorsal aspect. (D, E). pressed with cover slip. (D). full spore mass. (E). detail 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.

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–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).

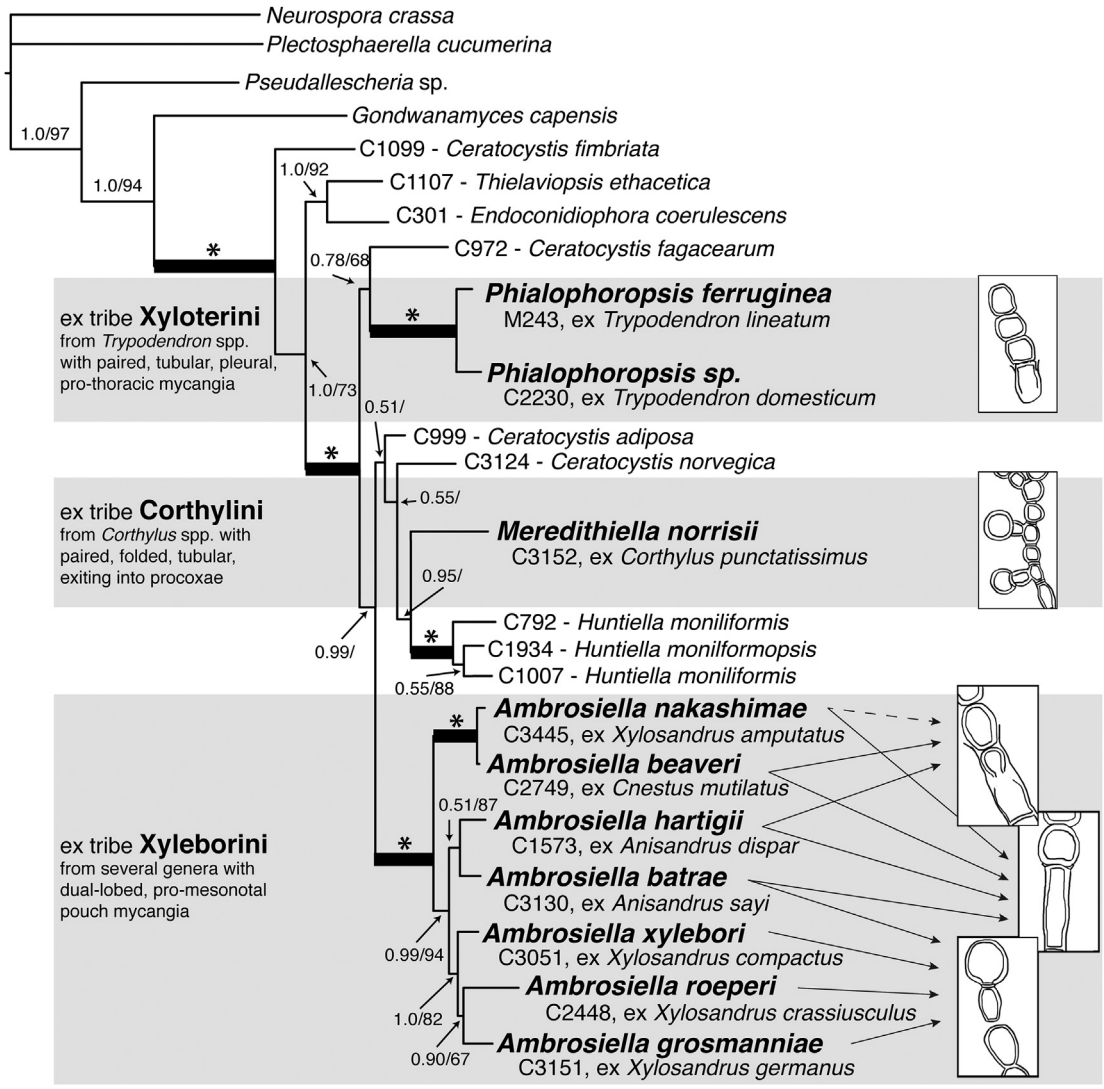

Fig 5 – Phylogenetic tree from Bayesian analysis of the combined *TEF1α* and SSU (18S) rDNA dataset of ambrosia beetle symbionts and representatives of the *Microascales*. Posterior probabilities from Bayesian analysis and bootstrap support values (>50 %) from maximum parsimony analysis are indicated 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*.

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).

Trypodendon lineatum beetles were trapped in Alaska using Lineatin lure and collection cups that were dry and contained the insecticide Vapona (Reich *et al.* 2014). Several female specimens were dried and shipped to Iowa for isolations, but no *Ambrosiella* sp. was isolated. Galleries in an infested log of *Picea* sp. collected in Colorado in 2014 were also examined. The galleries (BPI 893129, 893130) were packed with conidiophores with deep-seated phialides but no aleurioconidia (Fig 2K–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 *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 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* associates as a monophyletic clade, and all of these species formed aleurioconidia on branching conidiophores (Fig 5). The *Xylosandrus amputatus* associate had sequences similar to those of *Ambrosiella beaveri*, the *Anisandrus sayi* associate had sequences similar to those of *Ambrosiella hartigii*, and the *Xylosandrus 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.

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

MycoBank MB504757.

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 *Ambrosiella beaveri* (CBS 121750) and two isolates (C3180 and C3181) from *Cnestus 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, *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 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. *Aleurioconidiophores* (Fig 1E) hyaline to brown with age, simple to branched, 12–115 (155) μm long, composed of one or more cells, producing a single terminal aleurioconidium or a chain of aleurioconidia, conidiogenous cell with a collarette. *Aleurioconidia* (Fig 1F) globose to subglobose, hyaline to light brown with age, thick-walled, aseptate, 6–10 (14) × 6–10 μm. *Phialoconidiophores* uncommon, with moderately seated phialides, producing basipetal chains of conidia by ring-wall building. *Phialoconidia* ellipsoidal to globose, hyaline to light olive brown with age, becoming thick-walled, aseptate 6–10 (12) × 5–9 μm. *Protoperithecia* (Fig 2B, C) superficial, spherical, tan to brown, 55–70 μm diam. *Gallery growth* dense, flat, white to grey, becoming brown with age. *Aleurioconidiophores* (Fig 1C) similar to those in culture, 9–70 μm long, ending in a phialide that may have a distinct collarette. *Aleurioconidia* in galleries (Fig 1D) larger than in culture, terminal or in chains, globose to suboblate, thick walled, aseptate, hyaline, may become light brown with age, (8) 9–12.5 (15) × (7.5) 8.5–11.5 (16) μm.

Other cultures examined: **USA**: *Georgia*: Lowndes Co., 30° 49′ 06.6″ N, 83° 28′ 48.3″ W, *Xylosandrus amputatus* gallery in *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 aleurioconidiophores, but differs in growth rate (7–32 mm vs. 28–36 mm diam after 4 d at 25C), rarity of phialoconidiophores, and the production of protoperithecia. This is the first report of protoperithecia in ambrosia beetle symbionts, which are thought to be asexual (Harrington *et al.* 2010). No perithecia

or ascospores were seen, but the studied isolates were from galleries of a single infestation and may be of a single mating type. The beetle is native to Asia (Cognato *et al.* 2011b), and it is possible that only a single mating type of *A. nakashimae* was introduced. It also is possible that *A. nakashimae* is conspecific with *A. beaveri*, but phenotypic differences between the strains warrants distinction at this time.

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

MycoBank 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; Roeper *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 (Fig 1I) 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; Roeper *et al.* 1980; Roeper & French 1981) and *Xylosandrus germanus* (Weber & McPherson 1984; Roeper 1996), 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 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, 34° 53′ 40.91″ W, isolated from *An. sayi* caught in flight, July 2013, *R. Roeper*, holotype (dried culture, BPI 893131); ex-type living culture C3130 (CBS 139735).

Colonies on malt yeast extract agar 13–51 mm diam. after 4 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 1M, 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 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 aleurioconidia borne in chains (Fig 1J) and terminal aleurioconidia that tear away with conidiophore cells attached (Fig 1M, N).

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

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 *Corthylus columbianus* as *A. xylebori* (Batra 1967) is likely closely related to the new species we describe here from *Corthylus punctatissimus*, which also was previously reported to be *A. xylebori* (Roeper 1996).

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

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

MycoBank MB812573.

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 *X. germanus* caught in flight, 5 August 2013, *C. Mayers*, holotype (BPI 893132); ex-type C3151 (CBS 137359).

Colonies on malt yeast extract agar 45–60 mm diam. after 4 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 moniliod cells. *Aleurioconidia* produced terminally from a distinct subtending collarette, thick-walled, smooth, hyaline, aseptate, globose to subglobose, 7.5–12 × 7.5–12 μm, tearing away with attached conidiophore cells. *Gallery growth* a dense layer of aleurioconidiophores (Fig 1O), producing aleurioconidia Fig 1P, Q) as in culture. *Growth in mycangium* composed of arthrospore-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).

Other cultures examined: **Germany**: *Waldeck*: near Jena, beech forest, from *X. germanus* caught in flight, 2014, *P. Biedermann* (C3467). **d Netherlands**: *Gelderland:* near Wageningen, from *X. germanus* caught in flight, 2014, *L. van de Peppel* (C3466). **d USA**: *Georgia*: Clarke Co., Whitehall Forest, from *X. germanus* specimen caught in flight, 21 March 2014, *S. Fraedrich* (C3312). *Michigan*: Grand Traverse Co., Traverse City, Ashton Park, 44° 46′ 10.77″ N, 85° 38′ 59″ W, isolated from *X. germanus* taken from gallery (BPI 893133) in *A. saccharum*, 24 July 2013, *R. Roeper* (CBS 137358, C3149). *Missouri*: St. Louis County, from *X. germanus* specimen caught in flight, 30 April 2014, *S. Reed* (C3385). *New York*: Tompkins Co., Ithaca, from *X. germanus* mycangium, April 2009, *L. Castrillo* (C3127). *Ohio*: Wayne Co., isolated from *X. germanus* mycangium, May 2010, *B. Anderson* (C3126). *Tennessee*: Warren Co., McMinnville, from *X. germanus* mycangium, April 2011, *N. Youssef* (C3128). *Virginia*: Princess Anne Co., Virginia Beach, isolated from *X. germanus* mycangium, June 2009, *P. Schultz* (CBS 137357, C3125). **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 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. 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 MB812574.

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

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

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

Though only one species of *Meredithiella* is described at this time, an ITS sequence from *Corthylus consimilis* and Batra's (1967) illustrations of a similar fungus from *Corthylus columbianus* imply that there are other species in *Meredithiella* associated with *Corthylus* spp. The aleurioconidia of *Meredithiella* look similar to those of some *Ambrosiella* spp., but the aleurioconidiophores of the *C. punctatissimus* (Fig 2I, 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 25C, 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 2I, 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 monillioid hyphae, bearing a single, terminal aleurioconidium. *Aleurioconidia* terminal, subglobose to globose, thick-walled, aseptate, smooth, hyaline, 9–11 × 8–11 μm, breaking off with a conidiophiore cell attached (Fig 2H) or rarely singly (Fig 2G). *Gallery growth* with abundant aleurioconidiophores (Fig 2F) bearing terminal aleurioconidia, 9–12.5 × 7.5–13 μm.

Other specimens examined: **USA**: *Michigan*: Grand Traverse Co., Traverse City, Ashton Park, 44° 46′ 11.60″ N, 85° 38′ 34.89″ W, *C. punctatissimus* gallery in *A. 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 *A. 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. columbianus* (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. 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. Aleurioconidia 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 *Trypodendon lineatum* associate in *Ambrosiella*, both *P. trypodendri* and *Ambrosiella 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 MB336297.

Synonym: Ambrosiella trypodendri (L.R. Batra) T.C. Harr., Mycotaxon 111:355. 2010 MycoBank MB515299.

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, Trypodendron domesticum*, and *Trypodendron retusum* (Fig 3).

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

MycoBank MB812586.

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

MycoBank MB474947.

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

MycoBank MB326141.

Comments — An ambrosial fungus in the galleries of *T. lineatum* was observed by Hartig (1872) in Germany and later by Leach *et al.* (1940) in Minnesota. Mathiesen-Käärik (1953) described the fungus as *M. ferruginea*, and Batra (1967) moved the species to *Ambrosiella*. Other studies confirmed the relationship between *Phialophoropsis ferruginea* and *T. lineatum* (Funk 1965; Francke-Grosmann 1967), but this species was also thought to be the symbiont of *T. domesticum* and *T. retusum* (Batra 1967), *Trypodendron betulae* (Roeper & French 1981), and *Trypodendron rufitarsis* (French & Roeper 1972). We examined Batra's (1967) Oregon material (BPI 407710, Fig 2O) from *T. lineatum* galleries and saw palisades of conidiophores with deep-seated phialides bearing phialoconidia singly or in chains. Isolations were attempted from galleries made by *T. lineatum* in a log of *Picea* sp. from Colorado (BPI 893129, 893130), but the attempts were unsuccessful. Microscopic examination of this gallery growth found phialoconidiophores with deep-seated phialides and chains of conidia (Fig 2L, 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

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

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 *Phialophoropis* cultures, and *P. cambrensis* was not associated with an ambrosia beetle.

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

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 coevolutionary 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). 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 (Francke-Grosmann 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-Grosmann 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 *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 & Rudinsky 1969a) and have *Raffaelea* symbionts (Batra 1967; Funk 1970; Roeper & French 1981; Harrington *et al.* 2010). Finally, in *Xyloterini*, *Trypodendron* spp. carry *Phialophoropsis* in their complex, tubular, pleural-prothoracic mycangia (Francke-Grosmann 1956, 1967; Abrahamson *et al.* 1967; Batra 1967; Schneider & Rudinsky 1969b; French & Roeper 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 fagacearum,* 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. fagacearum,* and *C. adiposa* form phialoconidia in deep-seated phialides, but only *C. adiposa* forms aleurioconidia (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. fagacearum*, *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 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 (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 phialoconidiophores 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 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, Ambrosiella hartigii*, and *Ambrosiella xylebori*, but we have found only aleurioconidiophores with inconspicuous collarettes in *A. xylebori*. Moderately-seated phialides were observed in *Ambrosiella beaveri*, *A. hartigii*, and perhaps *Ambrosiella 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 *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, monillioid cells that may break off with attached, terminal aleurioconidia (Harrington *et al.* 2014). Distinctive, monillioid conidiophores have been illustrated in *A. xylebori* (Batra 1967; Brader 1964; von Arx & Hennebert 1965; Kaneko 1967), *Ambrosiella roeperi* (Kaneko 1967; Harrington *et al.* 2014), and *Ambrosiella 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 & Hennebert 1965), which likely do not disarticulate (Harrington *et al.* 2014).

Meredithiella norrisii produces terminal aleurioconidia on monillioid hyphae that tear away with one or more conidiophore cells attached, similar to the third type of *Ambrosiella* conidiophore. However, the *M. norrisii* aleurioconidia are borne on short side branches that arise from a central monillioid hypha, as opposed to the branched aleurioconidiophores of *Ambrosiella* spp.

Most species in the family *Ceratocystidaceae* are weak to aggressive plant pathogens (Harrington 2009, 2013), but the ambrosia beetle symbionts in this family appear to be strictly nutritional symbionts. Ambrosia beetles have a broad array of fungal associations and have been implicated as vectors of tree pathogens, but rather than acting directly as vectors, they more commonly facilitate spread of pathogens, such as *C. fimbriata*, via expelled frass containing aleurioconidia (Harrington 2009, 2013). An exception is the invasive *Xyleborus glabratus,* whose mycangial symbiont is the laurel wilt pathogen, *Raffaelea lauricola* (Fraedrich *et al.* 2008; Harrington *et al.* 2011). Other *Raffaelea* spp. and *Fusarium* spp. associated with ambrosia beetles may aid their beetles in killing trees, but these fungi are not wilt fungi and do not systemically colonize their hosts (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 *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 *Xyleborus affinis* using PCR. The amplified ITS sequence from *X. ferrugineus* matched most closely to the ITS sequence of *A. grosmanniae* from *Xylosandrus 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 *Xylosandrus 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 *X. germanus*, *X. crassiusculus*, and *Xylosandrus 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 *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 (Nakashima *et al.* 1987, 1992; Nakashima 1989; Kinuura 1995). *Microcorthylus* spp. apparently have mycangia similar to *Corthylus* 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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