Patterns of coevolution between ambrosia beetle mycangia and the *Ceratocystidaceae*, with five new fungal genera and seven new species

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14 new taxa
*Microascales*
*Ceratocystinaceae*
symbiosis
two new typifications

Abstract Ambrosia beetles farm specialised fungi in sapwood tunnels and use pocket-like organs called mycangia to carry propagules of the fungal cultivars. Ambrosia fungi selectively grow in mycangia, which is central to the symbiosis, but the history of coevolution between fungal cultivars and mycangia is poorly understood. The fungal family *Ceratocystidaceae* previously included three ambrosial genera (*Ambrosiella*, *Meredithiella*, and *Phialophoropsis*), each farmed by one of three distantly related tribes of ambrosia beetles with unique and relatively large mycangium types. Studies on the phylogenetic relationships and evolutionary histories of these three genera were expanded with the previously unstudied ambrosia fungi associated with a fourth mycangium type, that of the tribe *Scolytoplexytopodini*. Using ITS rDNA barcoding and a concatenated dataset of six loci (28S rDNA, 18S rDNA, tef1-α, tub, mcm7, and rpl1), a comprehensive phylogeny of the family *Ceratocystidaceae* was developed, including *Inodoromyces interjectus* gen. & sp. nov., a non-ambrosial species that is closely related to the family. Three minor morphological variants of the prontal disk mycangium of the *Scolytoplexytopodini* were associated with ambrosia fungus in three respective clades of *Ceratocystidaceae*: *Wolfgangiella* gen. nov., *Toshionella* gen. nov., and *Ambrosiella remansi* sp. nov. Closely-related species that are not symbionts of ambrosia beetles are accommodated by *Catunica adiposa* gen. & comb. nov. and *Sololoca norvegica* gen. & comb. nov. The divergent morphology of the ambrosial genera and their phylogenetic placement among non-ambrosial genera suggest three domestication events in the *Ceratocystidaceae*. Estimated divergence dates for the ambrosia fungi and mycangia suggest that *Scolytoplexytopodini* mycangia may have been the first to acquire *Ceratocystidaceae* symbionts and other ambrosial fungal genera emerged shortly after the evolution of new mycangium types. There is no evidence of reversion to a non-ambrosial lifestyle in the mycangial symbionts.

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INTRODUCTION

Multiple groups of wood-boring weevils, collectively known as ambrosia beetles, cultivate fungal gardens along the walls of their sapwood tunnels (‘gardens’) in a reciprocally obligate mutualism (Hulcr & Stelinski 2017). The dozen or more separately-evolved lineages of ambrosia beetles (Kirkendall et al. 2015, Hulcr & Stelinski 2017) avoid competition from phloem-feeding bark beetles and other insects in the nutritious inner bark by using fungal agriculture to exploit the nutrient-poor sapwood (Harrington 2005, Harrington et al. 2010). Reciprocally-obligate fungus farming is shared with only two other lineages of insects: the higher-attine ants (Mueller et al. 2018) and the *Termitomyces*-farming termites (Nobre et al. 2011), but ambrosia beetles are unique in their use of special fungus-carrying pockets called mycangia that allow cultivar persistence across generations (Francke-Gromsman 1956, 1963, 1967). The fungi benefit from targeted transport to fresh substrate in mycangia, and they are also protected from desiccation; ambrosia fungi appear to be unable to disperse and survive independently. Mycangia are central to the ambrosia beetle-fungus symbiosis, and they drive the evolution of both ambrosia beetles and the fungi that grow inside them.

The term mycangium is broadly applied to a range of separately-evolved physical adaptations (usually invaginations of the exoskeleton that form pockets, cavities, or crevices) on different parts of the beetles’ bodies for the storage and transport of fungal spores (Francke-Gromsman 1963, 1967, Beaver 1989, Six 2003). The most effective and selective mycangia are ‘glandular sac mycangia’ (Six 2003) associated with active gland cells that secrete nutrients into or near the mycangium lumen (Schedl 1962, Francke-Gromsman 1963, 1967). The secretions support the selective, asexual growth of fungal symbionts (Schneider 1975, Skelton et al. 2019), and the propagules overflow from the mycangium opening to inoculate galleries during sapwood excavation (Schedl 1962, Beaver 1989, Harrington et al. 2014). Most mycangia are small relative to the beetles’ body size (hereafter ‘small mycangia’) and are relatively simple in structure, such as the oral/preoral pouch mycangia con vergently developed by multiple ambrosia beetle lineages (Hulcr & Stelinski 2017). Certain other mycangia are larger internal cavities with more complex structure (hereafter ‘large mycangia’). Large mycangia are believed to represent
| Ambrosia fungus | Associated ambrosia beetle | Specimen type | Isolate or specimen ID | Location | Date collected | Collected by | ITS GenBank Accession No. |
|-----------------|-----------------------------|---------------|------------------------|----------|---------------|--------------|--------------------------|
| Ambrosiella | | | | | | |
| A. aff. Xylosandrus germanus | | | | | | |
| A. brevis | Xylosandrus germanus | Culture from female (in Lindera trioloba) | C409 | Aichi, Toyota, Inabu, Japan | July 2014 | H. Kajimura same as MG950182 |
| A. brevis | Xylosandrus germanus | Culture from female (in Lindera trioloba) | C408 | Aichi, Toyota, Inabu, Japan | July 2014 | H. Kajimura MG950181 |
| A. catenulata | Anisandrus apicalis | Culture from female | C3909 | Moroika, Iwate Prefecture, Japan | Apr. 2015 | H. Masuya same as MG950184 |
| A. catenulata | Anisandrus apicalis | Culture from female | C3910 | Moroika, Iwate Prefecture, Japan | Apr. 2015 | H. Masuya same as MG950184 |
| A. catenulata | Anisandrus apicalis | Culture from female | C3911 | Moroika, Iwate Prefecture, Japan | Apr. 2015 | H. Masuya same as MG950184 |
| A. catenulata | Anisandrus apicalis | Culture from female | C3912 | Moroika, Iwate Prefecture, Japan | Apr. 2015 | H. Masuya same as MG950184 |
| A. catenulata | Anisandrus apicalis | Culture from female | C3913 (CBS 142649) | Moroika, Iwate Prefecture, Japan | Apr. 2015 | H. Masuya MG950184 |
| A. grossmanniae | X. germanus | Culture from female | C3901 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya same as KR611324 |
| A. grossmanniae | X. germanus | Culture from female | C3902 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya same as KR611324 |
| A. grossmanniae | X. germanus | Culture from female | C3903 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya same as KR611324 |
| A. grossmanniae | X. germanus | Culture from female (in Lindera trioloba) | C4058 | Aichi, Toyota, Inabu, Japan | July 2010 | H. Kajimura MG950185 |
| Ambrosiella aff. Xylosandrus germanus grossmanniae NRgro1 | | | | | | |
| Xylosandrus germanus | Culture from female | C3986 | Iwate Prefecture, Moroika, Japan | Apr. 2015 | H. Masuya same as MG950186 |
| Xylosandrus germanus | Culture from female | C3999 (CBS 142648) | Iwate Prefecture, Moroika, Japan | Apr. 2015 | H. Masuya MG950186 |
| Xylosandrus germanus | Culture from female | C3900 | Iwate Prefecture, Moroika, Japan | Apr. 2015 | H. Masuya same as MG950186 |
| A. roeperi | X. crassiusculus | Culture from female (ethanol trap) | C39134 | Fushan, Taiwan | July 2014 | H.H. Shih MG950187 |
| A. roeperi | X. crassiusculus | Culture from female (ethanol trap) | C39139 | Fushan, Taiwan | July 2014 | H.H. Shih MG950187 |
| T. nipponensis | S. shogun | Culture from female (in unidentified tree) | C3904 (CBS 141492) | Tazawako, Aichi Prefecture, Japan | July 2014 | H. Masuya IO342064 |
| T. nipponensis | S. shogun | Culture from female (in unidentified tree) | C3905 | Tazawako, Aichi Prefecture, Japan | July 2014 | H. Masuya same as KX342064 |
| T. nipponensis | S. shogun | Culture from female (in unidentified tree) | C3906 | Tazawako, Aichi Prefecture, Japan | July 2014 | H. Masuya same as KX342064 |
| T. nipponensis | S. shogun | Culture from female (in unidentified tree) | C4064 | Hachimantai, Iwate Prefecture, Japan | July 2014 | H. Masuya same as KX342064 |
| T. nipponensis | S. shogun | Culture from female (in unidentified tree) | C3878 (CBS 141494) | Dounia, Kouchi Prefecture, Taiwan | Feb. 2014 | H.H. Shih IO342067 |
| T. taiwanensis | S. pubescens | Culture from female (ethanol trap) | C3886 | Jinhshan, Kaohsiung Prefecture, Taiwan | Feb. 2014 | H.H. Shih same as KX342066 |
| T. taiwanensis | S. pubescens | Culture from female (ethanol trap) | C3889 | Jinhshan, Kaohsiung Prefecture, Taiwan | Feb. 2014 | H.H. Shih same as KX342066 |
| T. taiwanensis | S. mikado | Culture from female (ethanol trap) | C3448 (CBS 141495) | Lienhuachih, Taiwan | Aug. 2014 | C. Wuest same as KX342066 |
| T. taiwanensis | S. mikado | Propagules in mycangium of female (ethanol trap) | M304 | Fushan, Taiwan | June 2014 | H.H. Shih same as KX342066 |
| T. taishanensis | S. mikado | Propagules in mycangium of female (ethanol trap) | M305 | Fushan, Taiwan | June 2014 | H.H. Shih same as KX342066 |
| T. transmara | S. shogun | Propagules in mycangium of female (unidentified tree) | C3908 (CBS 141493) | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya IOG342065 |
| T. transmara | S. shogun | Propagules in mycangium of female (unidentified tree) | C3908 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya IOG342065 |
| T. transmara | S. shogun | Propagules in mycangium of female (unidentified tree) | C3908 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya IOG342065 |
| T. transmara | S. shogun | Propagules in mycangium of female (unidentified tree) | C3908 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya IOG342065 |
| W. franzmeieri | Scolytaphytopus fasciatus | Propagules in mycangium of female in unidentified tree | C3908 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya IOG342065 |
| W. franzmeieri | Scolytaphytopus fasciatus | Propagules in mycangium of female in unidentified tree | C3908 | Hachimantai, Iwate Prefecture, Japan | June 2015 | H. Masuya IOG342065 |
| W. madagascarensis | S. rugosus | Propagules in mycangium of female in unidentified tree | M288 | Ambositra, Toliara Province, Madagascar | May 2015 | B. Jordal IOG342063 |
| W. madagascarensis | S. rugosus | Propagules in mycangium of female in unidentified tree | M288 | Ambositra, Toliara Province, Madagascar | May 2015 | B. Jordal IOG342063 |
| Wolfrangiella sp. | S. congorus | Propagules in mycangium of female | M683 | Cameroon | 2012 | B. Jordal MG54339 |
| Wolfrangiella sp. | S. congorus | Propagules in mycangium of female | M683 | Cameroon | 2012 | B. Jordal MG54339 |

Table 1: Collection information and ITS rDNA accession numbers for select cultures and specimens.

Includes isolate or specimen numbers from the culture collection of T.C. Huntington, Iowa State University (C or M), Westerdijk Fungal Biodiversity Institute (CBS), and U.S. National Fungus Collections (BPI).

Holotype specimens indicated by bold HT; e-holotype cultures indicated by bold eHT.
significant energy commitments by the beetles and are generally more specific in the fungi they carry (Harrington et al. 2014, Mayers et al. 2015, 2018, Skelton et al. 2019).

A variety of fungi are associated with ambrosia beetles (Harrington 2005, Skelton et al. 2018), but we define ‘ambrosia fungi’ in the strict sense (i.e., primary ambrosia fungi, after Batra 1985) as co-adapted symbionts that grow in ambrosia beetle mycangia, form a specially-adapted, dense layer of sporulation (‘ambrosia’) as food in the beetles’ tunnels, and show no evidence of living independently (Francke-Grosmann 1963, 1967, Harrington et al. 2010, 2014, Mayers et al. 2015). With a recent exception in the wood-decay basidiomycete Flavodon ambrosius (Kasson et al. 2016, Simmons et al. 2016), only six genera in three orders of ascomycetous fungi meet these requirements: the polyphyletic Rafaeella s.lat. and monotypic Afroraffaeella in the family Ophiostomataceae (Ophiostomatales) (Harrington et al. 2010, Dreden et al. 2014, Bateman et al. 2017); the Fusarium ambrosia clade (FAC) (Hypocreales) (Kasson et al. 2013, D’Oonnell et al. 2015); and Ambrosiella, Meredithiella, and Phialophoropsis in the family Ceratocystidaceae (Microscales) (Mayers et al. 2015, 2018). Each lineage was most likely domesticated from ubiquitous, free-living companions, commensals, or contaminants in wood, as evidenced by their close relatives.

The large mycangia of ambrosia beetles have so far only been associated with the three ambrosial genera of Ceratocystidaceae, and each of the three fungal genera is exclusively associated with a different type of large mycangium (Mayers et al. 2015, 2018). The long-established genus Ambrosiella (Brader 1964, Von Arx & Hennebert 1965, Harrington et al. 2010) was restricted by Mayers et al. (2015) to a monophyletic group containing Ambrosiella xylebori and other species carried in the mesonotal pouch mycangia of ambrosia beetles in the Xylosandrus complex (tribe Xyleborini) (Skelton et al. 2019). Ambrosiella currently comprises nine species, all of which produce large aleurioconidia on branching monilioid conidiophores adapted for beetle grazing (Harrington et al. 2014, Mayers et al. 2015, 2017, Lin et al. 2017). Meredithiella accommodates three symbionts of Corthylus ambrosia beetles (tribe Corthylini), which have a winding procoxal tube (prothoracic coil) mycangium (Mayers et al. 2015, 2018). Meredithiella has alternately-branching conidiophores that produce aleuroconidia that are similar to those of Ambrosiella (Mayers et al. 2015, 2018). Phialophoropsis accommodates two named species and other putative species associated with the prothoracic pleural mycangia of Trypodendron (tribe Xyletorini) (Mayers et al. 2015, Lehenberger et al. 2019). Phialophoropsis is distinguished by the production of long chains of phialoconidia from deep-seated phialides and the absence of aleuroconidia (Mayers et al. 2015). All three genera were classically assumed to be strictly asexual, but the recent discovery of a sexual morph in Ambrosiella cleistominuta suggests cryptic sex may be common in ambrosia symbionts (Mayers et al. 2017).

Despite recent phylogenetic analyses of the Ceratocystidaceae (De Beer et al. 2014, 2017, Mayers et al. 2015, Nel et al. 2017) and an upswing in interest in the ambrosia symbiosis (Hulcr & Stelinski 2017), the phylogenetic relationships of the ambrosia beetle symbionts to the rest of the family remains poorly resolved (De Beer et al. 2014, Mayers et al. 2015, 2018). This is largely due to the difficulty of acquiring these fungi in pure culture (Francke-Grosmann 1963, 1967, Batra 1985) and the taxonomic gaps in the known ambrosia fungi in the Ceratocystidaceae. The Scolytoplatypodini comprise a fourth ambrosia beetle lineage with large mycangia, and their uncharacterized fungal symbionts might provide missing links in the unresolved phylogenetic and evolutionary relationships of Ceratocystidaceae ambrosia fungi. The tribe Scolytoplatypo-
Species descriptions
Isolates were grown on malt yeast agar (MYEA; 2 % malt extract, Difco Laboratories, Detroit, MI, USA; 0.2 % yeast extract, Difco; 1.5 % agar, Sigma-Aldrich, St. Louis, MO, USA) at room temperature. Agar plugs cut with a #1 cork borer (3 mm diam) were transferred from the leading margin of growth to three new MYEA plates, incubated at 25 °C for 7 d in the dark, and the diameter of the colonies measured. Colors are per Rayner (1970). Representative cultures were deposited in the Westerdijk Fungal Biodiversity Institute (CBS), and representative dried specimens were deposited in the U.S. National Fungus Collections (BPI).

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

For routine species identification, amplification and sequencing of the nuclear rDNA ITS1-5.8S-ITS2 (internal transcribed spacer region, ITS barcode) (Schoch et al. 2012) used fungal primers ITS1F and ITS4 (Gardes & Bruns 1993, White et al. 1990). The Ceratocystidaceae-specific ITS primer sets Cerato-
1F/ITS4r3.7R and ITS4r3.5/ITS4 (Mayers et al. 2015) were also used to obtain sequences from DNA extracts from galleries, mycangia, and whole beetles. All ITS amplification used the same PCR conditions (85 °C for 2 m; 95 °C for 95 s; 36 cycles of 58 °C for 1 m, 72 °C for 80 s, and 95 °C for 70 s; 52 °C for 1 m; 72 °C for 15 m; and 4 °C hold).

Six nuclear genes were sequenced for multi-locus phylogenetic analyses. For nuclear large subunit ribosomal DNA (28S rDNA), primers LORL and LR5 were used for amplification with the same PCR conditions as the ITS barcode, and primers LORL and LR3 were used for sequencing (Vilgalys & Hester 1990, Rehner & Samuels 1994). For nuclear small subunit ribosomal DNA (18S rDNA) amplification and sequencing, a combination of general primers (NS1, NS3, NS6, NS7, NS8, SR1R, and SR9R) were used (Elwood et al. 1985, Vilgalys & Hester 1990, White et al. 1990), as well as a new primer designed for ascomycetes (‘NS4Asco’, 5’-CTTCCGTCATTATTCTTTAAG-3’, used in place of NS4) and two primers designed to specifically amplify fungal 18S rDNA from mycangia (‘NS4Cer’, 5’-CAGTATTTCGTAAGGAAG-3’, used in place of NS4; and ‘SR9Cer’, 5’-GGCATCAGTATTCAGCTG-3’, used in place of SR9R). All 18S rDNA PCR used the same PCR conditions (94 °C for 2 m; 36 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 m; 72 °C for 5 m; and 4 °C hold), but annealing temperature was lowered to 49 °C in some cases. For translation elongation factor 1-alpha (tef-1-a), primers EFCF1 and EFCF6 were used for amplification with the PCR conditions of Oliveira et al. (2015), though we generally replaced EFCF1 with a new primer designed to avoid a problematic intron in many isolates (‘EFCF1’, 5’-GGCYGACTGCGATGAGGYTC-3’). Internal primers EFCF2 (Oliveira et al. 2015) and another new primer (‘EFCer3’, 5’-CARACHGTGAACGAYCTGTC-3’) were occasionally used for supplemental internal sequencing. Amplification and sequencing of the beta-tubulin (tub) were used the primers (Bt1a and Bt1b) and conditions of Glass & Donaldson (1995). Amplification and sequencing of the 60S ribosomal protein L1 (rpl1) and the DNA replication licensing factor mcm7 (minichromosome maintenance complex component 7) used the primers (60S-506F/60S-908R and Cer-MCM7F/Cer-MCM7TR) and conditions of De Beer et al. (2014), but for some isolates the annealing temperature was reduced to 55 °C. Sequencing was performed by the Iowa State University DNA Sequencing Facility, and electropherograms of complementary and overlapping reads were examined, compared, and assembled using Sequence Navigator v. 1.0.1 or AutoAssembler v. 1.3.0 (Applied Biosystems, Foster City, California).

Molecular phylogeny
The ITS rDNA sequences of the Ceratocystidaceae have numerous indels that are ambiguously aligned, so for barcoding purposes, ITS sequences were manually aligned in two separate datasets: one for Ambrosiella and close relatives (TreeBASE URL: http://purl.org/phylo/treebase/phylo/phylo/phylistudy/TB2:S23518) and one for Phialophoropsis and close relatives (TreeBASE URL: http://purl.org/phylo/treebase/phylo/phylistudy/TB2:S23519). In addition to new ITS sequences (Table 1), alignments included ITS sequences newly generated from Ceratocystis adiposa, available from previous studies (Harrington et al. 2014, Mayers et al. 2015, 2017, 2018, Lin et al. 2017), obtained from selected Phialophoropsis isolates in another study (Mayers et al. unpubl.), or provided by the laboratory of J. Huiric (University of Florida), as detailed in Table S1. A separate maximum parsimony tree was created from each of the two alignments with PAUP v. 4.0b10 (Swofford 2002) using heuristic searches with gaps treated as fifth character (which preserved the important diagnostic signals present in indels), starting trees obtained via stepwise addition, and the tree-bisection-reconnection branch-swapping algorithm. Using C. adioposa and M. norrisii as a monophyletic sister group to the ingroup, the Ambrosiella alignment had 552 characters, of which 135 were parsimony-informative, 43 were variable but parsimony-uninformative, and 374 were constant. The Phialophoropsis alignment used Brettziella fagaracearum as an outgroup with midpoint rooting, with 509 characters, of which 45 were parsimony-informative, 30 were variable but parsimony-uninformative, and 434 were constant. A representative tree was chosen from the trees produced from both alignments for illustration, and branch support values were generated for both trees via 1000- replicate bootstrap maximum parsimony analysis in PAUP.

All multi-locus analyses used a manually-aligned, concatenated alignment (TreeBASE URL: http://purl.org/phylo/treebase/phylo/phylistudy/TB2:S22560) of 18S rDNA, tef-1-a, 28S rDNA, tub, mcm7, and rpl1. Accession numbers for newly-generated sequences and those from previous studies (De Beer et al. 2014, Mayers et al. 2015, 2017, 2018, Lin et al. 2017) are listed in Table S2. Representatives of all major lineages in the Cerato-
cystidaceae were included (De Beer et al. 2014, 2017, Nel et al. 2017), but a close relative of the family, Cornuvesica (Marinowitz et al. 2015), was not included in analyses due to inadequate sequence data. Sequences also included those of a Michigan isolate of an unnamed Microascales sp. (C3547 = CBS 142647) we obtained from a gallery of the ship timber beetle, Elaterio-
des lugubris (family Ly menacingidae), which is not an ambrosia beetle. Sequences for the six genes were also extracted from genome assemblies available in the NCBI database (National Center for Biotechnology Information, Bethesda, Maryland): Huntiella moniliformis JMSH0000000 (Van der Nest et al. 2014), Huntiella bhutanensis MJMS0000000 (Wingfield et al. 2016b), and Scedosporium boydii NJFT0000000 (Duvaux et al. 2017). The resulting six-gene alignment had 5276 characters. Introns were eliminated from tef-1-a, tub, mcm7, and rpl1, as were ambiguously-aligned regions from 18S rDNA and 28S rDNA (618 characters total). The final alignment consisted of 4658 characters, 3347 of which were constant, 261 were variable but parsimony-uninformative, and 1050 were parsimony-informative.
A Bayesian analysis of the six-gene alignment was performed with MrBayes v. 3.2.2 (Ronquist et al. 2012), with a single MCMC run with four chains (one cold, three heated) for 100,000 generations, which was sufficient to achieve an average standard deviation of split frequencies less than 0.002. Models and partitions were selected by PartitionFinder 2 (Lanfear et al. 2016) in ‘n mrbayes’ mode, using ‘aicc’ (converted Akaike Information Criterion) model selection and a greedy algorithm (Lanfear et al. 2012) and powered by PhyML (Guindon et al. 2010). As suggested by PartitionFinder 2, GTR+I+G was applied to the first partition (18S rDNA), fifth partition (28S rDNA and tub codon position 1), sixth partition (rpl1 codon position 2 and tub codon position 2), seventh partition (mcm7 codon position 1), tenth partition (rpf1 codon position 1), and eleventh partition (rpf1 codon position 3); GTR+G was applied to the third partition (tef1α codon position 3 and tub codon position 3) and ninth partition (mcm7 codon position 3); GTR+I was applied to the eighth partition (mcm7 codon position 2); and F81+I+G was applied to the second partition (tef1α codon position 2) and fourth partition (tef1α codon position 1). A consensus tree was generated using the function ‘sumt’ with a burn-in value of 150,000 and visualized with FigTree v. 1.4.0. In addition to posterior probability values provided by MrBayes, additional branch support values were generated for the tree via 1000-replicate bootstrap maximum likelihood analysis in RAxML (Mayers et al. 2018) and 1000-replicate bootstrap parsimony analysis in PAUP.

The resulting Bayesian inference tree was annotated with symbols representing the production of necked perithecia (Nag Raj & Kendrick 1975, Upadhyay 1981, De Beer et al. 2014), fertile cleistotheca or immature spherical ascosporas (Mayers et al. 2015, 2017, 2018), and aleuropoecid, as reported in the literature or observed in our available cultures and specimens.

Divergence date estimates

For the primary analysis (Analysis A), a time-calibrated Bayesian tree with estimates of divergence dates was generated using BEAST v. 2.4.7 (Bouckaert et al. 2014) and an .xml file created in BEAUti 2 from the same six-gene alignment used to create the Bayesian inference tree of the Ceratocystidaceae. Most genera were pruned to two representative species, and the sequences from three additional outgroup taxa were extracted from genomes of Aspergillus niger ASM285v2 (Pel et al. 2007), Sclerotinia sclerotiorum AAGT01000000 (Amselem et al. 2011), and Xylaria sp. JSS73 JWIU00000000 (Lee et al. unpubl. data). In the absence of fossils of Ceratocystidaceae, secondary calibrations were applied in uniform distributions (Schenk 2016) to the Leotiomyceses-Sordariomycetes crown (267–430 Ma) and the Sordariomycetes crown (207–339 Ma), defined as the 95% highest posterior density (HPD) range from the 5-fossil-calibrated analysis of Beimforde et al. (2014). Site models were unlinked, and the tree and relaxed log normal clock were linked for all partitions. The birth-death model (Gernhard 2008) was used for the tree prior. Preliminary runs using the same models and partitions selected by PartitionFinder 2 for the MrBayes analysis were examined with Tracer 1.6 (Rambaut et al. 2014), but these gave unacceptably low effective sample size values (ESS below 100) for both the posterior and prior distributions. This appeared to be due to certain base substitution rates tending towards zero, which we interpreted as over-parameterization (Surina et al. 2014). To mitigate this, the models for partitions 3, 6, 7, 9, 10, and 11 were relaxed from the GTR to the HKY model, which yielded acceptable ESS values. In the final analysis two separate MCMC runs of 25,000,000 generations were performed with pre-burn-ins of 150,000 and their output combined with LogCombiner 2.4.7. The combined output was analyzed with Tracer v. 1.6, resulting in all ESS values above 200 and convergence in the prior, likelihood, and posterior values. The tree files from both runs were combined and reduced to 10,000 trees with LogCombiner 2.4.7, then a maximum clade credibility tree was created and annotated with TreeAnnotator v. 2.4.7 with 15% burn-in and median height nodes, which was visualized with FigTree v. 1.4.0.

A secondary analysis (Analysis B) using a 28S rDNA molecular clock was performed as a comparison to Analysis A for selected nodes, after the methods of Berbee & Taylor (2010). The highest pairwise distance (Kimura 2-parameter model) on each side of the node was used from the 28S rDNA dataset of 1663 aligned characters. This pairwise distance was divided by two and multiplied by 100 to obtain percent base substitutions per lineage, which was multiplied by 100 Ma/1.25 %, considered to be a reasonable estimate for 28S substitution rate for the higher fungi (Berbee & Taylor 2010). The estimated fungal divergence dates were compared to the origins of their associated mycangia using the crown dates estimated by Gohli et al. (2017) and Pistone et al. (2018). Specifically, the mesonotal pouch mycangium clade included the monophyletic Xyloandrus complex (Johnson et al. 2018, Skelton et al. 2019) (i.e., Xyleborini genera with mesonotal mycangia: Anisodrus, Crestus, Eccoptopterus, Hadromedius, and Xylodrus); the prothoracic coil mycangium clade included Corthlyus (subtribe Cortlylinia); the prothoracic pleural mycangium clade included Trypodendron (tribe Xyloterini); and the pronotal disk mycangium clade included Remansius and Sclerotoplatypus (tribe Sclerotoplatypodini). Both Gohli et al. (2017) and Pistone et al. (2018) used four relevant fossils and BEAST to estimate divergence times for clades within the subfamily Sclerotinae. Gohli et al. (2017) used 5 genes from 305 species, whereas Pistone et al. (2018) used 18 genes from 182 species. The Pistone et al. (2018) estimates were used for tribe Sclerotoplatypodini and Trypodendron due to identical taxon coverage but far more gene data, and the Gohli et al. (2017) estimate was the only one available for Corthlyus.

For the Xyloandrus complex, which diverged early in tribe Xyleborini (Johnson et al. 2018) and should be only slightly younger than the tribe, the younger estimate by Pistone et al. (2018) of 14.3 Ma for the entire tribe Xyleborini was used. The older estimate of 22.5 Ma for the Xyloandrus complex by Gohli et al. (2017) is likely due to a combination of oversampling of Xyleborini taxa and a smaller number of genes, which resulted in inflated divergence estimates compared to other studies of the tribe (Jordal et al. 2000, Farrell et al. 2001, Jordal & Cognato 2012, Cognato et al. 2018, Pistone et al. 2018). Small discrepancies between the dates used in this study and those presented in Gohli et al. (2017) and Pistone et al. (2018) are due to our use of median node ages from raw data provided by the authors.

Ancestral state reconstruction

To infer the ancestral symbiotic state of nodes in the Ceratocystidaceae, FigTree v. 1.4.0 was first used to root the consensus tree produced by MrBayes to a single outgroup (S. boydii). Then, each species was coded as ‘ambrosial’ (0) or ‘not ambrosial’ (1), which included plant pathogens or putative saprophytes. Analysis was performed on the S. boydii-rooted tree with BayesTraits 2.0.2 in ‘MultiState’ mode with internal nodes defined with MRCA, and involved 10,000,000 Reverse Jump MCMC iterations, exponential priors with mean 30, and burn-in of 100,000 generations. The resulting ancestral probabilities were visualized as pie graphs in TreeGraph v. 2.14 by applying the log output from BayesTraits to the S. boydii-rooted Bayesian tree using ‘Import BayesTraits data...’ in TreeGraph. Selected pie graphs were transferred to the BEAST time-calibrated tree.
RESULTS

Fungus-beetle associations

We successfully identified putative species of Ceratocystidaceae from 10 species of Scolytoplatypodini representing the three recognised lineages of the tribe (Jordal 2013), including four species from the African/Malagasy lineage of Scolytoplatys (S. congonus, S. fasciatus, S. permirus, and S. rugosus), seven species from the Asian lineage of Scolytoplatys (S. daimyo, S. eutomoides, S. mikado, S. pubescens, S. raja, S. shogun, and S. tycan), and a species of Rumanus (R. mutabilis) (Table 1). Three different genera of Ceratocystidaceae appeared to be associated with the Scolytoplatypodini, and one of the fungus species was found to have a sexual morph. We also generated sequences from Ambrosiella symbionts of five Asian Xyleborini species (Anisandrus apicalis, Xylusandrus brevis, X. crassiusculus, X. germanus, and X. aff. germanus). Several Ceratocystidaceae species were associated with more than one beetle species, as reported previously (Lin et al. 2017, Mayers et al. 2018), but for the first time, individual ambrosia beetle species (in Asian Scolytoplatys) were associated with more than one species of Ceratocystidaceae.

African and Malagasy Scolytoplatys

Galleries of S. permirus from two different locations in Madagascar (Table 1) had thick, homogeneous, white-grey ambrosia growth, and the larval cradles were sealed from the main tunnels by frass and fragmented mycelium. The material was desiccated, and isolation attempts from the galleries and the females inside were unsuccessful. The DNA extracted from the ambrosia growth in both galleries yielded the same ITS sequence, which grouped within the Ceratocystidaceae and was most similar to but distinct from Phialophoropsis (Fig. 1). Phialidic conidiophores expected of Phialophoropsis were not seen, but the ambrosia growth contained monilioid branches of dissecting, irregular to globose, thallic-arthric propagules (Fig. 2n–r). A homogeneous mass of thallic-arthric fungal propagules that were solitary or in short chains (Fig. 2t) were found in the mycangium of a S. permirus female from a third location in Madagascar (Table 1). The DNA from this mycangial spore mass yielded an ITS sequence nearly identical (Fig. 1) to that obtained from the two galleries of S. permirus (but with a repeated ‘TC’ in the first variable region of ITS1).

A gallery of S. rugosus in Madagascar had ambrosia growth that appeared similar to that in galleries of S. permirus, and DNA extracted from the S. rugosus gallery yielded an ITS sequence nearly identical to sequences of the fungus associated with S. permirus (Fig. 1), though lacking a repeated ‘CC’ in the first variable region of ITS1. Isolations from the desiccated gallery and the single female inside were not successful. Conidiophores in the gallery of S. rugosus (Fig. 2g) were similar to those in galleries of S. permirus, but the S. rugosus galleries had spheroidal ascomata embedded in the homogenous mat of ambrosia growth. The ascomata lacked necks or ostioles and released large, banana-shaped ascospores when crushed (Fig. 2d).

DNA extracted from the mycangium contents of a South African S. fasciatus female preserved in ethanol (Table 1) yielded an ITS sequence identical to that recovered from the Malagasy S. rugosus gallery (Fig. 1). Isolations from the mycangia of six fresh South African S. fasciatus females collected using ethanol-baited traps yielded isolates of a green-grey, slow-growing fungus (Fig. 2a), and the ITS sequences derived from these cultures were identical to the one obtained from the preserved S. fasciatus specimen (Fig. 1). The six cultures produced branching, dissecting, thallic-arthric propagules (Fig. 2h–m) similar to those observed in the S. rugosus galleries, and the six cultures also produced spherical, immature ascomata. In two of the isolates (C4325 and C4328), the ascomata matured and produced large, banana-shaped ascospores (Fig. 2e, f) identical to those produced by the ascomata in the S. rugosus gallery. Single ascospores transferred from isolate C4328 to MYEA from fertile ascomata using a sterile needle (Mayers et al. 2017) germinated from both ends of the spores (Fig. 2f). The resulting colonies were similar in appearance to the parent culture and yielded identical ITS sequences, but the colonies did not produce ascomata and appeared debilitated in that they produced less surface growth, had a slower growth rate, and attained smaller maximum colony diameters.

ITS sequences were obtained from the mycangia of two female S. congonus caught in 2017 in Cameroon (MH454339).
from DNA extract M683) and Tanzania (MH454340 from DNA extract M685). The two sequences were similar to each other, but the Tanzania sequence has an extra repeated ‘C’ in ITS1, an A/G transition in ITS1, and a G/A transition in ITS2. Phylogenetic divergence was also observed between specimens of *S. congonus* from Cameroon and Tanzania (Jordal 2018). The fungal ITS sequences from the *S. congonus* specimens formed a distinct sister group to the ITS sequences from other African *Scolytoplatypus* (Fig. 1), mirroring the phylogenetic relationship of *S. congonus* and the other African species of *Scolytoplatypus* (Jordal 2018).

Asian *Scolytoplatypus*

Fungal isolates from the mycangia of *Scolytoplatypus* spp. in Japan and Taiwan (Table 1) had ITS sequences that were unique within the *Ceratocystidaceae* but were most similar to those of *Ambrosiella* (Fig. 3). Five Japanese *S. shogun* females yielded green-grey, slow-growing cultures (Fig. 4a, g, q) with an identical ITS sequence. All five isolates produced terminal, globose, thick-walled aleuriocidia (Fig. 4b–e) that were much larger than those observed in *Ambrosiella* (Mayers et al. 2015, 2017, Lin et al. 2017). The individual cells of the branching conidiophores occasionally became globose and thick-walled (Fig. 4c) and were difficult to differentiate from aleuriocidia.

A sixth isolate from *S. shogun* (C3908) had conidiophores and branching, disarticulating, thallic-arthric propagules (Fig. 4h–m), similar to those of the other *S. shogun* isolates, but C3908 had an atypical colony morphology (Fig. 4g) and a different ITS sequence. The DNA extracted from the mycangium of a Russian *S. tycon* (Table 1) yielded an ITS sequence that differed from C3908 at only one base position. Propagules in the *S. tycon* mycangium appeared to be thallic-arthric propagules (Fig. 4o–p), and in contrast to the propagules observed in mycangia of the Malagasy/African *Scolytoplatypus* spp., the propagules in the *S. tycon* mycangium appeared to sometimes branch, as illustrated in mycangia of *S. shogun* (Nakashima et al. 1987). Two *S. pubescens* females collected from different locations in Taiwan (Table 1) yielded isolates of a dark, olive-green fungus (Fig. 4q) with an ITS sequence that was most similar to those obtained from *S. shogun* and *S. tycon* (Fig. 3). Chains of aleuriocidia that formed in culture were generally longer and the conidiophores less branched compared to those in *S. shogun* isolates (Fig. 4r–v), and the *S. pubescens* isolates produced more aerial hyphae and unique vertical hyphae with branching, dark-brown chlamydospores (Fig. 4x–y). The DNA extracted from the mycangia of two *S. mikado* females and one isolate from a third Taiwanese *S. mikado* female yielded ITS sequences identical to that of the isolates from *S. pubescens* (Fig. 3). Isolate C3448 from *S. mikado* initially had a colony morphology similar to that of *S. pubescens* isolates, but C3448 later differentiated into a slower-growing form with a reddish tint. Propagules in the mycangia of *S. mikado* were single or chained, sometimes-branching and thallic-arthric (Fig. 4z).

Cultures isolated in 2002 from *S. daimio*, *S. mikado*, *S. shogun*, and *S. tycon* in Japan (Table 1) were lost, but partial to full ITS sequences were obtained in 2002 and compared to more recent sequences. The ITS sequences of the *S. daimio*, *S. mikado*, and *S. shogun* cultures grouped with sequences obtained from the new isolates from *S. shogun* (Fig. 3). The sequences from *S. tycon* isolates grouped more closely with those from the atypical *S. shogun* isolate C3908 and the *S. tycon* mycangium (Fig. 3). Small one- or two-bp differences among the 2002 ITS sequences may be due to ambiguities or errors encountered with older sequencing technology.

An ITS sequence provided by J. Skelton (U. Florida) from a spore mass oozing from the mycangium of a female *S. eutomoides* caught in Mu village, Chimbu Province, Papua New Guinea by J. Hulcr (U. Florida) was similar to the sequences of the Taiwan *Scolytoplatypus* spp., differing only in a 2-bp deletion in the variable region of ITS1 (Fig. 3). Two ITS sequences provided by You Li (U. Florida) from cultures isolated from the mycangia of two female *S. raja* caught in flight in mainland China (Guiyang, Guizhou, 2015 and Shiwanqashan, Guangxi, 2018) were identical to the sequences obtained from *S. pubescens* and *S. mikado* in Taiwan (Fig. 3).
Fig. 3  One of 12 most parsimonious trees produced from analysis of ITS rDNA sequences of cultures and specimens of Ambrosiella and close relatives from the collections at Iowa State University (C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), the UAMH Centre for Global Microfungal Biodiversity (UAMH), or other collections. Sequences in bold are new. Single asterisk (*) indicates sequence obtained from DNA extracted from whole beetle or mycangial spore mass; double asterisks (**) from DNA extracted from gallery growth. Country of origin of the beetle, gallery, or culture is indicated. Bootstrap support values > 50 % indicated on branches. Catunica adiposa and Meredithiella norrisi were used as outgroups. CI = 0.7823. An ’e’ indicates an ex-type culture; HT = holotype; IT = isotype; PT = paratype. — Scale bar = 8 changes (bp).
**Remansus**

Galleries of *R. mutabilis* collected in Madagascar (Table 1) appeared to be recently-initiated and devoid of ambrosia growth. The DNA extracted from the mycangium contents of a female taken from one of the galleries yielded an ITS sequence that closely matched sequences of *Ambrosiella* (Fig. 3). This mycangium was full of arthric-thallic propagules that were solitary or in short, linear chains (Fig. 5c–d) and were very similar to mycangium propagules of *Ambrosiella* (Harrington et al. 2014, Mayers et al. 2015, 2017). Isolations were unsuccessful from the desiccated galleries and from the mycangia of two other females.

**Asian Xyleborini beetles**

Isolations were made from several Xyleborini species in the *Xylosandrus* complex that were sympatric with the studied Asian *Scolytoplatypus* species. All of these *Xyleborini* had *Ambrosiella* symbionts, as expected (Mayers et al. 2015, 2017, Lin et al. 2017, Skelton et al. 2019) (Fig. 3). Three Japanese females identified as *Xylosandrus aff. germanus* yielded isolates of an undescribed species (*Ambrosiella aff. grosmanniae NRgr01*), which was morphologically distinct from *A. grosmanniae* and may coincide with cryptic diversity in *X. germanus* and its *A. grosmanniae* symbionts (Ito & Kajimura 2017). Two Japanese *X. brevis* and one Japanese *X. germanus* yielded isolates.
of what may be another undescribed species (*Ambrosiella aff. beaveri* NRbea1) in the *A. beaveri* complex (Fig. 3). Five Japanese *Anisandrus apicalis* yielded isolates of *Ambrosiella catenulata* (Fig. 3), the first report from this beetle species (Lin et al. 2017).

Prior to the discovery of the sexual morph of the *S. fasciatus* symbiont, *A. cleistominuta* was the only ambrosia beetle symbiont known to produce mature ascocarps (Mayers et al. 2017). As a morphological comparison, we sought ascomata of the type species, *Xylosandrus compactus*, because galleries of this twig borer are easily collected from the dead branch tips on living trees (Greco & Wright 2015). We collected 87 dead twigs with small gallery entrance holes on various living hosts in Mississippi and Louisiana, USA in June and July 2016. Many galleries were abandoned or contained only adult beetles and sparse ambrosia growth, but 25 galleries had healthy ambrosia growth that could be confirmed to be *A. xylebori* by morphology, direct ITS barcoding of gallery scrapings, and/or isolation of pure cultures, including CBS 144147. Twelve of the galleries with healthy ambrosia growth had neckless ascomata embedded in the ambrosia layer (Fig. 6a) that appeared very similar to the ascomata of *A. cleistominuta* (Mayers et al. 2017). Two of the ascomata were plated onto MEA and produced cultures of *A. xylebori*, but these cultures produced no ascomata. Some of the gallery ascomata contained ascospores (Fig. 6b–d) nearly identical to those of *A. cleistominuta* and similar in size to those of the perithecia-forming *C. adiposa* (Fig. 6e) and *C. norvegica* (Fig. 6f). In comparison to the ascospores of *Ambrosiella*, or any other *Ceratocystidaceae*, the ascospores of the *S. fasciatus* isolates are dramatically larger (Fig. 6g).

![Fig. 5 Mycangium of *Remansus mutabilis* and mycangium propagules of *Ambrosiella remansi*. a. Cross-section of pronotal disk mycangium showing mycangium wall (mw), reticulated pouches (rp), and fungal spores (fs); b. detail of mycangium wall, showing socketed seta (ss) on hollow cylindrical pedestal and reticulated cone (rc); c. propagules of *A. remansi* surrounding seta; d. propagules of *A. remansi* (all photos by Nomarski interference microscopy of BPI 910622, stained with cotton blue). — Scale bars: a = 100 µm; b–d = 10 µm.](image-url)
Scolytoplatypodini mycangia

Consistent with Schell’s (1962) illustrations, all observed mycangia of Scolytoplatypodini were disk-shaped cavities just under the cuticle on the dorsal side of the pronotum that emptied through a central, circular cuticular pore. Waxy masses of fungal propagules in an unidentified matrix generally plugged the pores and oozed out when pressure was applied to the dissected mycangium. As also seen in Schedl’s illustrations, the interior of the Scolytoplatypodini mycangia were lined with long setae that led towards the pore and presumably helped guide fungal propagules to exit. The morphology of these internal setae differed substantially between African and Asian Scolytoplatypodini species. The setae of African Scolytoplatypodini species (S. congonus, S. fasciatus, S. permirus, and S. rugosus) were of two types: shorter, thinner setae that grew directly from the sclerotized plates of the mycangium wall (Schedl’s ‘unechte Haare’ or ‘false hairs’), and longer, thicker setae that grew from short, cone-shaped sockets on the mycangium wall (Schedl’s ‘echte Haare’ or ‘true hairs’), as illustrated in the African species S. acuminatus (Schedl 1962). In contrast, the mycangia of Asian Scolytoplatypodini species (S. mikado, S. pubescens, and S. tycon) had only one type of setae, which were socketed on the apexes of tall reticulated cones that were extensions of the reticulated network of the mycangium wall. This is a new description for mycangia of S. pubescens, and the setae match previous illustrations of the setae of S. blandfordi, S. daimio, S. mikado, S. raja, S. shogun, and S. tycon (Berger & Cholodkovsky 1916, Schedl 1962, Nakashima et al. 1987, Beaver & Gebhardt 2006, Li et al. 2019).

Mycangia of the rare and recently-described (Jordal 2013) genus Remansus had not been illustrated previously. The mycangia of female R. mutabilis collected in Madagascar were prontal disks similar to those of Scolytoplatypus. However, the interior setae of the Remansus mycangium were of only one type, which were socketed on the apexes of tall, hollow, elongated, non-reticulated, cylindrical, and pigmented pedestals (Fig. 5a–b). Interspersed with these pedestals were tall reticulated cones (Fig. 5b) that did not support terminal setae but were otherwise similar to the cones supporting the interior setae of Asian Scolytoplatypus. The mycangium wall of R. mutabilis was composed of a large, reticulated network, between which hung more finely-reticulated, semi-spherical pouches (Fig. 5a). This reticulated network was more similar to the reticulated mycangium walls of Asian Scolytoplatypus than the sclerotized plates of African Scolytoplatypus (Schedl 1962). Both the setal pedestals and reticulated cones of R. mutabilis appeared to be contiguous with this reticulated network. Fungal propagules were found throughout the mycangium lumen as well as in the semi-spherical pouches (Fig. 5a).

Phylogenetic analyses

The recovered symbionts of the three Scolytoplatypodini lineages were members of the Ceratocystidaceae, but they did not form a monophyletic group. The ITS sequences of African Scolytoplatypus symbionts were most similar to Phialophoropsis. Parsimony analysis of the Phialophoropsis-affiliated alignment produced one tree (Fig. 1). The African Scolytoplatypus symbionts formed a distinct sister group to Phialophoropsis, with strong bootstrap support (Fig. 1). The ITS sequences of the mycangial symbionts of Asian Scolytoplatypus and R. mutabilis were more similar to Ambrosiella, and parsimony analysis of the Ambrosiella-affiliated alignment produced 84 trees (including Fig. 3) that differed only in the topology of the Japanese Scolytoplatypus symbionts that were sequenced in 2002. The
Fig. 7 Phylogenetic tree from Bayesian analysis of a six-gene dataset (18S rDNA, 28S rDNA, tef1-α, tub, mcm7, and rpl1) of ambrosia fungi and other representatives of the Microascales from the Iowa State University collection (C or M), Westerdijk Fungal Biodiversity Institute (CBS), U.S. National Fungus Collections (BPI), the Belgian Co-ordinated Collections of Micro-organisms (MUCL), the International Collection of Microorganisms from Plants (ICMP), or the UAMH Centre for Global Microfungal Biodiversity (UAMH). The family Ceratocystidaceae is indicated by a grey-shaded rectangle and the five ambrosia fungus genera are indicated by coloured rectangles. Symbols to the right of each taxon represent the presence of sexual sporocarps or aleuroconidia. Posterior probabilities from Bayesian inference (BI), bootstrap support values from 1000-replicate maximum likelihood analysis in RAxML (ML), and bootstrap support values from 1000-replicate maximum parsimony analysis in PAUP* (MP) are indicated in box at left margin with lowercase letters indicating the associated branch. Branches with BI ≥ 1.0, ML ≥ 100, and MP ≥ 100 support are labelled with an asterisk (*), and branches with BI = 1.0, ML ≥ 95, and MP ≥ 95 are indicated with thicker branches. Posterior probability values ≥ 0.995 and < 1.0 are represented as ‘0.99’, bootstrap support values ≥ 99.5 and < 100 are represented as ‘99’, and bootstrap support values ≤ 0.95 are indicated with two hyphens (‘--’). — Scale bar = 0.1 estimated substitutions per site.

Asian Scolytoplatypus symbionts formed a distinct sister group to Ambrosiella with strong bootstrap support, but the symbiont of *R. mutabilis* grouped with *Ambrosiella* s.str. with moderate support (Fig. 3). New ITS sequences of Xyloborin symbionts were placed within *Ambrosiella* s.str. (Fig. 3), as expected (Mayers et al. 2015, 2017, Lin et al. 2017).

Multi-gene Bayesian analysis confirmed the sister relationships of African Scolytoplatypus symbionts with Phialophoropsis and Asian Scolytoplatypus symbionts with Ambrosiella, as well as the placement of the *R. mutabilis* symbiont with *Ambrosiella* s.str., where it was placed as the first-diverging taxon (Fig. 7). Including Meredithiella, there were five distinct, well-supported clades of ambrosia fungi with high posterior probability, RAxML bootstrap, and parsimony bootstrap support values. The inferred relationships among genera in the Ceratocystidaceae were similar to those of previous analyses, with the ambrosia beetle symbionts, Huntiella, and three other species grouping separately from the other half of the family (De Beer et al. 2014, 2017, Mayers et al. 2015). The placement of *Huntiella* within the ambrosia half of the family was somewhat ambiguous. The genus was positioned under a short internode with 0.98 posterior probability support, but this branch collapsed to a polytomy when certain *Huntiella* taxa were added to or removed from analysis. The ambiguous phylogenetic placements of three lone taxa (*Bretziella fagacearum, Ceratocystis adiposa*, and *C. norvegica*) with respect to the ambrosia beetle symbionts were somewhat better resolved than in earlier studies (De Beer et al. 2014, 2017, Mayers et al. 2015, 2018). *Ceratocystis adiposa* was placed as sister taxon to Meredithiella with 1.0 posterior probability support, *B. fagacearum* formed a sister taxon to the Phialophoropsis/African Scolytoplatypus symbiont clade with moderate support, and *C. norvegica* was placed as
sister taxon to the Ambrosiella/Asian Scolytoplatypus symbiont clade with moderate support. Both C. adiposa and C. norvegica are clearly placed outside of Ceratocystis s.str. but are not accommodated by any currently-defined genera (De Beer et al. 2014, 2017, Mayers et al. 2015, Nel et al. 2017). Both of these species (and B. fagacearum) have no known close relatives, and they are biologically and morphologically distinct from each other and the ambrosia beetle symbionts.

**Taxonomy**

In recognition of their distinct morphological characteristics, and supported by clear phylogenetic separation from known taxa, two new genera are proposed for the respective symbionts of African and Asian Scolytoplatypus. The symbiont of *R. mutabilis* is described as a new species of Ambrosiella. Two new monotypic genera are created to accommodate the problematic non-ambrosial taxa *C. adiposa* and *C. norvegica*. An additional monotypic genus is created to accommodate a close phylogenetic relative to the *Ceratocystidaeae* isolated from a ship timber beetle gallery in the course of the study.

**Wolfgangiella**

*Wolfgangiella* C. Mayers & T.C. Harr., *gen. nov.* — MycoBank MB824930

**Etymology.** Feminine. After entomologist Wolfgang Dietrich Schell, who characterized the mycangia of *Scolytoplatypus* and made other significant contributions to our understanding of mycangium diversity.

Type species. *Wolfgangiella franznegeri.*

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

Notes — *Wolfgangiella* is most closely related to *Phialophoropsis* (Fig. 7), and its arthric-thallic conidia are somewhat similar to the disarticulating cells observed in *Phialophoropsis* (Mathiesen-Käärinki 1953). However, all studied *Phialophoropsis* spp. produce chained conidia from deep-seated phialides (Batra 1967, Mayers et al. 2015), which were not observed in *Wolfgangiella*. Cultures of the type species for *Wolfgangiella* produce significantly less pigment than *Phialophoropsis* spp.

**Wolfgangiella franznegeri** C. Mayers, T.C. Harr., & Roets, *sp. nov.* — MycoBank MB824932; Fig. 2a–m, 61

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

**Typus.** SOUTH AFRICA, Western Cape Province, Betty’s Bay, Harold Porter National Botanical Garden, S34°21’1.58” E18°55’37.03”, from mycangium of *Scolytoplatypus fasciatus* caught in flight, 21 Jan. 2017, F. Roets C4325; Western Cape Province, Betty’s Bay, Harold Porter National Botanical Garden, from mycangium of *S. fasciatus* caught in flight, 21 Jan. 2017, F. Roets five isolates from separate females (C4325, C4326, C4327, C4329, C4331).

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

**Wolfgangiella madagascarensis** C. Mayers, McNew, & T.C. Harr., *sp. nov.* — MycoBank MB824933; Fig. 2n–t

**Etymology.** After the country of origin, Madagascar.

**Typus.** MADAGASCAR, Ambahoantely Forest Reserve, ambrosia growth in gallery of *Scolytoplatypus perminus* in Ooctea sp. or Macaranga sp., May 2015, B. Jordal (BPI 910641 holotype).

Conidiophores composed of branching, disarticulating chains of thallic-arthric conidia. *Conidia* irregular or globose to sub-globose, 4–13(–17) µm diam, detaching singly or in chains. Growth in mycangium composed of irregular cells 4.5–12.5 x 3.5–7 µm in size, solitary or in branched or unbranched chains.

Additional specimens examined. MADAGASCAR, Ranomafana National Park, propagules in mycangium of *Scolytoplatypus perminus* caught in flight, 2012, B. Jordal sp; Andasibe-Mantadia National Park, ambrosia growth in gallery of *S. perminus* in Ooctea or Macaranga sp., May 2015, B. Jordal M399.

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

**Toshionella**

*Toshionella* C. Mayers & T.C. Harr., *gen. nov.* — MycoBank MB824934

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

Type species. *Toshionella nipponensis.*

*Auleuroconidia* terminal, thick-walled, globose, single or in basipetal chains, sometimes surrounded by a membranous sheath, on branching moniloid or simple conidiophores. Associated with ambrosia beetles, including *Scolytoplatypus* spp. in Asia. Phylogenetically classified in the *Ceratocystidaeae*.

Notes — One or more members of this genus were previously reported from Asian *Scolytoplatypus* and treated as putative species of *Ambrosiella* (Nakashima et al. 1987, 1992, Nakashima 1989, Kinuura et al. 1991, Kinuura 1995, Beaver & Gebhardt 2006). *Toshionella* is closely related to *Ambrosiella*.
(Fig. 7), and its conidiophores are similar, but its aleurioconidia are much larger. The phialidic conidiophores reported in Ambrosiella (Lin et al. 2017, Mayers et al. 2015, 2017) were not observed in Toshionella. The thin membranous sheath observed around the conidiophores in A. cleistominuta (Mayers et al. 2017) and C. adiposa (Hutchinson 1939, Bhat 1972, Hawes & Beckett 1977a, b). Though rare, Toshionella ambrosioides sometimes disarticulate with the penultimate conidiophore cell attached (Fig. 4e, j, v), as is common in Ambrosiella and Meredithiella (Harrington et al. 2014, Mayers et al. 2015, 2018). All Toshionella cultures produced wet and gooey mounds of thick-walled, globose to irregular, hyaline cells (Fig. 4f, n, w), which may be the ‘sproul cells’ previously reported forming a ‘slimy layer’ in galleries of Scolytoplatypus spp. (Nakashima 1989, Beaver & Gebhardt 2006).

**Toshionella nipponensis** C. Mayers, T.C. Harr., & H. Masuya, sp. nov. — MycoBank MB824935; Fig. 4a–f

*Etymology. After the country of origin, Japan (endonym Nippon).*

Typos. *JAPAN,* Akita prefecture, Tatamago, from mycangium of *Scolytoplatypus shogun* emerging from *Fagus crenata*, July 2014. H. Masuya (BPI 910635 holotype, dried culture), culture ex-type CBS 141492 (= C3904).

Conidiophores 8.5–35 µm long, single- or multiple-celled, hyaline becoming red-brown, branched, composed of thick-walled monilioid cells, bearing terminal aleurioconidia, conidiophore cells later becoming thick-walled and disarticulating into single cells. *Aleurioconidia* hyaline becoming faintly red-brown, globose to subglobose, ovoid, 18–26 × 17.5–25.5 µm, generally wider than tall, often flat on bottom, borne singly or in chains, terminally, from monilioid conidiophores, terminally or intercalary on simple hyphae, or rarely directly from the side of the conidiophore. In older cultures, thick-walled cells presumed to be aleurioconidia accumulate in moist mounds on the culture surface, globose to ellipsoidal, thick-walled, 7–25 µm diam.

Cultural characteristics — Colonies on MYEA 32–55 mm after 7 d at 25 °C, surface growth white, fluffy in patches, later covering surface and becoming chalky, pale olivaceous to olivaceous grey, reverse grey olivaceous to olivaceous black, becoming dark slate blue, margin hyaline, becoming greenish black. In older cultures, irregular buff sporodochia on surface, sometimes bearing red liquid drops, later center growth thick, dense, raised, and red-brown.

Additional cultures examined. *JAPAN,* Akita prefecture, Tatamago, from mycangium of *Scolytoplatypus shogun* emerging from *Fagus crenata*, July 2014. H. Masuya three isolates from separate females (C3905, C3906, C3907); Iwate prefecture, Hachimantai, from mycangium of *S. shogun* emerging from *Fagus crenata*, 7 July 2014. H. Masuya C4064.

Notes — *Toshionella nipponensis* was isolated from five *S. shogun* females in Japan, and the cultures had an identical ITS sequence that was nearly identical to those obtained from Japanese *S. daimio,* *S. mikado,* and *S. shogun* isolates in 2002 (Fig. 3). The fungus illustrated in galleries of Japanese *S. daimio,* *S. mikado,* and *S. shogun* (Nakashima et al. 1987, 1992, Nakashima 1989, Kunura et al. 1991, Kunura 1995) and informally described by Nakashima et al. (1987) is probably *T. nipponensis.* Sequences obtained from Japanese *S. mikado* by Ito & Kawasaki (2017), including sequences of 18S rDNA (LC140888), 28S rDNA (LC140895 and LC140896), and tub (LC140904), may also represent *T. nipponensis.* An atypical isolate from *S. shogun* that differed in phenotype, DNA sequences, and culture morphology is described below as a separate species.

**Toshionella transmara** C. Mayers, T.C. Harr. & H. Masuya, sp. nov. — MycoBank MB824936; Fig. 4g–p

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

Typos. *JAPAN,* Iwate prefecture, Hachimantai, from mycangium of *Scolytoplatypus shogun* emerging from *Fagus crenata*, June 2015, H. Masuya (BPI 910638 holotype, dried culture), culture ex-type CBS 141493 (= C3908).

Conidiophores 8–40 µm long, single- or multi-cellular, hyaline, branched, composed of thick-walled monilioid cells, bearing terminal aleurioconidia, conidiophore cells later becoming thick-walled, disarticulating into single cells. *Aleurioconidia* hyaline, thick-walled, globose to subglobose, ovoid, (8.5–)14–18 × (8.5–)14–19 µm, generally wider than tall, often flat on bottom, borne singly or in chains, terminal on monilioid conidiophores or on single-celled side branches, or rarely directly from the side of conidiophores. Thick-walled cells presumed to be aleurioconidia occasionally accumulate in moist mounds on the culture surface, globose to ellipsoidal, thick-walled, 10.5–30 µm diam. *Thalic-arthic conidia* thick-walled, 11–17–(19.5) × 9–13.5–(16) µm, usually ellipsoidal to globose, borne in branching monilioid chains on the surface of mature sporodochia, disarticulating singly or in chains. *Myccangial growth* of branching, thalic-arthic cells, 4.5–10 × 5–14 µm, solitary or in chains.

Cultural characteristics — Colonies on MYEA 40–63 mm after 7 d at 25 °C, surface growth superficial, greyish sepia to olivaceous grey, covered in randomly dispersed superficial, spherical, white to off-white sporodochia 1–1.2 mm diam, distributed randomly, or clustered around site of transfer, reverse greenish black, margin greenish black, aerial hyphae absent.

Additional specimens examined. *RUSSIA,* Vladivostok, propagules in mycangium of *Scolytoplatypus tycon* caught in flight, July 2008, B. Jordal sp1.

Notes — This species is closely related to *T. nipponensis* (Fig. 3) but can be differentiated by its faster rate of growth, scatted spherical sporodochia, and lack of chalky white surface growth. The single living representative (C3908 = CBS 141493) was isolated from *S. shogun* in Japan, but nearly-identical ITS sequences were obtained from the mycangium of a female *S. tycon* caught in Vladivostok, Russia (sp1) and three cultures from Japanese *S. tycon* sequenced in 2002 (Fig. 3). Nakashima et al. (1992) may have illustrated *T. transmara* in galleries of Japanese *S. tycon*.

**Toshionella taiwanensis** C. Mayers, T.C. Harr. & H.H. Shih., sp. nov. — MycoBank MB824937; Fig. 4q–z

*Etymology. After the location of first discovery, Taiwan.*

Typos. *TAIWAN,* Kaohsiung City, Douna, from *Scolytoplatypus pubescens* caught in flight, Feb. 2014, H.H. Shih (BPI 910637 holotype, dried culture), culture ex-type CBS 141494 (= C3687).

Conidiophores 15–115 µm long, hyaline or red-brown, branched, composed of thick-walled monilioid cells or thin-walled irregular cells, produced densely across the entire surface of the culture, bearing terminal aleurioconidia, conidiophore cells developing into thick-walled chlamydospores. *Aleurioconidia* hyaline, thick-walled, globose to subglobose, ovoid, 13.5–23 × 12.5–22 µm, generally wider than tall, often flat on bottom, produced terminally, singly or in chains, on long monilioid conidiophores or single-celled side branches or directly on simple hyphae. *Chlamydospores* of two types, borne terminally on branching, aerial, erect towers of simple hyphae, thick-walled, globose to subprolate, obovoid, deeply pigmented, 15.5–18 × 15–16 µm, generally taller than wide; or ellipsoidal to globar, lightly red-brown, thick-walled, disarticulating singly or in chains, borne in...
branching monilioid chains. In older cultures, thick-walled cells presumed to be aleurioconidia accumulate in moist mounds on the culture surface, globose to ellipsoidal, thick-walled, 10.5–26.5 μm diam. Mycangial growth branching, thallic-arthric, of thick-walled cells, 4.5–11.5 × 3.5–8 μm, solitary or in chains.

Cultural characteristics — Colonies on MYEA (22–)40–53 mm after 7 d at 25 °C, reverse greenish black, margins hyaline to buff, submerged, becoming white, dense, raised, fruitly smelt at 5 d, somewhat fading by 14 d, covered with a dense white to olivaceous gray carpet of aerial mycelium and conidiophores. Subsurface hyphal visible beneath the dendroid margin of surface growth, deeply pigmented with diffusible pigment rarely extending beyond surface growth, deep brown to greenish black.

Additional cultures examined. TAIWAN, Kaohsiung City. Jingdashan, from Scoytolyctus pubescens caught in flight, Feb. 2014, H.H. Shih C3688; Nantou County, Lienhuachih, from S. mikado caught in flight, Aug. 2014, C. Wuest CBS 141495 (= C3448; dried culture BPI 910636).

Additional specimens examined. TAIWAN, Nantou County, Lienhuachih, propagules in mycangium of Scoytolyctus mikado caught in flight, June 2014, H.H. Shih M304; Yilan County, Fushan, propagules in mycangium of S. mikado in Cinnamomum sp., June 2014, H.H. Shih M305.

Notes — This new species is associated with both S. pubescens and S. mikado in Taiwan and is related to the Japanese Toshionella species (Fig. 3). Sequences obtained in 2002 from Japanese S. mikado were of T. nipponensis rather than T. taiwanensis, implying geographic rather than host-species distinction of the two species (Fig. 3). Toshionella taiwanensis can be differentiated from the Japanese Toshionella by its dense, fluffy surface growth and the presence of uprigh, branching aerial towers bearing terminal, pigmented chlamydospores (Fig. 4x–y), which are unique among the studied ambrosia fungi but similar to the aleurioconidia produced by species of Ceratocystis s.lat. (Paulin-Mahady et al. 2002). The fungus isolated from S. pubescens in Taiwan by Lin (2016) and given the provisional name ‘Ambrosiopsis globoa’ is probably T. taiwanensis. The isolate from S. mikado (C3448 = CBS 141495) initially resembled the two isolates from S. pubescens, but C3448 later grew more slowly (32–36 mm after 7 d at 25 °C with a maximum growth diameter of 63–67 mm) and produced a diffusible red-brown pigment that was not observed in the other isolates. The changed culture also lacked fluffy surface growth, instead forming conidiophores in loose tufts across the surface and below the surface of the agar. Cultures obtained from S. raja caught on mainland China had ITS sequences identical to those of T. taiwanensis (Fig. 3), suggesting that the species is not restricted to Taiwan, but further study of the cultures is needed. A very similar sequence was obtained from a S. eutomoides female in Papua New Guinea (Fig. 3).

**Ambrosiella** Brader ex Arx & Hennebert emend. C. Mayers & T.C. Harr.

**Etyymology.** Brader (1964) named the genus for its ecological role as ‘ambrosia’, a term first coined by Schmidberger (1836).

Type species. Ambrosiella xylebori.

**Conidiophores** single to aggregated in sporodochia, hyaline, unbranched or sparingly branched, one-celled to septate, producing terminal aleurioconidia or chains of conidia from phialides. Ascomata, when present, cleistothecoid, textura intricata. Ascospores hyaline, aseptate, reniform, often thick-walled. Ascii not observed. Associated with ambrosia beetles. Phylogenetically classified in the Ceratocystidaceae.

Notes — Harrington et al. (2010) restricted Ambrosiella to ambrosia beetle symbionts related to Ceratocystis (i.e., classified in Ceratocystidaceae). Mayers et al. (2015) showed Ambrosiella to be polyphyletic and returned A. ferruginea and A. trypodendri to Phialophoropsis. Mayers et al. (2017) demonstrated a cleistothecoid sexual morph in A. cleistominuta, as we demonstrated here in the type species. A. xylebori. The generic description of Ambrosiella is here emended and follows the assexual description of Harrington et al. (2010), and is updated to include the morphology of its sexual morph. The ten current species include the seven Xyleborini-associated species reviewed by Mayers et al. (2015), as well as two other Xyleborini-associated species (Lin et al. 2017, Mayers et al. 2017) and the new species described below.

**Ambrosiella remansi** C. Mayers & T.C. Harr., sp. nov. — MycoBank MB824938; Fig. 5c–d

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

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

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

Notes — This species was found as propagules in the mycangium of R. mutabilis, the type species for the genus Remansus, which is only known from Madagascar (Jordal 2013). The ITS sequence obtained from this single specimen places it with Ambrosiella (Fig. 3), and the multigene phylogeny places the fungus as an early-diverging member of Ambrosiella (Fig. 7). Though its conidial morph is unknown, the mycangial growth of R. remansi is composed of non-branched, thallic-arthric mycangial propagules as expected of Ambrosiella (Harrington et al. 2014, Mayers et al. 2015, 2017), in contrast to the more irregular and sometimes-branched mycangial growth of Toshionella. The rarity of Remansus specimens (Jordal 2013) precludes further study at this time, but the novelty of the first Ambrosiella sp. not associated with the mesonotal mycangium of a host in the Xylodendro complex warrants the naming of A. remansi as a new species.

**Catunica** C. Mayers & T.C. Harr., gen. nov. — MycoBank MB824939

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

**Type species.** Catunica adiposa.

**Perithecia** black, with long necks, ostiolar hyphae hyaline to brown. Ascospores hyaline, half-moon-shaped, sometimes with a sheath. Conidiophores phialidic, hyaline to brown, producing chains of conidia that vary from hyaline to pale brown, cylindrical to doliiform, or with truncate to rounded ends, smooth-walled to verrucose. Aleurioconidiophores enteroblastic to holoblastic, pigmented, producing chains of dark brown to black aleurioconidia, ovoid to globose, thick-walled, with verrucose to fimbriate walls, often enveloped by a brown membranous sheath, detaching in chains, with or without sheath. Phylogenetically classified in the Ceratocystidaceae.

Notes — Catunica adiposa, the only known species of the genus, is a causal agent of sugar cane rot (Butler 1906, Sartorius 1927), has been implicated in human illness (Agarwal et al. 2014), and has been isolated from Prunus (Paulin-Mahady et al. 2002), Pinus (Talbot 1956), air (e.g., CBS 138.34), books (e.g., IMI 21285), and other substrates. Catunica has distinctive variation in conidium morphology, ranging from typical cylindrical conidia from deep-seated phialides (endoconidia) to thick-walled, dark, ornamented aleurioconidia (Fig. S1).
These conidial types were referred to as phialoconidia and chlamydospores by Nag Raj & Kendrick (1975). We agree with other authors (Butler 1906, Sartoris 1927, Davidson 1935, Moreau 1952, Hunt 1956, Talbot 1956, Upadhyay 1981) that both types of spores are produced from common conidiophores and change over time. It may produce the longest perithecium necks in the family (Malloch & Blackwell 1993) and also produces distinctive, half-moon-shaped ascospores with gelatinous sheaths. Its ecology and phylogenetic position are unique (Fig. 7), and its relationship to Meredithiella spp., which are obligate symbionts of ambrosia beetles (Mayers et al. 2018), is difficult to reconcile.

**Catuna adiposa** (E.J. Butler) C. Mayers & T.C. Harr., comb. nov. — MycoBank MB824940; Fig. 6a, S1a–S1e

_Basionym_. Sphaeroaena adiposum E.J. Butler, Mem. Dept. Agric. India, Bot. Ser. 1: 40. 1906.

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

Ophistoma adiposum (E.J. Butler) Nannf. in Melin & Nannf., Svenska Skogsv.-Fören. Tidskr. 32: 408. 1934.

_Endoconidiophora adiposa_ (E.J. Butler) R.W. Davidson, J. Agric. Res. 50: 802. 1935.

_Ceratocystis adiposa_ (E.J. Butler) C. Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.

_Ceratostomella major_ J.F.H. Beyma, Zentrabl. Bakteriol., 2. Abt. 91: 348. 1935.

_Ophistoma majus_ (J.F.H. Beyma) Gold., Boll. Staz. Patol. Veg. Roma 15: 158. 1935.

_Ceratocystis major_ (J.F.H. Beyma) C. Moreau, Rev. Mycol. (Paris) Suppl. Col. 17: 22. 1952.

_Etymology_. After the ‘fatty substance’ in which the ascospores are suspended (Butler 1906).

**Typus. CANADA.** Ontario, Cornwall, from hardwood chips, 14 Jan. 1969, J.K. Shields (BPI 910979, epitope designated here MBT386138, dried culture); culture ex-type CBS 183.86 (= C999 in collection of T.C. Harrington = C2518/C411 in collection of FPIInnovations/Forintek); — _India_. Bihar, Champaran, Seeraha, on Saccharum officinarum, 30 Oct. 1903, E.J. Butler (HClO 3513 lectotype designated here, MBT386139).

For descriptions, see Butler (1906), Sartoris (1927), Davidson (1935), Moreau (1952), Hunt (1956), Talbot (1956), Nag Raj & Kendrick (1975), and Upadhyay (1981).

_Additional cultures examined. FRANCE_. 1953, C. Moreau CBS 147.53 (= C905 = LCP 903). — _India_. from Saccharum officinarum, date unknown (deposited in IMI prior to 1927; deposited in CBS in 1927; deposited in MUCL in 1967), Subramanian CBS 127.27 (= C957 = MUCL 9507). — _JAPAN_. from Prunus persica, date unknown, P. Soentoro CBS 809.74 (C998). — _NEOTERLANDS_. from air, c. 1934, F.H. van Beyma CBS 138.34 (= C906 = MUCL 9518). — _USA_. North Carolina, from wood chips, 1967, R.A. Blanchette C299.

_Notes_. — Butler (1906) did not designate a type specimen, but he established Herbarium Cryptogamae Indianae Orientalis (HCIO) in 1905 (Maheswari et al. 2012) and deposited a specimen (HCIO 3531) under the name _Sphaeroaena adiposum_. The specimen is available in HCIO, which is maintained by the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India (pers. comm., HCIO). The collection information for HCIO 3531, 1903 in Bihar (Fig. S2a), matches his first discovery of the fungus (Butler 1906) and the specimen consists of an infected section of sugarcane with red-brown streaks and dark black fungal growth (Fig. S2b), consistent with Butler’s description of _S. adiposum_. This is the only specimen known to be directly identified by Butler as _S. adiposum_, it appears to be part of the protologue, and we designate HCIO 3531 as lectotype for the species (see Fig. S3 and caption for discussion of IMI 21355 and IMI 21285, the only reasonable alternatives). We did not examine HCIO 3531, but six isolates of _C. adiposa_ from six different countries (Table S1) had identical ITS sequences (Fig. 3) and displayed the characteristic conidiophores and conidia of _C. adiposa_ (Fig. S1), as illustrated by Butler (1906), Hunt (1956) and Nag Raj & Kendrick (1975). We designate a dried culture of CBS 183.86 from Canada as epitype, and it was used to represent _C. adiposa_ in our multi-locus phylogenetic analyses and produced sexual and asexual structures in culture. Isolate CBS 127.27 (= MUCL 9507) was collected in India on Saccharum officinarum, and it shares the common ITS sequence of the other isolates, but our culture of CBS 127.27 was sterile and debilitated. A draft genome (ASM164068v1) is available for _C. adiposa_ CBS 136.34 (Wingfield et al. 2016a), and an NCBI BLASTn search against this genome using our ITS sequence found a perfect match (460/460 bp identical).

**Solaloca** T.C. Harr., _gen. nov._ — MycoBank MB824941

_Etymology_. Feminine. An arbitrary construction based on the Latin sola (adj), feminine form of solus, ‘solitary’; and locus (n), ‘place/location’.

_Type species_. Solaloca norvegica.

_Perithecial_ bases and necks black, ostiolar hyphae hyaline. Ascospores hyaline, thick-walled, cylindrical to rarely curved. _Conidiophores_ not observed. Phylogenetically classified in the _Ceratocystidaceae_.

_Notes_. — Solaloca norvegica was isolated from a single collection of galleries of _Ips typographus_ in Picea abies in Norway in 1974, but the new species was not described until 2010 (Reid et al. 2010). Conidia were not seen, and we failed to find conidia in our isolate (C4124) of the ex-type strain UAMH 9778 (= UAMH 11187). Phylogenetically, Solaloca is placed within the family _Ceratocystidaceae_ (Fig. 7) with no obvious relatives. In morphology (perithecia and ascospores) and habitat, it resembles the conifer-inhabiting members of _Endoconidiophora_ (Harrington & Wingfield 1998), and it shares no obvious characters with ambrosia beetle symbiotes other than the association with the subfamily Scolytinae.

**Solaloca norvegica** (J. Reid & Georg Hausner) T.C. Harr., _comb. nov._ — MycoBank MB824942; Fig. 6f

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

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

_Typeus. NORWAY_. Ostfold, near Sandem, from gallery of _Ips typographus_ in _Picea abies_, Sept. 1973, J. Reid (UAMH 11187 holotype, dried culture), ex-type culture under same accession.

_For description, see Reid et al. (2010)._

_Additional cultures examined. NORWAY_. from Sandem, from gallery of _Ips typographus_ in _Picea abies_, Sept. 1973, J. Reid UAMH 9778.

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

**Inodoromyces** C. Mayers & T.C. Harr., _gen. nov._ — MycoBank MB828237

_Etymology_. Masculine. After the Latin inodorus (adjective, ‘without scent’) and -omyces (‘fungus’).

_Type species_. Inodoromyces interjectus.

_Conidia_ terminal, chained or clustered, from phialidic conidiophores on simple hyphae. Phylogenetically classified in the _Microascales_. Sexual morph unknown.

_Notes_. — Inodoromyces lacks the odour and pigmentation characteristic of much of the _Ceratocystidaceae_, to which it forms
an incertae sedis sister group (Fig. 7), but it shares phialidic conidiogeny with the Ceratocystidaceae. The genus is based on only one isolate, but its unique phylogenetic position between the Ceratocystidaceae and the Gondwanamycetaceae (e.g., *Knochaviasia*) merits its description. Although our phylogenetic analyses did not include the mycophilic Microascales genus *Cornuvesica*, which is also family incertae sedis and related (on a long branch) to both the Ceratocystidaceae and Gondwanamycetaceae (Marincowitz et al. 2015), *Cornuvesica* spp. cannot grow independently and the 18S sequence of *I. interjectus* C3547 differed significantly from that of *Cornuvesica falcata* (1545/1661 bases, 93 % match with AY271797) (Hausner & Reid 2004).

**Inodoromyces interjectus** C. Mayers & T.C. Harr., sp. nov. — MycoBank MB828238; Fig. 8

**Etymology.** Based on the Latin interjectio (noun, alternative form of interjecto, ‘interjection’), after the terminal pairs of conidia in early conidiophores (Fig. 8c–d) that often resemble an exclamation mark (!).

**Typus.** USA, Michigan, Montcalm County, Alma College Ecological Tract, from gallery of *Elateroides lugubris* larvae in *Populus* sp., Dec. 2014, M. Bunce (BPI 910978 holotype, dried culture), culture ex-type CBS 142647 (= C3547).

Conidiophores hyaline, simple to many-branched, scattered across surface and embedded in media, bearing terminal conidia from deep phialides in chains, forming clusters. *Conidia* hyaline, 3–12(–30) × 1.5–4.5 µm, globose becoming oblong (rarely bacciliform) with increasing size, often with one or two conspicuous polar vacuoles, rarely septate. *Gallery growth* sparse tufts of conidiophores, similar to those in culture.

**Cultural characteristics — Colonies** on MYEA 30–48 mm diam after 4 d at 25 °C, surface growth superficial, hyaline, margin dendroid, irregularly branched, submersed, extending further than surface, lacking odour, reverse hyaline.

Notes — We interpret *I. interjectus* as a transient contaminant rather than a symbiont of the ship timber beetle *E. lugubris*. We also isolated an *Alloascoidea* sp. (CBS 142651) with a 28S sequence (MK025991) similar to *A. hylecoeti* (563/589 bp, 96 %, match with NG_055394; Kurtzman & Robnett 2013) that dominated growth in the gallery and is the presumptive mycangial symbiont of *E. lugubris* (Batra & Francke-Großmann 1961, 1964, Kurtzman & Robnett 2013).

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**Table 2** Comparison of divergence date estimates generated for selected fungal clades and available divergence date estimates for the beetle clades with large mycangia.

| Clade                  | Beetle divergences | Fungal Analysis A | Fungal Analysis B |
|------------------------|--------------------|-------------------|-------------------|
| Ceratocystidaceae      |                    | 80.3 (53.7, 115.5) | 103.9             |
| Ambrosia common ancestor (node 1) |            | 62.4 (41.1, 91.3)  | 51.8              |
| **Scolytoplatypodini** (pronotal disk mycangium) |                  |                   |                   |
| Mereditheilla/Toshionella/Ambrosiella (node 2) | 53.8 (48.7, 61.2)  | 46.7 (30.0, 69.6)  | 46.4              |
| **Scolytoplatypus** (split of African/Asian setal types) |                   |                   |                   |
| Wolfgangiella/Philophoropsis (node 3) | 41.1 (36.3, 50.7)  | 31.2 (16.2, 52.4)  | 19.6              |
| Toshionella/Ambrosiella (node 4) |                  | 30.5 (18.1, 47.4)  | 34.1              |
| Ambrosiella including A. remansi (node 5) |                  | 18.1 (8.7, 29.6)   | 4.8               |
| **Trice Xyleborini** |                    |                   |                   |
| Ambrosiella excluding A. remansi |                  | 12 (5.1, 21.2)     | 2.4               |
| **Corthylus** (prothoracic coil mycangium) |                  | 16.4 (5.6, 29.7)   | 19.4              |
| Mereditheilla |                   | 16.6 (7.3, 30.7)   |                   |
| **Trypodendron** (prothoracic pleural mycangium) |                | 11.6 (9.6, 13.3)   |                   |
| Philophoropsis |                   | 5 (1.9, 10.3)      | < 0.04             |

1. Estimates from 29-partition analysis of Pistone et al. 2018, except *Corthylus* from Gholt et al. 2017, median crown age (low 95 % HPD, high 95 % HPD).
2. Analysis A: Six-locus BEAST analysis; median crown age (low 95 % HPD, high 95 % HPD).
3. Analysis B: 18S fixed rate analysis; crown age at 1.25 %/100 Ma.
4. Estimate is for entire tribe Xyleborini; the *Xylosandrus* complex (mesonotal mycangium origin) would be slightly younger.

**DIVERGENCE DATE COMPARISONS**

The topology of the Ceratocystidaceae in the time-calibrated tree produced by BEAST (Analysis A, Fig. S4) was identical to that of the MBayes tree (Fig. 7). Median age estimates and 95 % HPD ranges were produced for all nodes in the tree using secondary fossil dates (Fig. S4, Table 2). Most of the estimates produced by Analysis B (fixed molecular clock based on 18S sequences at 1.25 % substitutions per 100 Ma) fell within the 95 % HPD ranges of Analysis A, with the exception of crown ages of genera, where 18S rDNA variation was very limited. Analysis B gave an older estimate for the crown of the family Ceratocystidaceae (Table 2), but otherwise Analysis B estimates were younger or approximated those in Analysis A, and the chronological order of the node dates were similar in the two analyses. The estimated crown age for the Ceratocystidaceae at 80.3 Ma using Analysis A was somewhat older than the estimate of 61.9 Ma by Van der Nest et al. (2015), who used a similar secondary analysis and calibration points but many fewer taxa.

The clade accommodating all ambrosia fungi in the family (node 1, Fig. 9) had an estimated crown age of 62.4 Ma, older than the
estimate for the origin of the oldest large mycangium (i.e., the pronotal disk mycangium of the *Scolytoplatypodini*) at 52 Ma (Pistone et al. 2018), though the 95 % HPD ranges overlapped (Table 2). The estimated crown age for the clade containing all ambrosia fungi with aleuroconidia, that is, *Ambrosiella, Meredithiella*, and *Toshionella* (node 2, Fig. 9), was 46.7 Ma, which is somewhat younger than the Pistone et al. (2018) estimated age for the pronotal disk mycangium (*Scolytoplatypodini*) but is much older than the estimated ages for the mesonotal (*Xylosandrus* complex) and prothoracic coil (*Corythina*) mycangia. The estimated crown age for the *Wolgangiella* and *Phialophoropsis* split (node 3, Fig. 9) at 31.2 Ma is somewhat younger than the estimated African-Asian *Scolytoplatypus* split at 41.1 Ma but much older than the estimated crown age for *Trypodendron* at 11.6 Ma, allowing for the possibility that an ancestral African *Scolytoplatypus* symbiont was horizontally transferred to *Trypodendron*. The estimate for *Phialophoropsis* (5 Ma) postdates the origin of the *Trypodendron* mycangium (11.6 Ma). Likewise, the crown age for the *Toshionella/Ambrosiella* clade (node 4, Fig. 9) at 30.5 Ma allows for an origin of the clade following the African-Asian *Scolytoplatypus* split and horizontal transfer of *Ambrosiella* to the *Xylosandrus* complex. The estimated crown age of *Ambrosiella* including the *Remansus* symbiont (*A. remansi*) (node 5, Fig. 9), is 18.1 Ma, which is older than the estimated origin of the entire tribe *Xyleborini* (including the mesonotum mycangium clade) at 14.3 (Pistone et al. 2018), but the crown of *Ambrosiella* minus *A. remansi* (i.e., the mesonotum mycangium symbionts) is younger at 12 Ma. The estimated crown age of *Meredithiella* (16.6 Ma) closely approximately the purported origin of the prothoracic coil mycangium of *Corythys* (16.4 Ma according to Gohli et al. 2017), which would be consistent with the hypothesis that this was a third origin of ambrosia symbiosis in the *Ceratocystidaceae* (Mayers et al. 2015, 2018).

**ANCESTRAL STATE RECONSTRUCTION**

Phylogenetic analyses (Fig. 7) and divergence date comparisons between the fungal symbionts and their respective mycangia (Fig. 9) suggested three evolutionary events leading to ambrosia symbiosis, resulting in three ambrosial clades: *Wolgangiella*/*Phialophoropsis*, *Toshionella/Ambrosiella*, and *Meredithiella*. Ancestral state reconstruction further indicated that each of the three groups were likely derived from non-ambrosial ancestors (Fig. 9). For the common ancestor of *Ambrosiella, Toshionella*, and *Meredithiella* (node 2, Fig. 9), there was similar support for an ambrosial lifestyle vs a non-ambrosial lifestyle, but the former would require the ambrosial lifestyle to have been reversed at least twice (for *C. adiposa* and *S. norvegica*).

**DISCUSSION**

This study represents the most comprehensive molecular phylogeny of ambrosia fungi in the *Ceratocystidaceae* and the first to compare the evolutionary history of these fungi to the evolutionary history of their associated mycangia. It is also the
first to fully characterise the mycangia and mycangial symbionts of the ambrosia beetle tribe Scolytoplatypodini, which appear central to the origin of ambrosia beetle symbiosis in the Ceratocystidaeae. Two novel genera were needed to accommodate the symbionts of Scolytoplatypus, raising the number of symbiotic genera in the Ceratocystidaeae to five: Ambrosiella, Meredithiella, Phialophoropsis, Toshionella, and Wolfgangiella. With the exception of A. remansi, each genus is strictly associated with a single mycangium type, but there appears to be only limited species–level coevolution within fungal genera. We further propose that the Scolytoplatypodini must have domesticated Ceratocystidaeae symbionts at least twice, that the Xylosandrus complex horizontally acquired their ancestral Ambrosiella cultivar from the Scolytoplatypodini, and that Meredithiella was separately domesticated by Corthylus. The origin of Phialophoropsis cultivation by Trypodendron remains unclear.

The form and evolution of Scolytoplatypodini mycangia

The Scolytoplatypodini mycangium was first illustrated by Berger & Cholodkovsky (1916), was hypothesized to carry fungal spores by Nonberg (1951), and described in detail by Schedl (1962). The mycangium is a large, disk-shaped cavity in the prothorax, lined with internal setae that help guide spores through a small conspicuous pore on the dorsal pronotum. The mycangium is surrounded with abundant gland cells whose secretions support the growth of fungal propagules inside the cavity (Schedl 1962, Beaver & Gebhardt 2006). Gross mycangium morphology of the three lineages in the tribe Scolytoplatypodini is similar, but we confirm the observation of Schedl (1962) that the interior setae of the mycangia are dramatically different in African and Asian Scolytoplatypus. Interestingly, the mycangium of R. mutabilis had a third form of setae socketed terminally on cylindrical pedestals, as well as reticulated finger-like cones similar to the reticulated cones of Asian Scolytoplatypus but lacking terminal setae. In both Remansus and Asian Scolytoplatypus the reticulated cones may contain active gland cells because the area between the reticulations stained deeply with cotton blue (implying living cytoplasm) and Berger & Cholodkovsky (1916) illustrated nucleated cells between the reticulations. Alternatively, the cones might serve as channels for glandular secretions from gland cells outside the mycangium wall (Schedl 1962). In either case, the cones may help secreted nutrients penetrate into the dense mass of fungal propagules in the mycangium. The cones may also fill space in the mycangium so that a smaller fungal mass is required to squeeze inoculum from the dorsal pore, which is accomplished by a simple movement of the beetle’s head and thorax muscles (Schedl 1962). African Scolytoplatypus lack the cones and would appear to have the more primitive mycangium type.

The similarity of the highly developed reticulated mycangium walls and cones in Remansus and Asian Scolytoplatypus, and the close phylogenetic relationship of their symbionts (Ambrosiella and Toshionella), could be explained by the two beetle lineages forming a group sister to a more primitive African Scolytoplatypus, as implied by Schedl (1975). The mycangium wall of the Asian S. daimio was reported by Schedl (1962) to be unreticulated and composed of rigid plates, similar to the sclerotized walls of the African species, and reticulations may be a more advanced character in some but not all Asian species. However, phylogenetic analyses (Jordal 2013, 2018, Gehli et al. 2017, Pistone et al. 2018) place Remansus sister to a monophyletic Scolytoplatypus. Under this assumption, African Scolytoplatypus and Remansus either:

1. independently and convergently evolved the complex reticulations of their mycangium walls and the reticulated cones; or

2. the African lineage of Scolytoplatypus underwent a reduction in mycangium complexity and lost its reticulated cone structures.

Further complicating the matter, Toshionella and Ambrosiella, the symbionts of the Scolytoplatypodini with reticulated cones, appear to be superior ambrosia fungi as they form lush ambrosia growth with large, lipid-filled aleurioconidia and disarticulating conidiophore cells, believed to be advantageous for insect grazing (Nakashima et al. 1992, Harrington et al. 2014, Meyers et al. 2015, 2017). Wolfgangiella, the symbiont associated with the simpler African mycangium type, produces less luxuriant ambrosia with columns of relatively small propagules that apparently lack lipid bodies. The tribe Scolytoplatypodini likely had an Afro-tropical ancestral origin (Pistone et al. 2018), and only species in the African lineage of Scolytoplatypus are found in Africa today (Jordal et al. 2013, 2018). Asian Scolytoplatypus are strictly Asian and Remansus is known only from Madagascar, where the African lineage of Scolytoplatypus is also found. Interestingly, the African species of Scolytoplatypus that we studied in Madagascar were associated with the putatively primitive fungal symbiont, Wolfgangiella, rather than the Ambrosiella symbiont of the sympatric R. mutabilis. The absence of Remansus and Asian Scolytoplatypus from the putative ancestral origin in Africa, the apparently-derived nature of their mycangia, and their closely-related and advanced fungal symbionts would be best explained by these two lineages forming a sister group to a basal African Scolytoplatypus. However, this is in conflict with the phylogenetic analyses of the Scolytoplatypodini.

Mycangium morphology is often underappreciated in taxonomic treatments of ambrosia beetles. The mycangium setae in Scolytoplatypus are one of several morphological traits separating the Asian and African clades of the genus (Schedl 1975, Beaver & Gebhardt 2006, Jordal 2013) and help support a separate genus for the Asian clade (the African S. perminus is the type species of Scolytoplatypus). The subgenera Spongocerus and Taeniocerus were previously proposed for Japanese Scolytoplatypus spp. and are available, but they were not generally accepted (Wood 1983, Beaver & Gebhardt 2006). Beaver & Gebhardt (2006) list seven Asian Scolytoplatypus species that reportedly do not have pronotal mycangium openings in females, and Knížek (2008) described an eighth species. Bright (1994) noted that in one of these species (S. reticulatus) the females instead have a smooth, circular spot in place of the mycangium opening. These species are unusual and should be examined further to determine if they represent a true loss of mycangia – which would be novel in ambrosia beetles except for possible analogous cases in a Camptocerus subclade that reverted to phloem feeding (Smith 2013) and the mycokleptic Diuncus of the Xylosandrus complex (Hulcr & Cognato 2010, Skelton et al. 2019) – and if they still obligately feed on fungi through alternative strategies, e.g., as mycokleptics. Otherwise, both the ambrosia beetle lineages and their fungal symbionts appear to be on irreversible evolutionary tracts.

Rejection of a single ancestral ambrosia fungus

Previous phylogenetic analyses showed that the ambrosial genera are interspersed with the non-ambrosial genera Huntuella, Bretziella fagacearum, Catunica adiposa, and Solatoca norvegica (De Beer et al. 2014, 2017, Meyers et al. 2015, 2018), and these anomalous placements are more strongly supported in this study. These taxa include wound colonizers (Huntuella), plant pathogens (B. fagacearum and C. adiposa), and a bark beetle associate with an unknown ecological role (S. norvegica) (Reid et al. 2010, De Beer et al. 2014). The five ambrosial genera share derived and apparently convergent traits conducive to their role as obligate ambrosia beetle symbionts, including
dimorphic (mycangial and mycelial) forms, easily-grazed conidiophores, dense and pure ambrosia growth in galleries, and (in Ambrosiella, Meredithiella, and Toshionella) large, lipid-filled aleuroconidia. The non-ambrosial taxa of the Ceratocystidaceae with known sexual morphs form long-necked perithecia with sticky ascospore masses at the top to facilitate dispersal on insect exoskeletons (Cain & Weresub 1957, Malloch & Blackwell 1993, Harrington 2008). Surprisingly, the ascocoma of _A. cleistomnuta_ (Mayers et al. 2017), _A. xylebori_, and _W. africana_ are necklace (cleistothecia) and lack sticky ascospore drops. Similar immature ascocoma in _Meredithiella_ were thought to be unfertilized cleistothecia (Mayers et al. 2018). The sexual morph of these ambrosia fungi may be more important in fostering genetic diversity than in dispersal on the exoskeleton, and the ascospores may be eaten and pass through the gut (Mayers et al. 2017). Rather than a cleistothecious ancestor and four reversions from cleistothecia back to necked perithecia, it seems more likely that the ancestors of these ambrosial genera had long-necked perithecia and that necklace ascocoma arose concomitantly in the absence of evolutionary pressure on cuticular dispersal of ascospores.

If the common ancestor of the five ambrosial genera was itself an ambrosia fungus, then the non-ambrosial taxon would need to represent multiple reversions from derived ambrosia beetle mutualists back to free-living species, which has been considered highly unlikely in fungi domesticated for insect agriculture. Possible reversions are not apparent in the ambrosial genus _Raffaelea_, which is polyphyletic and appears to be the result of multiple domestication events (Vanderpool et al. 2018). Escape from symbiosis has not been identified in the cultivars of fungus-farming termites that are specialized to their symbiosis (Aanen et al. 2002, Nobre et al. 2011). Escape from symbiosis may have occurred in some ‘lower-attine’ cultivars that have no significant adaptation for cultivation and are able to live freely (Vo et al. 2009), but reversions are not seen in the truly domesticated ‘higher-attine’ cultivars (Schultz & Brady 2008, Mikheyev et al. 2010, Mueller et al. 2018). Cryptic sexual morphs could allow cultivars to more easily escape symbiosis (Mueller 2002, Aanen 2006, Mikheyev et al. 2007, 2010), but in contrast to the widely wind-dispersed basidiospores of the agaric symbionts of both _Ambrosiella_ and _Wolfgangiella_ have similar condiophores and their mesonotal mycangia (Hulcr & Remaneus) are confined to tunnels in wood that are not conducive to independent dispersal.

There are significant morphological differences among the ambrosial genera, most notably the presence of aleuroconidia and the thick ambrosia growth of _Toshionella, Ambrosiella_, and _Meredithiella_. In contrast, _Phialophoropsis_ and _Wolfgangiella_ lack aleuroconidia (as does their nearest neighbour, the non-ambrosial _B. fagacearum_) and have sparser ambrosia growth. _Phialophoropsis_ produces relatively unremarkable but quickly-replenished phialoconidia, and _Wolfgangiella_ only produces fragmenting arthroconidia, neither of which was observed to have the conspicuous lipid bodies of the genera with aleuroconidia. The ascospores of _Ambrosiella_ and _Wolfgangiella_, despite being produced in cleistothecious ascocoma, differ significantly in size (those of _Wolfgangiella_ are dramatically larger) and shape, further supporting convergent adaptations to neckless cleistothecia. Ancestral state reconstruction also supported a non-ambrosial ancestor for the three clades of ambrosial fungi.

Accepting the estimated divergence dates produced in our analysis, the crown age of tribe _Scolytoplatypodini_ (52 Ma by Pistone et al. 2018) is too young to accommodate a single ambrosial common ancestor of both _Wolfgangiella_ and _Toshionella_ at 62.4 Ma. The ancestral mycangial symbiont of tribe _Scolytoplatypodini_ is unclear, and the estimated splits of _Wolfgangiella_ and _Phialophoropsis_ (node 3, Fig. 9) at 31.2 Ma and of _Toshionella_ and _Ambrosiella_ (node 4, Fig. 9) at 30.5 Ma both follow the split of the African and Asian clades of _Scolytoplatypus_ at 41.1 Ma (Pistone et al. 2018). Either a _Toshionella_-like or a _Wolfgangiella_-like symbiont could have been the _Scolyto_ platypus symbiont before the African-Asian split. Regardless, the data at hand suggest that the first Ceratocystidaceae farmer was an ancestral _Scolytoplatypodini_.

Conclusions could change if _Platypodinae_ ambrosia beetles, a lineage of ambrosia beetles outside of the _Scolytinae_ (Mugy et al. 2018), were found to associate with _Ceratocystidaceae_ symbionts. The entirely-ambrosial _Platypodinae_ may have begun fungus farming at 86 Ma or earlier, long before the fungus-farming ambrosia beetle lineages in _Scolytinae_ (Vanderpool et al. 2018). The early _Platypodinae_ symbionts probably gave rise to the _Raffaelea_ cultivars that dominate the associations with ambrosial _Scolytinae_ (Vanderpool et al. 2018). The studied _Platypodinae_ have _Raffaelea_ as their primary symbionts (Harrington et al. 2010, Li et al. 2018a), but the thick, white ambrosia growth and black rind in galleries of the platypone _Cros-sotarsus niponicus_ (Nakashima et al. 1987) are notably similar to the ambrosia growth of _Ambrosiella_ and _Meredithiella_ (Mayers et al. 2015, 2017, 2018). The mycangium of _C. niponicus_ is uniquely complex compared to other _Platypodinae_ (Nakashima 1975, 1979), and its primary symbiont remains unclear; the yeasts, mucoromycete fungi and molds implicated as its ambrosia symbionts (Nakashima 1971, Nakashima et al. 1987, 1992) may have been misattributed. Regardless, even an older _Ceratocystidaceae_ symbiont of _C. niponicus_ would not resolve the phylogenetic and morphological conflicts supporting a single domestication in the family _Ceratocystidaceae_.

A single domestication for _Ambrosiella_ and _Toshionella_ _Toshionella_ and _Ambrosiella_ have similar condiophores and aleuroconidia with large lipid bodies, and they form a monophyletic group of ambrosia fungi with a crown age of 30.5 Ma (node 4, Fig. 9). This follows the split of Asian and African _Scolyto_ platypus at 41.1 Ma (Pistone et al. 2018), i.e., the origin of the Asian _Scolytoplatypus_ mycangium, which may or may not have harbored a _Toshionella_-like ancestor at that time. _Toshionella_ are carried by all studied Asian _Scolytoplatypus_, but the related _Remansus_ is found only in Madagascar (Jordal 2013) and _R. mutabilis_ was associated with an _Ambrosiella_ symbiont rather than a species of _Toshionella_. _Ambrosiella_ have otherwise been associated only with members of the _Xylosandrus_ complex, an early-diverging lineage of tribe _Xyleborini_ (Johnson et al. 2018), a tribe with a crown age of only 14.3 Ma in the estimate of Pistone et al. (2018). We estimated an older _Ambrosiella_ crown age at 18.1 Ma with the _R. mutabilis_ symbiont (_A. remansis_) included, whereas the clade of _Ambrosiella_ associated with the _Xylosandrus_ complex had a younger crown age of 12 Ma. A single transfer of an _Ambrosiella_ ancestor from tribe _Scolytoplatypodini_ (e.g., _Remansus_) to the _Xyleborini_ may have prompted the diversification of the monophyletic _Xylosandrus_ complex and their mesonotal mycangia (Hulcr & Cognato 2010, Hulcr & Stelinski 2017, Johnson et al. 2018, Skelton et al. 2019), as well as their _Ambrosiella_ symbionts (Mayers et al. 2015, 2017, Lin et al. 2017). However, we are severely limited by sequence analysis of the mycangial contents of just a single specimen of _Remansus_, and it is just as likely that _Remansus_ obtained its symbiont from the _Xyleborini_, which has been proposed to be Afro-Asian in origin and is now distributed worldwide (Cognato et al. 2011). The evolutionary history of _Toshionella_ and _Ambrosiella_ remains unclear, but it is reasonable to speculate that they were derived from a single ancestral domestication by _Remansus, Scolytoplatypus_, or an ancestral _Scolytoplatypodini_. Study of the mycangia and sym-
bionts of additional species of Remansus (Jordal 2013) and Central and South Asian Scolytoplatypus (Beaver & Gebhardt 2006, Mandelshtam & Petrov 2010) may prove enlightening, and it may become apparent that Ambrosiella and Tashionella are part of a continuous spectrum of symbionts rather than two clearly-distinct genera.

A separate origin for Meredithiella

Despite similar aleuroconidia and fairly close phylogenetic placement, Meredithiella may not share a common ambrosial ancestor with Ambrosiella and Tashionella. The clade accommodating these three fungal genera also contains the non-ambrosial taxa C. adiposa and S. norvegica. A single domestication by the Scolytoplatypodini followed by horizontal transfer is problematic because Meredithiella are only associated with Corthylus (Mayers et al. 2018), a strictly New World genus not thought to have been sympatric with the Old World Scolytoplatypodini (Pistone et al. 2018). The estimated divergence of Meredithiella and Ambrosiella at 46.7 Ma is post-Gondwanan (De Oliveira et al. 2009) and long after the divergence of New World Corthylus and Old World Scolytoplatypodini at ~ 100 Ma (Pistone et al. 2018). The estimated crown ages of Meredithiella and Corthylus are quite similar, however, and an independent domestication of Meredithiella by Corthylus (Mayers et al. 2015) at ~ 16 Ma in the New World is a reasonable hypothesis.

Unstudied genera of Corthalyna could help support or refute the exclusive association of Corthylus with Meredithiella. Amphicranus may be sister to Corthylus (Pistone et al. 2018), but its mycangia and symbionts are unknown. Microcorthylus have mycangia similar to those of Corthylus (Schedi 1962) and have been associated with Geosmithia symbionts (Kolak & Kirkendall 2010), but mycangial symbionts of Microcorthylus have not been confirmed. The well-studied Corthalyna genera Gnathotrichus and Monarchrum (Gohli et al. 2017, Johnson et al. 2018) have Raffaelea mycangial symbionts (Harrington et al. 2010) and small procoxal mycangia (Francke-Gromann 1967) that may represent precursors to the Corthylus mycangium. Perhaps the domestication of Meredithiella replaced a Raffaelea cultivar and led to the development of the larger prothoracic coi mycangium of Corthylus.

The ambiguous domestications of Wolfgangiella and Phialophoropsis

Wolfgangiella and Phialophoropsis lack aleuroconidia and form a monophyletic ambrosial clade that is distinctly related to other ambrosia beetle symbionts in the family. Wolfgangiella and Phialophoropsis are also distantly related to one another and to their closest relative, Bresciella. The three genera have no known close relatives and share few common morphological characters, other than the absence of aleuroconidia. The crown age of Phialophoropsis and Wolfgangiella (node 3, Fig. 9) was estimated at 31.2 Ma in our analysis. One possibility is that Wolfgangiella and Phialophoropsis are derived from a single domestication, perhaps by African Scolytoplatypus after its split from Asian Scolytoplatypus at 41.1 Ma (Pistone et al. 2018), which was followed by horizontal transfer to an ancestor of Trypodendron (crown age 11.6 Ma, Pistone et al. 2018) in Asia. Genetic diversity in the studied species of Wolfgangiella and Phialophoropsis is very low, resulting in very young crown age estimates for both genera, but the ITS sequences recovered from mycangia of central African S. congonis suggest more diversity (and an older crown age) in Wolfgangiella than was found in our multi-gene analyses. Central and Northern African Scolytoplatypus (Brown 1971), especially the phylogenetically distinct S. unipilus (Jordal 2018), may yield even more divergent symbionts. Trypodendron and Phialophoropsis have estimated divergence dates at 11.6 Ma and 5 Ma, respectively, which could be consistent with horizontal transfer from African Scolytoplatypus or a separate, novel domestication. However, Trypodendron is only one of the three ambrosia beetle genera that make up tribe Xyloterini, and the crown age for the Xyloterini is much older at 50.4 Ma, nearly as old as the estimated crown age of tribe Scolytoplatypodini at 53.8 Ma (Pistone et al. 2018). The ambrosia symbionts of the other two Xyloterini genera (Xyloterinius and Indocyphalus) need further study. The distinct mycangia and symbionts of Xyloterinius (Abrahamson & Norris 1966, 1969) suggest a much different symbiosis, and the distinct mycangium openings of Indocyphalus (Cognato et al. 2015) suggest that its mycangia may differ from those of Trypodendron, as may its unstudied ambrosia fungi.

Evolution between Ceratocystidaceae and large mycangia

Despite a wide variety of ubiquitous saprophytes and superficial associates available for domestication, the scolytid ambrosia beetles with large mycangia are only known to be tightly associated with ambrosia fungi in a single family, Ceratocystidaceae. These cultivars appear to be derived from at least three novel domestications, despite the ubiquitous presence of earlier-domesticated cultivars of Raffaelea s.l., which have apparently undergone host switching between beetle species, genera, tribes, and subfamilies for more than 80 million years (Vanderpool et al. 2018). Ceratocystidaceae ambrosia fungi must provide some superior benefit to their farmers, such as luxuriant ambrosia growth, grazing efficiency, an abundance of specific nutrients, or more effective exclusion of weed fungi. It is noteworthy that the conidiophores and conidia of many of the ambrosia symbionts of the Ceratocystidaceae contain an abundance of lipid bodies, and fats (especially sterols such as ergosterol that the insects cannot produce) have been implied as an important nutrient source for bark and ambrosia beetles (Norris et al. 1969, Kok 1979, Six 2003, Bents & Six 2006). Presumably, the Ceratocystidaceae are rich in some beneficial nutrient (Huang et al. 2019) or have another feature that has allowed for multiple mycangium-specific associations over the more common Raffaelea spp., or perhaps they are better able to select against or compete with deleterious weed fungi (Castillo et al. 2016, Ranger et al. 2018).

There must also be a reason that mycangia that carry Ceratocystidaceae convergently evolved to be relatively large in relation to the beetles’ size and have complex forms such as winding tubes or folded pouches with reticulated glandular walls. Raffaelea generally freely associate with different types of small mycangia in unrelated ambrosia beetle species, and multiple Raffaelea spp. can co-occur within the mycangia of a single adult beetle (Harrington & Fraedrich 2010, Harrington et al. 2010, 2011, 2014, Li et al. 2018a, Skelton et al. 2018), whereas large mycangia harbour pure cultures of a specific lineage of Ceratocystidaceae. These mycangia are ‘large’ and ‘complex’, but these aspects alone cannot explain how they selectively maintain strict symbioses with a specific genus of ambrosia fungus. Differential selection of specific Ambrosiella cultivars has been demonstrated to occur in mesonotal mycangia (Skelton et al. 2019), and an abundance of gland cells often lines the walls of large mycangia. Specificity is unlikely to be strictly through the action of antifungal or antibacterial secretions because the compounds would have to be exceedingly broad in their targets and potent in their effect while leaving the mycangial symbiont unharmed. It is more likely that some specific nutrient produced by these mycangia is uniquely or preferentially metabolised by Ceratocystidaceae, perhaps an unusual amino acid or oil. Trypodendron mycangia were shown to be sparsely filled and to carry propagules of weed fungi in the off season, but they were filled to capacity with homogenous masses of fungal propagules when mycangial glands enlarged.
and activated (Schneider & Rudinsky 1969). Schneider (1975) observed that in Trypodendron and other ambrosia beetles, mycangium gland cells swelled and became activated when fungal propagules entered the mycangium. In response to their secretions, the fungus switched to a mycangial morph and grew and divided to fill the mycangium, which eventually overflowed with fungal propagules. When the beetles were tending the garden or in diapause, the glands became inactive and fungal growth stopped. Some component of the mycangial secretions most likely provides a competitive edge for the growth of Ceratocystidaceae, filling the mycangium with their propagules and pushing other fungi out of the mycangium.

Mycangial wall reticulations may be important in reinforcing delicate layers of gland cells, such as those in Asian Sclerotytoplatypus, Remansus, Trypodendron (Francke-Grosman 1956, Schneider & Rudinsky 1969), Cortylus (Giese 1967, Mayers et al. 2018), and at least Cnestus in the Xylosandrus complex (Stone et al. 2007). However, African Sclerotytoplatypus mycangia do not have reticulated walls (Schedi 1962). We have also not observed reticulations in the mesonotal mycangia of Xylosandrus (Harrington et al. 2014, Mayers et al. 2015) or Anisandrus (Mayers et al. 2017), which have a dense concentration of gland cells situated on the part of the scutellum that is contiguous with the mycangium wall (Francke-Grosman 1963, 1967); in these beetles, the rest of the mycangium wall is thin, transparent, and flexible, and it stretches as the mycangium lobes enlarge with fungal growth (Li et al. 2018b).

Reciprocal co-adaptation and diffuse co-evolution

Fungus farming represents a major evolutionary feature in the Scolytinae (Gohl et al. 2017), and mycangia play an essential role in the upkeep, survival, and transmission of vital symbiotic fungi, which are themselves likely under appreciable evolutionary pressure. Mycangia (especially the large organs that carry Ceratocystidaceae) and their secretions represent a significant energy investment by the beetle for the long-term maintenance of their domesticated fungal cultivars. Especially in the haplo-diploid Xyleborini (Van de Peppel et al. 2018), clonal fungal cultivars appear to be maintained by nearly-exclusive vertical transfer, with some caveats (Skelton et al. 2019). With such obligate mutualism and evolutionary feedback between mycangium secretions and fungal growth, a strict species-level co-evolution might be expected (Mayers et al. 2015). However, patterns of ambrosia beetle symbiosis in general, and with the Ceratocystidaceae in particular, appear to be more diffuse. With the exception of Remansus, each Ceratocystidaceae genus is constrained to a specific mycangium type, yet sympatric beetle species with the same mycangium type sometimes carry the same fungal species, as has been demonstrated here in Woffgangiella and Toshionella and previously in Ambrosiella (Lin et al. 2017, Skelton et al. 2019), Meredithiella (Mayers et al. 2018), and Phialophorophytes (Lehenberger et al. 2019). There may be gradients of specificity (i.e., coevolution) and of host-symbiont phylogenetic congruence within these ambrosial clades, as in the well-studied Ambrosiella, where host switching is only clearly evident in the basal A. beaveri complex and in A. catenulata (Lin et al. 2017, Skelton et al. 2019). The secretions of each ambrosia beetle’s mycangium may preferentially support growth of its co-evolved fungus but still allow the growth of closely-related species in the absence of competition from the co-evolved symbiont (Skelton et al. 2019).

Reciprocal co-adaptation with diffuse co-evolution has been seen in the other fungus-farming insect systems (Nobre et al. 2011, Mueller et al. 2018). Obligate fungus-farming originated in attine ants about 55–65 Ma (Branstetter et al. 2017), and they farm five lineages of fungi in three separate clades of Agaricales (Mueller et al. 2018). Many of the attine ants are farmers of ‘lower-attine’ cultivars that are closely related and may themselves be free-living fungi (Mueller et al. 2018), but four ant genera (the higher-attines) in a monophyletic sublineage derived from the lower-attine ants farm two sister clades (‘Clade-A’ and ‘Clade-B’). These two clades are truly domesticated ‘higher-attine’ fungi and produce ‘gongylidia’ structures, which are special adaptations for ant grazing that arose an estimated 22–25 Ma (Branstetter et al. 2017, Mueller et al. 2018). Higher-attine ants may farm both higher- and lower-attine cultivars in the same nest (Mueller et al. 2018). The apparent superiority of gongylidia-associated cultivars over lower-attine symbionts (Mueller et al. 2018) is similar to the superiority of domesticated ambrosia fungal cultivars that produce ambrosia growth (e.g., Ambrosiella and Raffaelea) over undomesticated bark and ambrosia beetle symbionts (Batra 1985, Harrington 2005). Fungus-farming originated in termites about 31 Ma (Roberts et al. 2016), and they farm a single fungal genus, Termitomyces (Agaricales) (Aanen et al. 2002, Nobre et al. 2010, 2011). Similar to the large mycangium-Ceratocystidaceae associations, there is broad but strict lineage-level specificity between termites and their cultivars, but closely-related termite species commonly trade closely-related fungal cultivars (Nobre et al. 2011).

The reciprocal co-adaptation of mycangia and ambrosia fungi, and their irreversible life-styles, presents an intriguing model system for the evolution of obligate mutualisms. Recent studies suggest that patterns of interdependence, specificity, and co-adaptation vary greatly across ambrosia beetle lineages and their fungi, and these patterns may correspond with the diversity of mycangia. Each ambrosia beetle lineage associated with the Ceratocystidaceae appears to have originated with a new mycangium and domestication or horizontal transfer of a fungal symbiont. The mycangia and fungi have presumably adapted in response to one another over their shared evolutionary histories, though species-level co-evolution is not readily apparent. In the near future, genomic investigations are likely to provide answers to deeper questions, perhaps concerning metabolic adaptations or losses, host-cultivar signalling, and whether or not there are other molecular signatures of coevolution, on both sides of the symbiosis. Regardless, there remains a uniquely strong fidelity between each type of large and complex mycangium and the genus of its Ceratocystidaceae partners.

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conidia; c–e. basipetal aleurioconidia with membranous sheaths (a–b. isolate C871 (CBS 600.74) from Japan; c. isolate C906 (CBS 138.34) from The Netherlands; d–e. isolate C299 from USA). — Scale bars = 10 µm.

**Fig. S2** HCIO 3531, designated as lectotype for *Catunica adiposa* (= *Sphaeronomema adiposum*). a. Specimen label, which reads: ‘*Sphaeronomema adiposum Butl.*,’ ‘on Saccharum officinarum,’ ‘Seeraha, Champaran, Bihar,’ ‘Oct. 30th 1903,’ and ‘E.J. Butler’; b. *Saccharum officinarum* infected with *Catunica adiposa*; photographs courtesy of, and reproduced with permission from, Herbarium Cryptogamae Indiae Orientalis (HCIO), Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India.

**Fig. S3** Contents of specimens IMI 21355 (a) and IMI 21285 (b) of *Catunica adiposa* held by Royal Botanic Gardens Kew, the only known alternatives to HCIO 3531 to serve as type for the species. Nag Raj & Kendrick (1975) cited IMI 21355 as holotype for *C. adiposa* without explanation. IMI 21355 consists of dried cultures isolated from sugarcane in India on an uncertain date and prepared slides. This specimen was deposited to the Imperial Bureau of Mycology on 15 January 1927 by W. McRae, the then-Imperial Mycologist of India and a colleague of E.J. Butler’s. A letter included by McRae, which probably explained the specific origin of the material, could not be located (pers. comm., CABI and Royal Botanic Gardens, Kew). The available collection information for the specimen (ex sugarcane in Pusa) matches Butler’s original description, and presumably McRae was familiar with appropriate material to submit as representative of the species. Handwritten notes on envelopes and slides suggest that IMI 21355 was examined by Sartoris (1927), Hunt (1956), and Nag Raj & Kendrick (1975). IMI 21285 includes envelopes labelled ‘type’ and ‘ex-type’, but this specimen was not published as type material and was collected in England decades after the species description (pers. comm., CABI and Royal Botanic Gardens, Kew). Photographs courtesy of, and reproduced with permission from, CABI and Royal Botanic Gardens, Kew.

**Fig. S4** Time-calibrated tree produced by secondary-calibrated analysis in BEAST of the six-gene tree dataset (18S rDNA, 28S rDNA, *tef1*-α, *tub*, *mcm7*, and *rpl1*) with median node ages (Analysis A). Horizontal bars indicate 95 % Highest Posterior Density (HPD) ranges. Branch support values are Bayesian posterior probability from BEAST analysis. Scale in millions of years ago (Ma). *Aspergillus niger* was used as the outgroup.