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ORIGINAL PAPER

Ambrosiella beaveri, sp. nov., Associated with an exotic ambrosia beetle, *Xylosandrus mutilatus* (Coleoptera: Curculionidae, Scolytinae), in Mississippi, USA

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Abstract *Xylosandrus mutilatus* is an Asian ambrosia beetle that has recently established in Mississippi, Texas, Alabama, and possibly Florida, USA. We investigated the fungi associated with the mycangia (specialized fungus-transporting structures) of *X. mutilatus* in Mississippi. Mycangia consistently yielded an *Ambrosiella* sp. which was subsequently found to be closely related to, but distinct from, other *Ambrosiella* species affiliated with *Ceratocystis*. This *Ambrosiella* is described herein as *Ambrosiella beaveri* sp. nov. Also isolated were *Geosmithia lavendula*, *G. obscura,* and a yeast, *Candida homelintoma*. It is likely *Ambrosiella beaveri* was introduced along with the beetle into North America.

Keywords Ambrosia fungi · *Candida homelintoma* · *Geosmithia lavendula* · *Geosmithia obscura* · Scolytinae

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Introduction

Ambrosia beetles (Coleoptera: Curculionidae, Scolytinae) are cryptic insects that typically colonize the inner tissues of woody plants, and consequently, are easily transported from one region to another in logs and raw wood products (Oliver and Mannion 2001). The ability to be transported in wood has greatly facilitated their introduction into new areas where they can pose considerable threats to native forest ecosystems. Most ambrosia beetles are not tree-killers and primarily colonize stressed or recently killed plants. However, a number of species are aggressive, and some exhibit increased aggressiveness to trees and other plants in new areas of introduction (Weber 1978, 1982). For example, three *Xylosandrus* species exotic to North America, *X. germanus* (Blandford), *X. compactus* (Eichhoff), and *X. crassiusculus* (Motschulsky), all attack apparently healthy trees in areas where they have been introduced and cause considerable economic damage (Ngoan et al. 1976; Wood 1977; Weber 1982). Another exotic ambrosia beetle, *Xyleborus glabratus* (Eichoff), along with its symbiotic fungus, has recently been implicated in the death of large numbers of native red bay, *Persea borbonia* L. (Fraedrich et al. 2007).

Along with the introduction of exotic ambrosia beetles comes the added risk of introductions of exotic fungi. Ambrosia beetles are associated with complexes of fungi. The term ambrosia refers to the dense fungal gardens the beetles cultivate on the walls of their

galleries and upon which the beetles feed as an exclusive food source (Batra 1967; Beaver 1989; Kok et al. 1970). The gardens are composed mainly of a primary symbiotic fungus which is typically transported in the adult beetle's mycangia (specialized, highly specific exoskeletal structures) (Six 2003) and which supports growth of the beetle in the tree. In addition to the primary symbiont, a number of less consistently associated, often non-mycangial, secondary fungi are also associated with ambrosia beetles (Beaver 1989; Kok et al. 1970). The obligate nature of ambrosia beetle-fungus mutualisms suggests that when an exotic beetle has successfully established in a new location, its primary fungus has also established. Along with the primary fungus, secondary associates may also be introduced with a beetle and remain associated with it in its new habitat. It is also possible that new associations may form between native fungi and exotic beetles (Jacobs et al. 2004).

The fungi most commonly associated with ambrosia beetles occur in the anamorphic genera, *Ambrosiella* and *Raffaelea* (Batra 1967; Cassar and Blackwell 1996; Jones and Blackwell 1998). Molecular studies have revealed that *Ambrosiella* and *Raffaelea* are each polyphyletic and multiply derived out of both *Ophiostoma* (Ophiostomatales) and *Ceratocystis* (Microascales) (Spatafora and Blackwell 1994; Cassar and Blackwell 1996; Jones and Blackwell 1998; Rollins et al. 2001).

Xylosandrus mutilatus (Blandford) is an ambrosia beetle of Asian origin that has recently established in the United States in Mississippi, Texas, Alabama, and possibly Florida (FCAPSP 2003; Schiefer and Bright 2004; Haack 2006; Cognato et al. 2006; Stone and Nebeker 2007). In China, the beetle is a major pest on Chinese chestnut, *Castanea mollisima* Bl. (Tang 2000). In the US, the beetle attacks a wide range of plants including *Acer rubrum* L., *A. saccharum* Marsh., *A. palmatum* Thunb., *Ostrya virginiana* (Mill.) K. Koch., *Cornus florida* L., *Fagus grandifolia* Ehrh., *Liquidamber styraciflua* L., *Carya* spp., *Liriodendron tulipifera* L., *Melia azedarach* L., *Pinus taeda* L., *Prunus serotina* Ehrh., *Pr. americana* Marsh., *Ulmus alata* Michaux, and *Vitus rotundifolia* Michaux. (Stone et al. 2007). The economic impacts of this beetle in North America remain unknown. However, the beetle appears to favor stressed host plants such as those affected by herbicide spraying and prescribed burning (Stone et al. 2007).

Adult female *X. mutilatus* carry fungi in highly specific paired mesonotal mycangia (Kajimura and Hijii 1992, 1994; Stone et al. 2005). In Asia, the beetle has been reported to be associated with an *Ambrosiella* sp. (likely the primary symbiotic fungus), but also with *Paecilomyces* sp. and *Candida* sp. which grow abundantly in older galleries. Unfortunately, the fungi isolated from the beetle and its galleries in Asia were never fully described. In addition, while the distribution and host range of the beetle in the US has been the focus of several recent studies (FCAPSP 2003; Schiefer and Bright 2004; Haack 2006; Cognato et al. 2006; Stone and Nebeker 2007), the fungi introduced with the beetle into the United States remain uninvestigated. Our objective was to identify the fungi associated with the mycangia of *X. mutilatus* in Mississippi where the beetle is best established.

Materials and methods

Collection of insects and isolation of fungi

Adult female *X. mutilatus* were collected from vines of wild muscadine grapes (*V. rotundifolia*) located on a privately owned upland hardwood forest in Winston Co., Mississippi, USA. Collections were made from several vines on two dates, 18 July and 22 September, 2005.

Isolations of fungi made from beetles collected in July were conducted using a method modified from Woolfolk and Inglis (2004). Short sections of infested grape stems were cut from individual vines. Immediately following cutting, the sections were placed on ice in a sealed plastic bag, transported to the laboratory, and stored for 24 h at 5°C. Stems were split to expose galleries containing individual insects; five female beetles were located. Each beetle was submerged in 1% sodium hypochlorite solution containing 0.01% Tween 80 (decreases surface tension and thus allows more effective surface sterilization of insect cuticle) (Sigma Chemical Co, St. Louis, MO, USA) for 1 min, transferred to 1% sodium thiosulfate for 1 min to neutralize the sodium hypochlorite, and rinsed twice in sterile distilled water. The beetles were chilled in 50 mM phosphate buffer containing 0.01% Tween 80 and then dissected to obtain the mycangia. Each mycangium was placed in a 1.5 ml sterile

microcentrifuge tube containing buffer and homogenized using a micropestle. Mycangial homogenates were diluted in a tenfold dilution series in duplicate and aliquots of 100 µl from each dilution were then spread evenly onto Sabouraud's Dextrose Agar with yeast extract (SDAY) (65 g SDA and 10 g yeast extract in 1 l of distilled water) amended with 0.02 g/l cycloheximide (selective for Ophiostomatales). Plates were held at room temperature for 5 days. Colonies of fungi were taken from dilutions yielding 30–300 colonies per dish. Five randomly selected fungal colonies from the appropriate dilution for each beetle were then subcultured onto SDAY for storage until used in morphological assessments, PCR, and DNA sequencing.

For beetles collected in September, individuals were removed from grape stems in the same manner, except that in this collection, 12 female beetles were located, each from a separate gallery from different vines. Using methods modified from Weber and McPherson (1984) and Kajimura and Hijii (1992) developed specifically for isolating from mycangia, individual beetles were immediately rinsed in sterile distilled water and then each aseptically transferred to two drops of sterile distilled water in a sterile Petri dish. The intact mycangium was aseptically removed and transferred directly to dishes containing SDAY. In previous isolations, the use of cycloheximide in the growth medium may have inhibited some mycangial associates (Microascales). Therefore, in this set of isolations, cycloheximide was not added. Cultures were held at room temperature for 5 days. From these dishes, 14 fungal colonies were subcultured onto SDAY for growth and subsequent identification.

Cultures of isolates from this study have been deposited in the culture collection (DLS) of the senior author at The University of Montana, Missoula. MT, USA, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). Herbarium specimens representative of the novel taxon were deposited in the South African Collection of Fungi (PREM), National Collection of Fungi, Agricultural Research Council, Plant Protection Research Institute, Pretoria, South Africa.

Morphology

Isolates were grouped by cultural and microscopic characteristics (*Ambrosiella* Batra 1967; *Geosmithia* Pitt 1979; Kolařík et al. 2005). For each morphological grouping, up to five isolates were then selected for DNA sequencing (Table 1).

For description of the *Ambrosiella* sp. isolated from *X. mutilatus* mycangia, cultures of four isolates were grown on Oatmeal Agar (OA) (15 g oats powder, 20 g agar and 1,000 ml deionized water) and malt extract agar (MEA) (2% malt extract agar) in the dark at 25°C. Micromorphological characteristics were described from mycelium and conidiophores from 10 days old cultures mounted in lacto phenol containing cotton blue. Color descriptions of cultures were made using Rayner (1970). Photomicrographs were made with a Zeiss Axioscop microscope and an attached Canon digital camera. For SEM photomicrographs, agar plugs containing *A. beaveri* were fixed for 8 h at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. After fixation, specimens were rinsed in several changes of 0.1 M phosphate buffer and left overnight in the same buffer. Specimens were post-fixed in 2% OsO_4 in 0.1 M phosphate buffer for 4 h and rinsed for 20 min each in three changes of distilled water. The specimens were dehydrated in a graded ethanol series, critical-point dried in a Polaron E 3000 CPD (Quorum Technologies, Newhaven, UK), mounted on aluminum stubs with silver paste, and sputter coated with gold–palladium (5–7 nm). Specimens were observed and digitally captured using a JEOL JSM-6500F Field Emission SEM at 5 kV (JEOL USA, Inc, Peabody, MA).

DNA extraction, PCR, sequencing, and phylogenetic analysis

To extract DNA, a small amount of mycelium and conidia was scraped from the surface of young relatively unpigmented colonies from single spore isolations growing on MEA. The mycelium was macerated in 200 µl PrepMan Ultra (Applied Biosystems, USA), incubated at 95°C for 10 min, and then centrifuged. The supernatant containing the extracted DNA was then removed and used in PCR.

PCR amplification for the *Ambrosiella* sp. carried out using the primer pairs *ITS3* (White et al. 1990) and *LR3* (Vilgalys and Hester 1990), and *Bt2b* (Glass and Donaldson 1995) and *T10* (O'Donnell and Cigelnik 1997) to amplify a portion of the ribosomal RNA encoding region and partial *β*-tubulin gene, respectively. For the *Geosmithia* species, the primer

Table 1 Culture collection and GenBank accession numbers for strains of fungi used in this study

Species	DLS	CBS	Other numbers	Beetle	Origin	GenBank no.	
						LSU/ribosomal RNA	β-tubulin
Ambrosiella beaveri	1,624	CBS 121753		*Xylosandrus mutilatus*	Mississippi, USA		
	1,625	CBS 121752		*X. mutilatus*	Mississippi, USA		
	1,626	[T]CBS 121751	PREM 59819	*X. mutilatus*	Mississippi, USA	EU825650	EU825656
	1,627	CBS 121750		*X. mutilatus*	Mississippi, USA		
A. ferruginea		CBS 460.82	CMW25522; Butin A-241			EU825651	EU825652
A. ferruginea		CBS 408.68	CMW26232; DSM 1500; MUCL 14520; UWE-101			AF275505	EU825653
A. hartigii		CBS 403.82	No. A239			AF275506	
A. hartigii			CMW20920; TUB4276				EU825654
A. xylebori		[T]CBS 110.61	DSM 1502; MUCL 8065; C1650			AF275508; DQ470979	EU825655
Geosmithia lavendula	1,628	CBS 121748		*X. mutilatus*	Mississippi, USA	EU807968	
G. obscura	1,629	CBS 121749		*X. mutilatus*	Mississippi, USA	EU807967	
Candida homelintoma	1,630			*X. mutilatus*	Mississippi, USA	EU807969	

DLS, culture collection of D.L. Six at the University of Montana, USA; CBS, Centraalbureau voor Schimmelcultures; PREM, National Collection of Fungi, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa

[T] Ex-type culture

pairs *ITS1F* and *ITS4* were used. For the yeast, the primers *ITS3* and *LR3* were used. Each PCR reaction mixture (50 µl total volume) consisted of 25 µl Master Mix (Promega, USA), 0.5 µl of each primer (10 pmol concentration), 24 µl molecular grade water and 1 µl of the DNA extract. PCR conditions for *ITS3/LR3* and *ITS1F/ITS4* were: one cycle of denaturation at 92°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and one final cycle of extension at 72°C for 8 min. PCR conditions for *Bt2b/T10* were: one cycle of denaturation at 95°C for 3 min, followed by 36 cycles of denaturation at 95°C for 45 s, annealing at 48°C for 45 s, and extension at 72°C for 1 min, and one final cycle of extension at 72°C for 4 min.

Amplicons were purified using a High Pure PCR Product Purification Kit (Roche, Germany) and sequencing was performed on an ABI 3130 automated sequencer (Perkin–Elmer Inc, USA) at the Murdock Sequencing Facility at the University of Montana. DNA sequences of representative isolates were deposited in GenBank (Table 1). In addition to fungi isolated from beetles in this study, we also sequenced isolates of *A. xylebori* Brader, *A. hartigii* Batra, and *A. ferruginea* (Math.-Kaärik) Batra (Table 1).

Contigs of forward and reverse sequences obtained with each primer pair, were constructed in MEGA4 (Tamura et al. 2007). BLAST searches were done with sequences of each isolate in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and published sequences of relevant and related species were incorporated into the data sets. Data sets were aligned online using the E-INS-i strategy in MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). The aligned data sets were then analyzed using distance (neigbour-joining) and parsimony methods in PAUP 4.0b10 for Microsoft Windows (Sinauer Associates Inc, Massachusetts, USA). DNA substitution models used in neigbour-joining analyses were determined using ModelTest 3.7 (Posada and Crandall 1998). Maximum parsimony (MP) was conducted using a heuristic search with tree-bisection-reconnection (TBR) branch-swapping. From the resulting trees a strict consensus tree was obtained. For both MP and NJ analyses, confidence values were estimated using bootstrapping (1,000 replicates). In addition, Bayesian phylogenetic inference was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Substitution models for Bayesian analyses were determined using MrModeltest 2.2 (http://www.abc.se/~nylander/). The Bayesian analyses were run for 10,000,000 generations, sampled every 100 generations. All analyses used four chains and a random starting tree. Burn-in was determined for each analysis by estimating the point where chains converged. Posterior nodal probabilities were obtained from the Markov Chain Monte Carlo results and summarized by generating trees in MEGA4.

Results

Fungi associated with *X. mutilatus mycangia*

Based on culture morphology, isolates obtained from mycangia of *X. mutilatus* could be separated into four taxa: three filamentous fungi and one yeast species. Microscopic investigation confirmed that one of the filamentous fungi was an *Ambrosiella* species. Mycangia of all beetles ($n = 17$) yielded the *Ambrosiella* species regardless of collection date or method. The remaining two species had penicillately branched conidiophores resembling those of *Penicillium* and *Geosmithia* species and were only isolated on medium not containing cycloheximide. These species were isolated from only one beetle each.

DNA sequencing, phylogenetic analysis, and SEM

Amplicons obtained from the LSU regions of all the *Ambrosiella* species sequenced in this study were approximately 540 bp in length. The data set, after alignment with related *Ceratocystis* and *Gondwanamyces* species, had to be trimmed at the ends resulting in a final length of 449 characters. Of these, 102 were parsimony informative, 326 characters were invariable, and 21 were variable but not parsimony informative. MP analysis resulted in 44 trees. From these trees we produced a strict consensus tree. From the NJ analyses a phylogram was produced. Tree topologies (not shown) and bootstrap values were similar for MP and NJ trees. Two independent Bayesian analyses were run, each starting from a random tree. Posterior probabilities and topologies were similar in both analyses. One Bayesian tree is

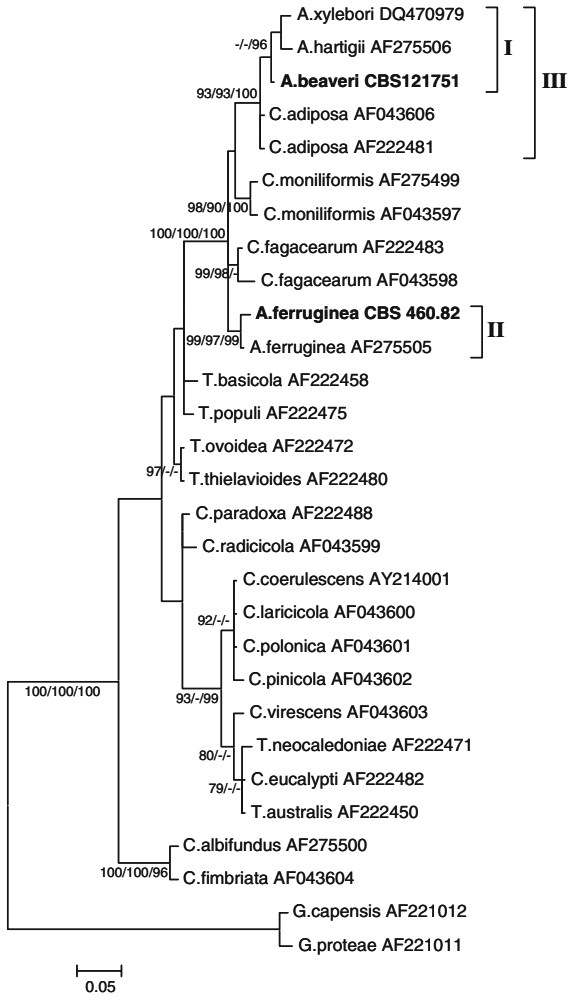

Fig. 1 Phylogram obtained with a Bayesian Monte Carlo Markov (MCMC) analysis of 449 bp from the nLSU of *Ambrosiella* and *Ceratocystis* species. The 50% majority rule consensus tree was obtained from 199,802 trees. *Numbers* at nodes indicate bootstrap support values (1,000 replicates) for neigbour-joining (NJ) and maximum parsimony (MP) analyses, as well as posterior probabilities obtained from Bayesian analyses (MrB) as follows: NJ/MP/MrB. Only bootstrap values above 75% and posterior probabilities above 95% are shown. *Bold* type indicates sequences obtained in this study. *Ambrosiella* species formed two well supported groups (I and II) within a monophyletic group (III) containing several *Ceratocystis* species

presented (Fig. 1). Only posterior probabilities >95% are shown. Overall, there was strong agreement on topology and node support among all three analyses.

Ambrosiella species included in the analyses of LSU sequences formed two well-supported groups (I and II), separated from each other by groups containing *Ceratocystis* species (Fig 1). The isolate obtained from *X. mutilatus* in this study (CBS 121751) clustered with *A. xylebori* (the type species for this genus) and *A. hartigii* to form group I (Fig. 1). Two isolates of *A. ferruginea* formed group II. Group I was part of a larger, well-supported group (III) that included two isolates of *C. adiposa*. Group III was separated from group II by two lineages consisting of, respectively, *C. moniliformis* and *C. fagacearum* isolates. Other, more distantly related monophyletic groups contained *Thielaviopsis* species, species of the *C. coerulescens* complex, and those belonging to the *C. fimbriata* complex.

The β-tubulin sequences varied significantly more between the *Ambrosiella* and *Ceratocystis* species than did the LSU sequences. These differences in length resulted primarily from the variability of the β-tubulin introns (Table 2). Few sequences for this region of the β-tubulin gene were available in GenBank for *Ceratocystis* species. From the *Ceratocystis* sequences that were available, species in the *C. coerulescens* complex all contained introns 2, 3, 4, and 5 (Table 2), while the *A. ferruginea* isolates (Group II) and *C. adiposa* possessed introns 2, 4, and 5, but lacked intron 3. *Ambrosiella hartigii*, *A. xylebori* and the *Ambrosiella* species isolated in the present study, all possessed introns 2 and 4, but lacked introns 3 and 5.

For analyses of the β-tubulin data set, all introns were ecluded from the analysis because they were un alignable. Exon 2 was lacking in several isolates (Table 2) and was thus also excluded, and exon 6 was trimmed at the 3′ to the length of the shortest fragment in the data set (95 bp). The final data set thus contained the complete exons 3, 4, 5, and the 5′ end of exon 6, totaling 219 characters. Of these, 64 were variable sites of which 52 were parsimony informative. MP analysis resulted in seven trees with a length of 98 steps. From these seven trees, a strict consensus tree was calculated. A phylogram was obtained from NJ analyses. Topologies (not shown) and bootstrap support values were similar for both NJ and MP analyses. Two independent Bayesian analyses were run, each starting from a random tree. Posterior probabilities and topologies were similar in both analyses. One tree is presented (Fig. 2). *Ambrosiella* species included in the analyses formed two distinct groups (I & II). The *Ambrosiella* obtained in this study again grouped closely, but distinct from *A. xylebori* and *A. hartigii,* and differed from both

Table 2 Presence and absence of introns and exons in the β-tubulin genes of *Ceratocystis* and *Ambrosiella* species included in the phylogenetic analyses, and their sizes in basepairs (bp)

Group	Species	↓ Genbank/isolate no Primer binding sites →	EXON 2 T10	INTRON 2	EXON 3 Bt2a	INTRON 3	EXON 4	INTRON 4	EXON 5	INTRON 5	EXON 6 Bt2b
C. coerulescens complex	*C. coerulescens*	AY140943/C313	24	133	27	131	42	196	57	264	>453
	C. pinicola	AY140937/206-291	24	135	27	131	42	189	57	265	>453
	C. douglasii	AY140944/C324	24	135	27	131	42	219	57	263	>453
	C. polonica	AY140939/93.208.10	24	135	27	131	42	210	57	265	>453
	C. resinifera	AY140946/D53/5A3	24	135	27	130	42	215	57	265	>453
	C. rufipenni	AY142242/C609	24	134	27	106	42	220	57	266	>453
C. adiposa	*C. adiposa*	AY140936/152-5-1	24	104	27	–	42	160	57	186	>453
Group II	*A. ferruginea*	CMW25522_CBS460.82	>18[a]	94	27	–	42	150	57	213	>157
	A. ferruginea	CMW26232_CBS208.68	>18	94	27	–	42	161	57	214	>157
Group I	*A. hartigii*	CMW20920_TUB4276	24	74	27	–	42	145	57	–	>163
	A. beaverii sp. nov.	CMW121751		>39	27	–	42	143	57	–	>95
	A. xylebori	CBS110.61_T		>66	27	–	42	143	57	–	>128

[a] Fragment sizes for introns or exons given as > xx, indicate that only a part of the sequence (xx bp) was obtained for that specific intron or exon

species in 9 bp and 24 bp positions in the exons, respectively. Based on β-tubulin data, *C. adiposa* was the *Ceratocystis* species closest to the four *Ambrosiella* species.

Comparisons of both LSU and β-tubulin sequences of the *Ambrosiella* species from this study with sequences of all other *Ambrosiella* species known to be allied with *Ceratocystis* (*A. hartigii, A. ferruginea* and *A. xylebori*) revealed that it is very closely related to these fungi, but distinct. Therefore, this fungus is described here as a new species, *Ambrosiella beaveri* sp. nov.

The two other filamentous fungi isolated from *X. mutilatus* mycangia were identified as *Geosmithia lavendula* (Raper & Fennell) Pitt. and *G. obscura* M. Kolařík, Kubátová & Pažoutová (Hypocreales). Both fungi provided strong matches to morphological descriptions of these species and comparisons of their DNA sequences to sequences for ex-type cultures of *G. lavendula* (AF033385) and *G. obscura* (AM181460) matched at 100% (Table 1). Scanning electron micrographs of *G. lavendula* (Fig. 5) show the cylindrical rough walled phialides with broad collula typical of members of this genus.

For the yeast, sequences matched 100% with a sequence for the ex-type of *Candida homilentoma* Van der Walt & Nakase (AB018166) as well as a sequence from a culture isolated from the gut of a fungus-feeding endomychid beetle collected in Panama (Meredith Blackwell, personal communication, Suh et al. 2005).

Description of *Ambrosiella beaveri* Six, Z. W. de Beer & W. D. Stone sp. nov. (Figs. 3–5)

Coloniae in agaro ex farina avenacea: effundo, obscure canus murinus, margine sepia canus. Sporodochia abundantia albae et olivaceo-luteae. Odoro graveolens. Conidiophora in area canus fuscare ramosus vel non ramosus, (2–5), 11–81 (–146) µm longis, 3–8 µm latae. Cellulae conidiogenae terminal, 8–21 (–27) µm longis, 2–11 µm latae. Conidia muritus crassa, gnatus sum aleurosporae blastosporis, globosae, solitariae vel catenatae, hyalina vel laete brunneae, laevia, 7–14 µm latae, 8–14 µm longis. Sporodochia conformo continuatus teges, infra masses cellae irregularis, (2–) 7–20 × 6–18 µm, effectus densa valli hyaline, ramosus vel non ramosus, conidiophorae phialidis, (19) 25–196 (–225) µm

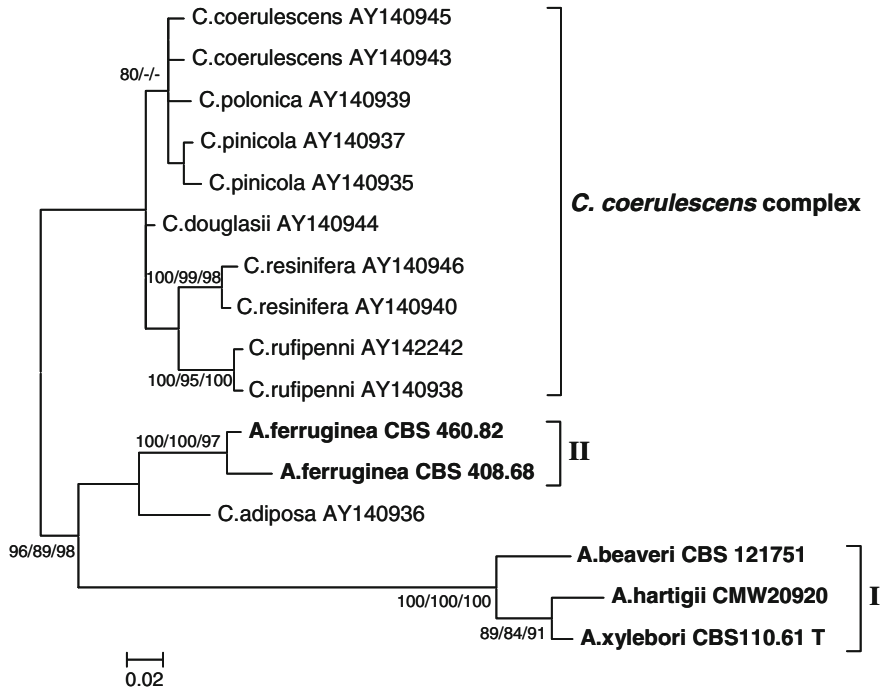

Fig. 2 Unrooted phylogram obtained with a Bayesian Monte Carlo Markov (MCMC) analysis of 222 bp consisting of exons 3, 4, 5 and part of exon 6 of the β-tubulin encoding gene for *Ambrosiella* and *Ceratocystis* species. The 50% majority rule consensus tree was obtained from 18053 trees. Numbers at nodes indicate bootstrap support values (1,000 replicates) for neigbour-joining (NJ) and maximum parsimony (MP) analyses, as well as posterior probabilities obtained from Bayesian analyses (MrB) as follows: NJ/MP/MrB. Only bootstrap values above 75% and posterior probabilities above 95% are shown. Bold type indicates sequences obtained in this study. *Ambrosiella* species formed two well supported groups (I and II) closely related to *Ceratocystis adiposa*

longis. Phialoconidia muritus tenuis, ovi-cylindrata, basi truncata, solitariae vel catenatae, hyalinae, laeves, 7–24 µm longis, 5–19 µm latae.

Etymology

Named for Roger A. Beaver, in recognition of his extensive and exceptional work with ambrosia beetles.

Morphology

10-days old colonies on oatmeal agar at 25°C 45 mm in diameter, effuse, dull Dark Mouse Grey with Greyish Sepia margins with abundant White to Buff sporodochia. Sporodochia becoming Olivaceous Buff after 14 days. Droplets of clear, glistening, brown liquid scattered sparsely across surface. Numerous, unevenly melanized fascicles forming from loose strands of aerial hyphae, extending up to 8 mm above colony surface. Underside of colony Pale Mouse Grey. Agar below colonies stained deep brown. Odor strong, unpleasant, similar to butyric acid. Colonies on MEA similar, but producing few fascicles and with most sporodochia confined to the center of the colony.

Microscopic characteristics

Mycelium superficial or immersed, composed of melanized, light brown, septate, repeatedly branched hyphae, with cells 6–25 µm long and 3–10 µm thick. Conidiphores in grey (non-sporodochial) areas, melanized, light brown, macronematous, mononematous, unbranched or branched (2–5), 11-81 (−146) µm long and 3–8 µm wide (Fig. 3). Conidiogenous cells terminal, 8–21 (–27) µm long and 2–11 µm wide. Conidia, thick-walled, originating as globose conidia, solitary or catenate, initially hyaline turning light brown, smooth, thick-walled, 8–14 µm wide and 8–14 µm long (Fig. 3). Hyphae forming thick-walled, globose intercalary chlamydospores, either singly or

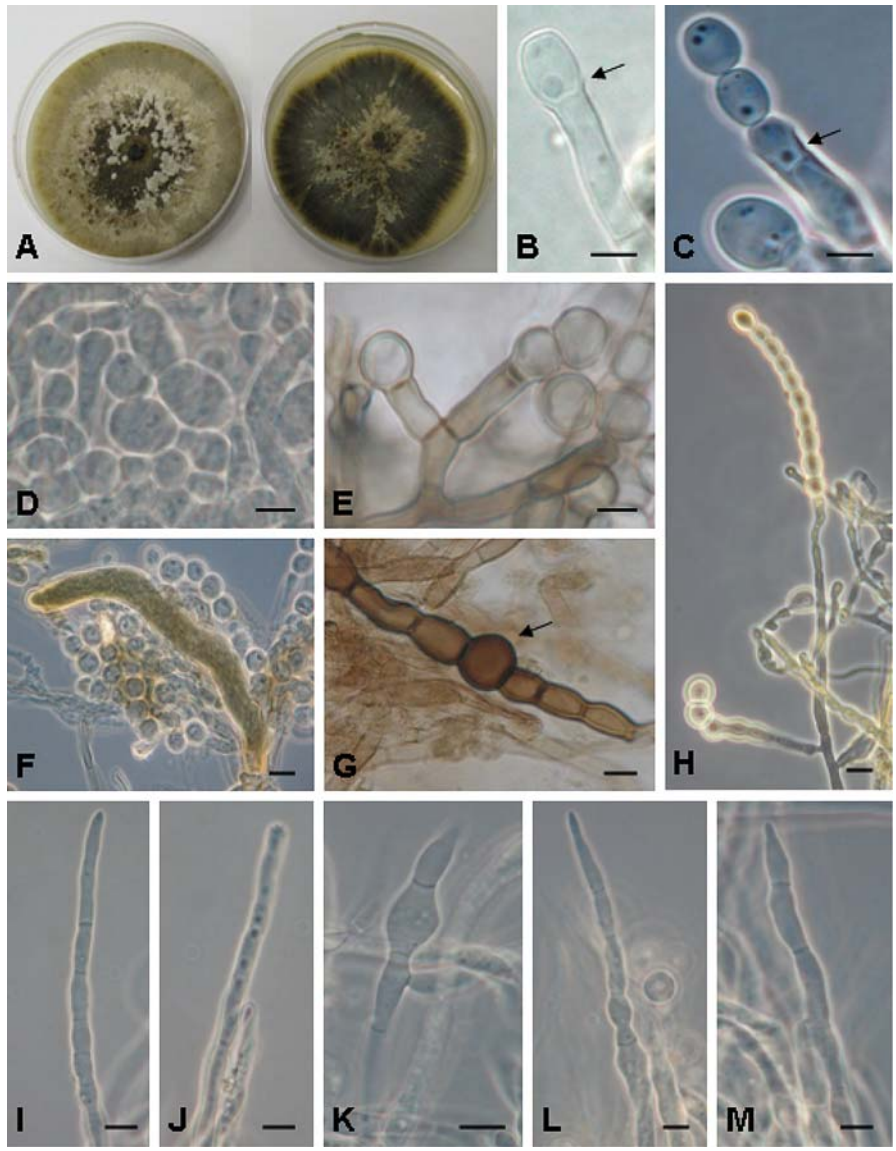

Fig. 3 *Ambrosiella beaveri*. **a** Colonies on oatmeal agar (*left*) and malt extract agar (*right*) after 10 days; **b**, **c** phialophores from sporodochia (*arrows* indicate phialidic conidiogensis); **d** sprout cells from sporodochia; **e** blastoconidiophores from *grey* (non-sporodochial) area of colony; **f** Sausage-shaped cells from fascicles; **g** intercalary chlamydospore (*arrow*) from *grey* area; **h** phialiconidiophore with catenate conidia; **i–m** hyphal filaments (**i**, smooth, **j**, granular; **k**, pointed and branched with swollen cell; **l**, **m** pointed, unbranched). (scale bars, 10 μm)

in chains (Fig. 3). Conidiophores occasionally interspersed by thin 3–5 (−14) μm wide, 53–108 (−133) long, unbranched, septate or non-septate hyphal filaments granular at the immediate tip (Fig. 3), or by unbranched, septate hyphal filaments, 3–6 (−7) μm wide and 51–219 μm long, with smooth tips (Fig 3).

Sporodochia consisting of interwoven melanized stromatic hyphae forming a discrete continuous mat covered by a mass of irregular sprout cells measuring (2−) 7–20 × 6–18 μm (Fig. 3) and giving rise to a dense palisade of hyaline unbranched or branched (2–3) conidiophores. Conidiophores, maconematous, mononematous, septate, (19) 25–196 (–225) μm in length, with conidiogenous cells 5–37 (–46) μm in length and 4–14 μm in width (Figs. 3, 4). Conidia thin-walled, oval–cylindrical with truncate ends, solitary or catenate, hyaline, smooth-walled, measuring 7–25 μm long and 5–20 μm wide (Figs. 3, 4). Rarely, globose conidia forming from stipe of conidiophore (Fig. 3). Conidiophores occasionally interspersed by branched or unbranched, septate hyphal filaments often possessing swollen cells near the middle or base and distinctly attenuated tips (Fig. 3). Filaments 52–244 μm long and 3–12 μm wide with apical cells measuring (3–) 13–42 μm long.

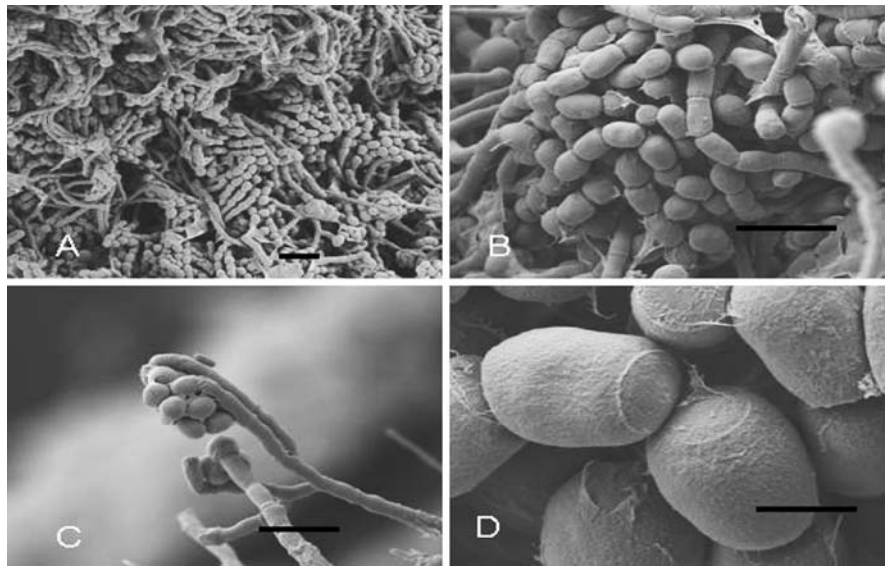

Fig. 4 Scanning electron micrographs of *Ambrosiella beaveri*. **a–c** Conidiophores showing catenate conidia (scale bars, 25 μm) D. Conidia (scale bar, 5 μm)

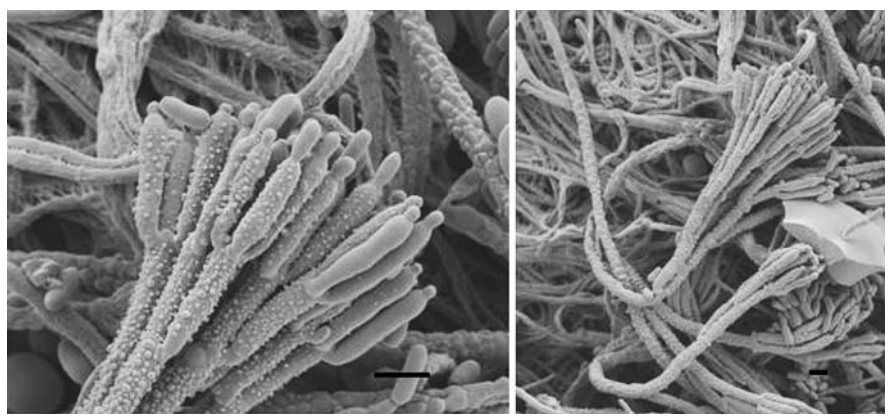

Fig. 5 Conidiophores of *Geosmithia lavendula* (scale bars, 5 μm)

Fascicles developing from loose strands of septate aerial hyphae from both sporodochia and non-sporodochial portions of the culture (cells 6–33 (–45) μm long and 1–9 μm wide), producing along their length, conidiophores and conidia similar to those produced in grey areas, as well as thick, densely-packed, thickened, intercalary sausage-shaped cells 6–15 μm in width and 29–144 (–279) μm in length (Fig. 3).

Ambrosiella beaveri is most similar to *A. hartigii,* but tends to have longer conidiophores and larger conidia. In addition, chlamydospores, which were abundant in our cultures of *A. beaveri*, have not been described for *A. hartigii*.

HOLOTYPE: USA. Winston County, Mississippi, isolated from mycangium of the scolytine ambrosia beetle, *Xylosandrus mutilatus,* infesting *Vitus rotundifolia*, (PREM 59819; Ex-type culture CBS 121751).

PARATYPES: USA. Winston County, Mississippi, isolated from mycangium of *Xylosandrus mutilatus* infesting *Vitus rotundifolia* (CBS 121750, CBS 121752, CBS 121753).

Discussion

Through phylogenetic analysis of ITS/LSU and β-tubulin DNA sequences, the *Ambrosiella* species was found to be closely related to, but distinct from, all other described *Ambrosiella* species with affinities to *Ceratocystis*. Therefore, we described this fungus as a new species, *A. beaveri*. *Ambrosiella beaveri* is closely related to *A. hartigii*, a fungus associated with *X. germanus* and *Xyleborus dispar* (F.), and to *A. xylebori,* a fungus associated with *X. compactus*

and *Corthylus columbianus* Hopkins. These results support previous work indicating that *Ambrosiella* is not only polyphyletic with derivations out of both *Ophiostoma sensu lato* and *Ceratocystis,* but also polyphyletic within *Ceratocystis* (Cassar and Blackwell 1996, Paulin-Mahady et al. 2002).

The isolation of two *Geosmithia* species from *X. mutilatus* was surprising. *Geosmithia* is a polyphyletic group with affinities to hypocrealean (Hypocreales: Bionectriaceae) and eurotialian (Eurotiales: Trichocomaceae) fungi. All known species associated with Scolytinae, including those isolated from *X. mutilatus* in this study, however, are in the Hypocreales. These fungi are similar in appearance to *Penicillium* but differ in their possession of cylindrical, rough-walled phialides and a lack of narrowed collula and green spores. *Geosmithia* were initially reported from a number of substrates including wood, seeds, soil, mushroom compost, bagasse, and leaves (Pitt 1979), but more recently Kolařík et al. (2004, 2005) have reported consistent associations of a number of *Geosmithia* with bark beetles (also Scolytinae, but which construct galleries in phloem instead of sapwood) inhabiting angiosperms in Europe and Asia. While bark beetles appear to be the most common vectors of these fungi, five species of ambrosia beetles in the tropics of America, Asia, and Australia have also been found to be associates (Kirschner 2001; Kolarík et al. 2007). It is surprising that these dry-spored fungi were acquired from mycangia as mycangia do not typically acquire dry spores, and are often highly specific to ophiostomatoid (sticky-spored) fungi. However, it is possible that isolations of these fungi resulted from the transfer of spores present on the external surface of host material to isolation plates. While *A. beaveri* was acquired from all mycangia, *Geosmithia* species were isolated from only two beetles, further indicating they may not be mycangial associates. As no bark beetles or other insects were found in the plant material from which our beetles were collected, we can assume that these fungi were vectored into the vines by *X. mutilatus*, and that their presence with *X. mutilatus* was not the result of cross-contamination with fungi from another source.

The yeast, *C. homilentoma,* was first described from larval galleries of bostrichid beetles in South Africa (van der Walt and Nakase 1973), but has subsequently been found associated with a number of other mycophagous insects including fungus-gardening attine ants in Brazil (Carreiro et al. 1997) and fungus-feeding endomychid beetles in Panama (Meredith Blackwell, personal communication, Suh et al. 2005). This yeast, therefore, appears to have a broad distribution and association with fungus-feeding insects. However, its role with these insects, if any, is unknown.

Given that ambrosia fungi are typically specific to their vector beetles, our results suggest that *A. beaveri* was likely introduced along with *X. mutilatus* into North America. It is unclear whether *G. lavendula* and *G. obscura* were introduced with this beetle, or acquired by the beetle once it established in its new environment. *Geosmithia lavendula* is apparently widespread in distribution and has been reported as a contaminant and an elm bark beetle associate in the US (Pitt 1979), as well as in association with bark beetles in the Mediterranean region, and *Ficus*-infesting bark beetles in particular (Kolařík et al. 2007). *Geosmitha obscura* has, heretofore, been reported only from *Scolytus* bark beetles in Europe (Kolařík et al. 2005). As more Scolytinae are surveyed for *Geosmithia* associates we will gain a fuller understanding of their geographic and host ranges and their potential roles with their hosts.

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