Ambrosiella roeperi sp. nov. is the mycangial symbiont of the granulate ambrosia beetle, *Xylosandrus crassiusculus*

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Abstract: Isolations from the granulate ambrosia beetle, *Xylosandrus crassiusculus* (Coleoptera: Curculionidae: Scolytinae: Xyleborini), collected in Georgia, South Carolina, Missouri and Ohio, yielded an undescribed species of *Ambrosiella* in thousands of colony-forming units (CFU) per individual female. Partial sequences of ITS and 28S rDNA regions distinguished this species from other *Ambrosiella* spp., which are asexual symbionts of ambrosia beetles and closely related to *Ceratocystis* spp. *Ambrosiella roeperi* sp. nov. produces sporodochia of branching conidiophores with disarticulating swollen cells, and the branches are terminated by thick-walled aleurioconidia, similar to the conidiophores and aleurioconidia of *A. xylebori*, which is the mycangial symbiont of a related ambrosia beetle, *X. compactus*. Microscopic examinations found homogeneous masses of arthrospore-like cells growing in the mycangium of *X. crassiusculus*, without evidence of other microbial growth. Using fungal-specific primers, only the ITS rDNA region of *A. roeperi* was amplified and sequenced from DNA extractions of mycangial contents, suggesting that it is the primary or only mycangial symbiont of this beetle in USA.

Key words: *Ambrosiella hartigii*, *Anisandrus dispar*, *Cnestus mutilatus*, *Trypodendron*, Xyleborini, *Xylosandrus amputatus*, *X. germanus*

INTRODUCTION

Most ambrosia beetles (*Coleoptera: Curculionidae: Scolytinae* and *Platypodinae*), including the important and highly invasive plant pest *Xylosandrus crassiusculus* (Motschulsky), are closely tied to fungal symbionts. Larvae and adults of ambrosia beetles typically feed on the growth and sporulation of fungi in the nutrient-poor sapwood of dead or dying trees, in contrast to most bark beetles, which feed primarily in the nutrient rich inner bark (Harrington 2005). Adult ambrosia beetles bore into the sapwood and inoculate the galleries with fungal symbionts that grow within sacs, called mycangia (Batra 1963, 1967; Francke-Grosmann 1967; Beaver 1989). Excretions into the mycangium apparently support growth of the fungi, and the overflow of spores continuously inoculates the galleries as they are being excavated over a period of weeks or months. A wide variety of fungi are found sporulating in galleries, but a narrower group of fungi are found growing in mycangia. Most of the identified mycangial inhabitants are in the genera *Raffaelea* or *Ambrosiella*, which are closely related to *Ophiostoma* and *Ceratocystis* respectively (Cassar and Blackwell 1996; Farrell et al. 2001; Massoumi Alamaouti et al. 2009; Harrington et al. 2010, 2011). *Raffaelea* spp. are the most common mycangial symbionts, whereas *Ambrosiella* spp. have been associated with species in three tribes of Scolytinae: *Xylosandrus* and allied genera (*Anisandrus* and *Cnestus*) in Xyleborini, *Corthylus* in Corthylini and *Trypodendron* in Xyloterini (Harrington et al. 2010).

Members of the tribe Xyleborini that have *Ambrosiella* symbionts, including *X. crassiusculus*, have a relatively large mycangium below the mesonotum of adult females (Francke-Grosmann 1967, Kinuura 1995). *X. crassiusculus*, which is native to Asia and Africa but is now established in Hawaii and eastern USA (Rabaglia e al. 2006), has a mycangial symbiont that was reported by Brader (1964) to be similar to *A. xylebori* Brader ex Arx & Hennebert and as *Ambrosiella* sp. 2 by Kinuura (1995). Gebhardt et al. (2005) identified the *X. crassiusculus* symbiont as *A. xylebori*, the mycangial symbiont of *X. compactus* Eichh. and *Corthylus columbianus* (Hopkins) (Batra 1967). Hulcr and Dunn (2011) reported that both *A. xylebori* and an unidentified *Fusarium* sp. were mycangial symbionts of *X. crassiusculus*.

Female, adult *X. crassiusculus* were collected in Georgia, South Carolina, Missouri and Ohio for examination of mycangial contents and isolation of symbionts. An *Ambrosiella* sp. was recovered in high numbers of CFU in all collections and was detected in spore masses from mycangia using molecular tech-

niques. It is shown to be distinct from *A. xylebori* and is described here as a new species.

MATERIALS AND METHODS

Beetle collection and fungal isolations.—Only females of *X. crassiusculus* can fly and have mycangia, and only adult females were studied. The beetles initially collected in South Carolina and Georgia were part of a study on potential vectors of the laurel wilt pathogen, *Raffaelea lauricola* TC Harr, Fraedrich & Aghayeva (Fraedrich et al. 2008, Harrington et al. 2008). Female adult *X. crassiusculus* were collected near Winokur, Georgia, as they landed on wounded stems of redbay (*Persea borbonia*) or clothing on a warm, late-winter day (12 Mar 2007), the same day in which *Xyleborus glabratus* Eichh. was collected for isolation of *Raffaelea* spp. (Harrington and Fraedrich 2010). Other female adults were excavated from tunnels in *Litsea aestivalis* with laurel wilt near Clyo, Georgia, (Fraedrich et al. 2011) or in sassafras (*Sassafras albidum*) trees with laurel wilt on Hunting Island, South Carolina, in Apr 2007.

Five adult *X. crassiusculus* were trapped in May 2013 in Boone County, Missouri, in Lindgren traps baited with ethanol, with water in the collection cup. Other adult *X. crassiusculus* were similarly trapped in Barrow County and Clarke County, Georgia, in Aug and Sep 2013. After collection, beetles were kept in individual vials and refrigerated (4 C) until isolations were attempted. Two female adult *X. crassiusculus* were collected in ethanol-baited Lindgren traps in Adair County, Missouri, on 12 Mar 2012 with polypropylene glycol in the collection cup. The beetles were preserved in ethanol at room temperature. Voucher specimens were placed in the Enns Entomological Museum at the University of Missouri.

Quantification of fungal propagules (CFU) per beetle was conducted as in studies for *Xyleborus glabratus* (Harrington and Fraedrich 2010, Harrington et al. 2011). An individual beetle was ground with glass tissue grinders and serially diluted on SMA (1% Difco malt extract, 2% agar and 100 ppm streptomycin sulfate added after autoclaving). For each tested dilution (10×, 100×, 1000×), three replicate plates were used. Some beetles were surfaced-sterilized for 3 min or 1 min in 0.6% sodium hypochlorite with 0.1% Tween 80, followed by rinsing in sterile distilled water before grinding.

DNA sequencing and phylogenetic analyses.—The rDNA sequences of isolates of the new *Ambrosiella* sp. were compared to four of the five described species of *Ambrosiella* (Harrington et al. 2010) and representatives of the other major clades of *Ceratocystis* (TABLE I), but no culture or sequence from *A. trypondendri* (Batra) TC Harr was available. Most isolates were grown on MYEA (2% Difco malt extract, 0.2% Difco yeast extract, 2% agar) 4–10 d at room temperature for DNA extraction with PrepMan™ Ultra (Applied Biosystems, Foster City, California). Amplification of a portion of the D1 and D2 domains of the nuclear ribosomal large subunit (LSU, 28S) was performed with primers LROR and LR5, and the amplified products were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility with primers LROR and LR3

(Paulin-Mahady et al. 2002). Full ITS rDNA sequences were generated with primers ITS1-F and ITS4 (Paulin-Mahady et al. 2002). In some cases, a *Ceratocystis*-specific primer designed from the 5.8S rDNA gene (Cerato3.7, 5′–CTGTCCGAGCGTCATTTCAC) was used in conjunction with the general reverse primer ITS4.

Aligned LSU rDNA sequences were analyzed for maximum parsimony (MP) with PAUP 4.0b10 (Swofford 2002). Gaps were treated as a fifth base, all characters had equal weight and heuristic searches used simple stepwise addition and tree-bisection-reconnection. Bootstrap analyses were conducted in PAUP with 1000 replications, but, because of the large number of most parsimonious trees found in some replications, the maximum number of trees was set to 100.

A likelihood tree and posterior probability (Bayesian) estimates for branches were determined with Mr. Bayes 3.2.1 (Ronquist and Huelsenbeck 2003), in which gaps are treated as missing data. The settings included a general time-reversible (GTR) model and gamma distribution, 1 200 000 generations, a diagnosis frequency of 5000, and a sample frequency of 500. The first 25% of samples were discarded (burninfrac = 0.25), and Bayesian posterior probability estimates were calculated by majority rule consensus of the trees after burn-in.

Species descriptions.—Cultures were grown on MYEA and malt extract agar (MEA, 1.5% Difco malt extract and 2% agar) at 25 C in the dark. Colors of cultures on MYEA followed the nomenclature of Rayner (1970). Representative cultures were deposited in the Centraalbureau voor Schimmelcultures, and herbarium specimens were deposited in the U.S. National Fungus Collections (BPI) and the Iowa State College Herbarium (ISC).

Mycangial contents of female beetles.—One of the Missouri *X. crassiusculus* adults stored in ethanol was used for microscopic examination of squashed mycangial contents with cotton blue in lactophenol, and the other beetle was used for DNA extraction and detection of *Ambrosiella* spp. with PCR. Six female adult *X. germanus* (Blandford) were collected similarly in Missouri and preserved in ethanol; four were examined for the presence of fungal spores in the mycangium, and DNA was extracted from two other female *X. germanus.* Individual, whole beetles were placed in 200 µL PrepMan™ Ultra extraction buffer and ground in sterile tissue grinders, followed by DNA extraction. The extracted DNA was concentrated with an Amicon Ultra 0.5 mL 100 K centrifugal filter (Millipore Ireland Ltd., Cork, Ireland) and used in PCR reactions with primers Cerato3.7 and ITS4.

Eleven adult females trapped in Georgia were placed in 20% lactic acid in a deep well slide for dissection. A cut was made through the elytra and down to the meso-metathorax juncture, and the scutellum and attached mycangial sac were teased out from under the pronotum with forceps and scalpel. The elytral fragments were removed and scutellum/mycangium transferred to a new slide and either mounted whole in a drop of cotton blue or the mycangial contents were forced out with forceps. A coverslip was placed over the drop of cotton blue and the mycangial contents observed at 500×. In some cases, the coverslip was removed later and the mycangial contents retrieved and placed in 100 or 200 µL

TABLE I. Origin of isolates and ambrosia beetle associates, if any, of *Ambrosiella, Ceratocystis* and *Thielaviopsis* spp. and outgroup taxa used for LSU rDNA sequencing and phylogenetic analysis

Species	Culture or specimen Nos.	Ambrosia beetle associate	Location	LSU rDNA accession No.
A. beaveri	C2749 (CBS 121750)	*Cnestus mutilatus* (mycangium)	Mississippi, USA	KF646765
	CM01	*Xylosandrus amputatus* (gallery)	Georgia, USA	
A. ferruginea	C2230 (CBS 460.82)	*Trypodendron domesticus*	Germany	KF646766
	C1571 (CBS 408.68)	*T. retusum* (mycangium)	Wisconsin, USA	AF275505
A. hartigii	C1573 (CBS 404.82)	*Anisandrus dispar* (mycangium)	Germany	
	C1574 (CBS 403.82)	*A. dispar* (discolored wood)	Germany	AF275506
A. roeperi	C2411, C2413, C2414, C2415 (CBS 135863), C2448 (CBS 135864), C2451 (CBS 135865), C2453	*X. crassiusculus* (beetles)	Georgia, USA	KF646767
	C2454	*X. crassiusculus* (beetle)	South Carolina, USA	
	C3041, C3042, C3043, C3044	*X. crassiusculus* (beetles)	Missouri, USA	
	C3129	*X. crassiusculus* (beetle)	Ohio, USA	
A. xylebori	C1650 (CBS 110.61)	*X. compactus* (gallery)	Ivory Coast	AF275508
	C2455, C2456, C2458	*X. compactus* (beetle)	Georgia, USA	
C. adiposa	C998 (CBS 600.74)		the Netherlands	AF222481
C. albifundus	C1048		South Africa	AF275500
C. bhutanensis	C2243 (CBS 112904)		Bhutan	KF646768
C. cacaofunesta	C1004 (CBS 153.62)		Ecuador	AF222486
C. coerulescens	C301 (ATCC12859, CBS 100.198)		Minnesota, USA	AF275510
C. eucalypti	C457		Australia	AF222482
C. fagacearum	C1305		Iowa, USA	AF222483
C. ficicola	C1393 (IFO 32969)		Japan	AF222485
C. fimbriata	C1390 (IFO 30501)		Japan	AF222484
C. moniliformis	C1008 (CBS 155.62)		Costa Rica	AF275499
C. moniliformopsis	C1934 (DAR 74609)		Australia	KF646769
C. paradoxa	C1001 (CBS 601.70)		Brazil	AF275498
C. pirilliformis	C2239 (CBS 118128)		Australia	KF646770
C. platani	C1317 (CBS 115162)		North Carolina, USA	KF646771
C. polychroma	C2241 (CBS 115776)		Indonesia	KF646772
C. radicicola	C869 (CBS 114.47)		California, USA	AF275513
C. variospora	C1963 (CBS 135862)		Iowa, USA	KF646773
C. virescens	C74 (CBS128997)		New York, USA	AF222489
T. australis	C448		Australia	AF222450
T. basicola	C1372 (CBS 414.52)		the Netherlands	AF222459
T. ovoidea	C1375 (CBS 136.88)		the Netherlands	AF222472
T. populi	C1368 (CBS 484.71)		Belgium	AF222475
T. thielavioides	C1377 (CBS 130.39)		Italy	AF222480
Gondwanamyces capense	C1960 (CMW 997)		South Africa	AF221012
G. proteae	C2235 (CBS 486.88)		South Africa	AF221011
Colletotrichum kinghornii	A100 (CBS 198.35)		United Kingdom	AF275496

PrepMan™ Ultra extraction buffer for DNA extraction, followed by DNA concentration and PCR as described above.

RESULTS

Isolations of the new species from Xylosandrus crassiusculus.—A filamentous fungus with microscopic characters (FIG. 1A–E) matching the descriptions of *Ambrosiella* spp. (Batra 1967) was consistently isolated from *X. crassiusculus* females with dilution plating. Four female adults were collected as they landed near freshly wounded redbay trees at Winokur, Georgia. Two of these beetles were ground after they were surface-sterilized for 3 min and yielded approximately
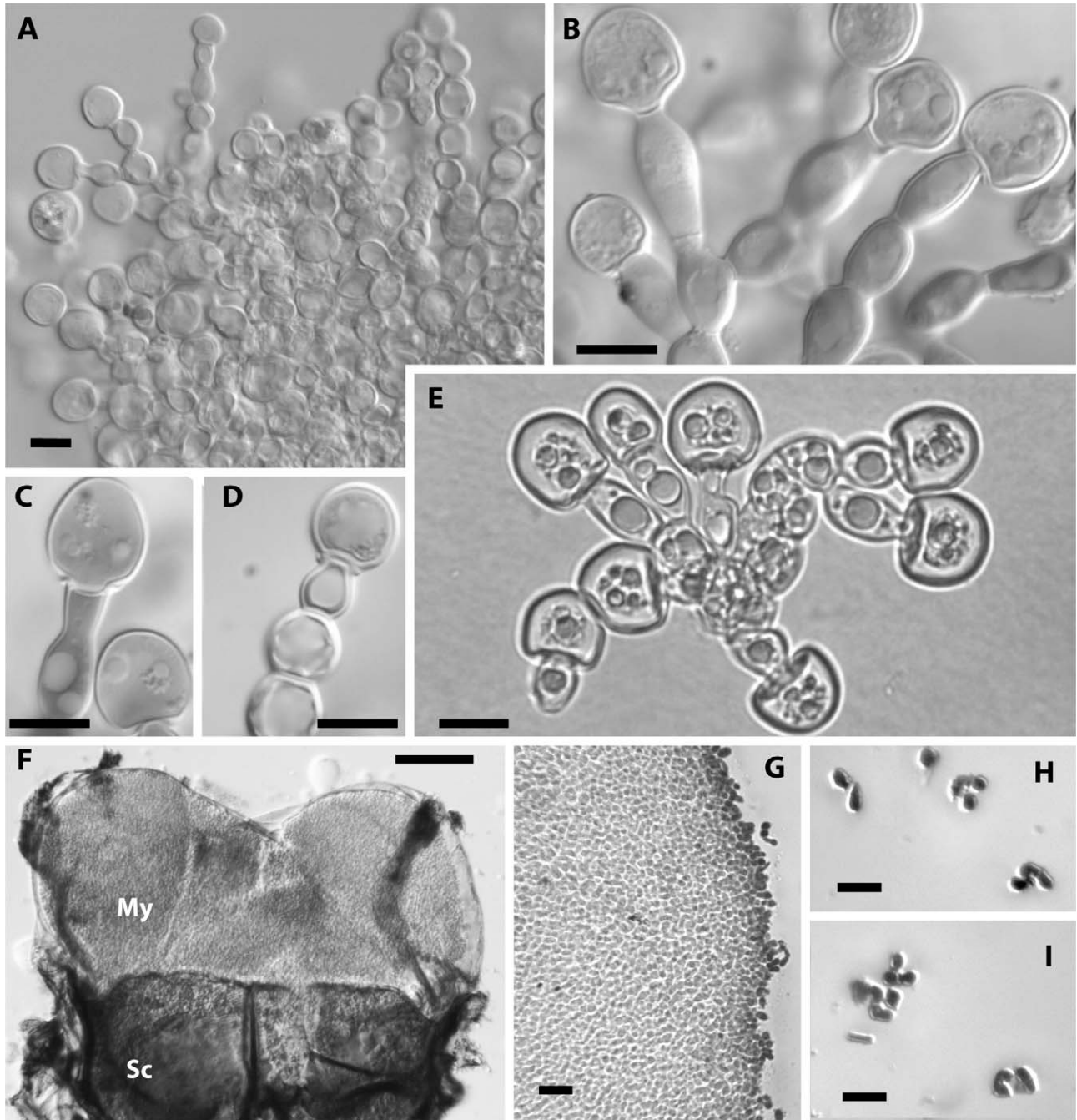
FIG. 1. Aleurioconidia and conidiophores of *Ambrosiella roeperi* (A–E), mycangium of *Xylosandrus crassiusculus* (F), and mycangial spores of *A. roeperi* (G-I). A. Sporodochium with branched conidiophores of swollen cells and terminal aleurioconidia. B, C. Developing aleurioconidia on swollen conidiophore cells. D. Mature, thick-walled aleurioconidium and swollen cells of conidiophore. E. Detached aleurioconidium with subtending conidiophore cell (lower left) and detached, multi-branched conidiophore with terminal aleurioconidia, taken with light microscopy to highlight lipid-like bodies. F. Bilobed mycangium (My), consisting of a transparent cuticle with a mass of spores inside, attached to the dark scutellum (Sc), dissected from the pronotum/mesothorax of a female *X. crassiusculus*. G. Homogeneous mass of conidia squeezed from the inside of a mycangium. H, I. Apparently dividing conidia from mycangium of *X. crassiusculus*. A–E from ex holotype culture (C2448, CBS 135864). All photos except 1E by Nomarski interference microscopy of stained material (cotton blue). Bar = 10 μm, except for 1F (= 100 μm).

1300 and 1000 CFU of the *Ambrosiella* sp. respectively, and no other filamentous fungus was isolated. The two beetles that were not surface-sterilized yielded 400 and 600 CFU of the *Ambrosiella* sp. per beetle respectively, again with no other filamentous fungi isolated. Yeasts also were isolated from three of these four beetles. The yeast colonies from two of these beetles had LSU rDNA sequences that matched most closely (535 out of 542 bp matching) the LSU sequence of *Cyberlindnera xylebori* Ninomiya, Mikata, Kajimura & Kawasaki (AB534167), and the yeast from the third beetle had a LSU sequence that matched exactly (523 bp) that of *Candida silvanorum* van der Walt, Klift & DB Scott (U71068).

One of two *X. crassiusculus* excavated from tunnels in *Litsea aestivalis* with laurel wilt in Clyo yielded 1700 CFU of an *Ambrosiella* sp. (as well as yeasts), but a second beetle from Clyo yielded only yeasts at more than 1000 CFU. A single *X. crassiusculus* female excavated from a gallery in a sassafras tree with laurel wilt on Hunting Island, South Carolina, yielded more than 400 CFU of the new *Ambrosiella* spp. and approximately 700 CFU of a yeast. No attempt was made to identify the yeasts.

Isolations were attempted from five *X. crassiusculus* females that were trapped May 2013 in Boone County, Missouri. The first two beetles were not surfaced-sterilized before grinding and plating, and these dilution plates had high numbers of bacterial colonies, presumably because the beetles were left in water in the field for up to 1 wk until collection. Nonetheless, one of these beetles yielded more than 2200 CFU of an *Ambrosiella* sp. The second beetle yielded numerous colonies of an *Ambrosiella* sp., but the colonies were too numerous to count on 100× dilution plates (> 5000 CFU per beetle). Three *X. crassiusculus* similarly trapped at the same location 2 wk later were surface-sterilized for 60 s before grinding, and two of these yielded an *Ambrosiella* sp. at 333 and 500 CFU per beetle respectively and no other fungus, while the third beetle yielded no fungus.

Analysis of rDNA sequences.—The aligned LSU rDNA dataset was analyzed to place the new species among other *Ambrosiella*, *Thielaviopsis* and *Ceratocystis* species (FIG. 2). In the alignment of 587 characters (including gaps), 439 characters were constant, 31 were parsimony uninformative and 117 were parsimony informative. There were 17 most parsimonious trees of 257 steps, with a homoplasy index (HI) of 0.2996 using all characters (HI = 0.3407 excluding uninformative characters), retention index (RI) = 0.9007 and rescaled consistency index (RC) = 0.6357. One of the 17 trees is illustrated (FIG. 2). The likelihood tree (not shown) from the Bayesian

analysis was similar in respect to the *Ambrosiella* spp., *C. adiposa* (EJ Butler) C. Moreau, *C. fagacearum* (TW Bretz) J Hunt and the Moniliformis complex. Bootstrap values and posterior probability estimates both indicated strong support for the above grouping as well as for the branch grouping *C. adiposa* with all *Ambrosiella* spp. except *A. ferruginea* (Math.-Käärik) LR Batra (FIG. 2). The LSU sequences of two isolates of *A. ferruginea* from two species in tribe Xyloterini were similar to each other and differed somewhat from those of the other *Ambrosiella* spp.

Each of the five studied *Ambrosiella* spp. had a unique LSU sequence. The new species was found to be closely related to other symbionts of the Xyleborini, which are native to Africa, Asia and/or Europe but are all established in eastern USA: *A. xylebori* (from *X. compactus*, a native of Asia and Africa), *A. hartigii* LR Batra (from *Anisandrus dispar* [Frabricius], a native of Europe) and *A. beaveri* Six, de Beer & WD Stone (from *C. mutilatus* [Blandford], a native of Asia). The DNA extracted from dense fungal sporulation in a gallery of *X. amputatus* (Blandford) (another Asian species, Cognato et al. 2011) collected in Georgia also was used for PCR and sequencing, and the generated ITS and LSU sequences were identical to the sequences of the *A. beaveri* culture taken from the holotype (Six et al. 2009).

The ITS rDNA sequences of 11 tested isolates of the new species were identical except for a series of Ts at the end of the ITS2 region. There was a repeat of seven Ts in most isolates (KF669871), but isolate C2415 had eight Ts and isolates C2451 (KF669872) and C2454 had nine Ts. The ITS sequences of the new species were similar to but distinct from those of *A. xylebori* (KF669874), *A. hartigii* (KF669873), *A. beaveri* (KF669875) and *C. adiposa* (AF275545). Compared to the *A. xylebori* ITS sequence (KF669874), the ITS sequence of the new species differed in only three single-base substitutions in ITS1, but the two species differed at 14 and eight base positions associated with indels in ITS1 and ITS2 respectively. Because most of the differences among *Ambrosiella* spp. were due to indels of mononucleotide repeats or larger indels, and attempted alignments of ITS sequences had much ambiguity, there was little reliable phylogenetic signal, and analysis of ITS rDNA sequences is not presented. Nonetheless each of the studied *Ambrosiella* spp. had unique and diagnostic ITS sequences.

Fungal spores in mycangia.—The mycangial contents dissected from the mesothorax of a female *X. crassiusculus* trapped in Missouri was a mass of arthrospore-like cells, 5.5(4.0–9.0) × 4.0(3.5–5.0) μm (FIG. 1H–I). The cells appeared to elongate at one or both ends with subsequent delimitation of
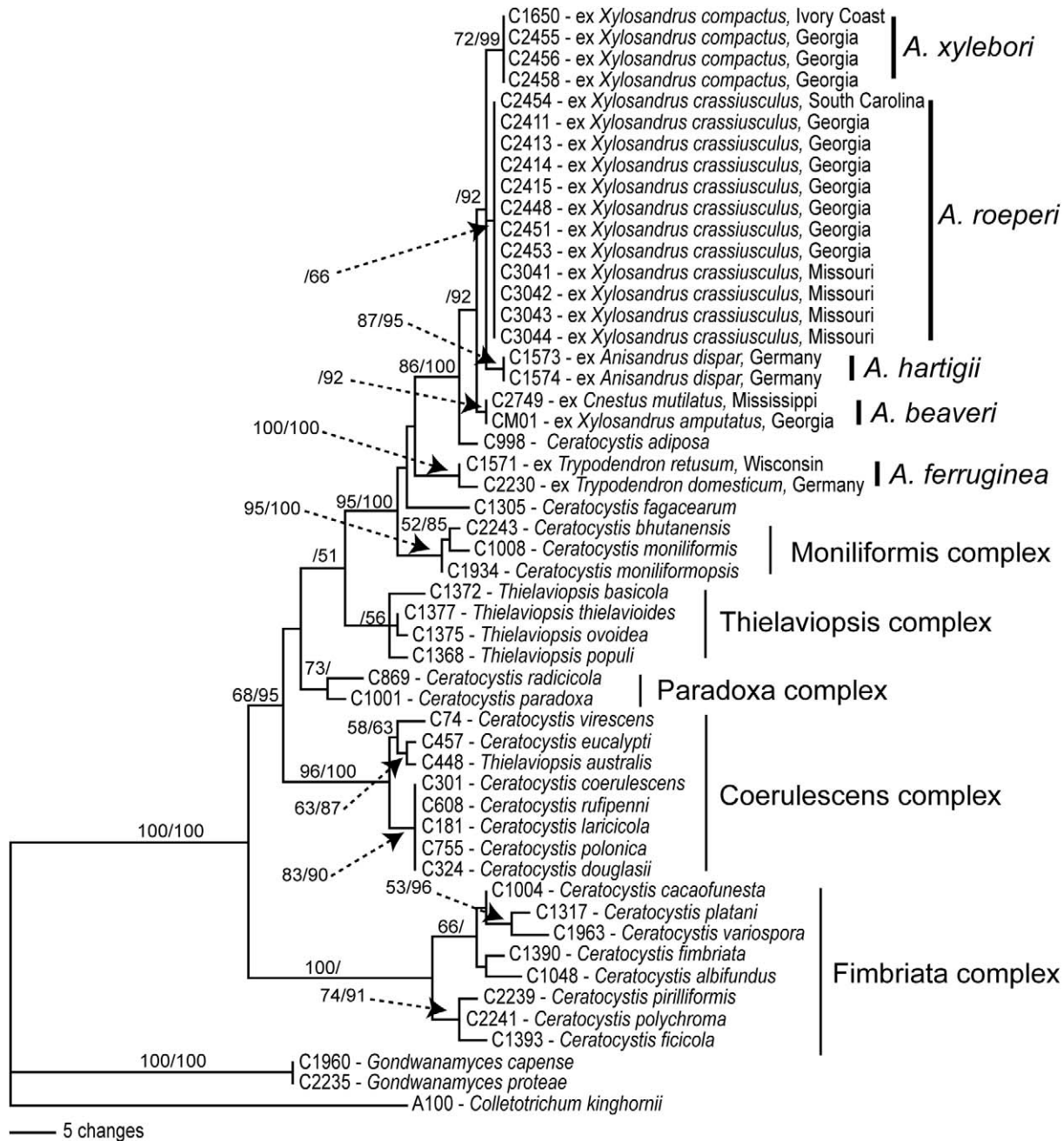
FIG. 2. One of 17 most parsimonious trees of representative *Ceratocystis*, *Thielaviopsis* and *Ambrosiella* spp. based on partial sequences of 28S (LSU) rDNA. Numbers associated with branches indicate support (> 50%) based on bootstrap replications/ posterior probability estimates.

cross walls, and up to four cells appeared to be linearly connected. Individual cells often were curved at one end, perhaps where the cell was expanding, and the wall less rigid. The mycangia of the two *X. germanus* beetles were similarly packed with fungal cells, although these cells were slightly larger: 6.5(4.5– 9.0) × 5.0(3.5–7.0) μm.

Partial sequences of the 5.8S rDNA and the entire sequences of ITS2 were obtained with extracted DNA

of a second ethanol-preserved specimen of *X. crassiusculus* and two specimens of *X. germanus* from Missouri using the *Ceratocystis*-specific primer Cerato3.7 and ITS4. The ITS2 sequence of the *X. crassiusculus* specimen was identical to that of the pure cultures of the new species of *Ambrosiella*. The ITS2 sequences from the two *X. germanus* specimens were identical and closely matched two GenBank accession numbers (HQ538467, HQ670423) of a

Ceratocystis sp. from *X. germanus* in Korea, whose ITS1 and ITS2 sequences differed somewhat from those of isolates CBS 403.82 and CBS 404.82 of *A. hartigii* from *Anisandrus dispar* (KF669873).

Microscopic examination of the mycangium or mycangium contents of 11 *X. crassiusculus* trapped in Georgia in 2013 revealed fungus-like material in nine of these (FIG. 1F–G). The tightly packed fungal material was homogeneous and appeared identical to the contents of the mycangium of the beetle trapped in Missouri. No bacterium-like cells were observed. After microscopic examination, DNA was extracted from relatively large mycangium spore masses recovered from six of the beetles. Using the fungal-specific primer ITS1-F and ITS4, four of these six DNA extractions yielded PCR products that were visible in ethidium bromide-stained agarose gels. Attempts to sequence these four products using the same primers produced easily read electropherograms of a single sequence (no minor peaks seen) in three of the cases, while the fourth yielded insufficient DNA for clear sequencing. The three sequences matched the ITS rDNA sequence of the new species.

TAXONOMY

Ambrosiella roeperi TC Harr. & McNew, sp. nov.

FIG. 1

MycoBank MB805798

Colonies 65–70 mm diam after 6 d at 25 C on malt yeast extract agar, leading margin smoke gray, center white to buff to vinaceus buff, with scattered light brown to rust-colored liquid drops on the surface of the mycelium, which become dark brown to black with age. Underside of colony dark brown to black, odor strong and sweet at 4–10 d, fading in older cultures. Sporodochia (FIG. 1A) sometimes forming on gray mycelium, superficial, white to buff, with a brown liquid on surface. Conidiophores borne singly or arising in small groups from sprout cells, hyaline to light brown, 10–80 μm tall, composed of unbranched or branched chains of swollen cells that may be filled with a rust-brown material and thick-walled at maturity; branches ending with a single, terminal aleurioconidium (FIG. 1A–D). Aleurioconidia terminal, solitary, enteroblastic, from an inconspicuous, subtending collarette (FIG. 1C), obglobose to globose, thick-walled, smooth, aseptate, hyaline to light brown with age, may be filled with rust-brown material, 9–15(18) × 9.5–14(17) μm, thick-walled at maturity (FIG. 1D). Aleurioconidia breaking from the conidiophore, attached to one or two swollen conidiophore cells (FIG. 1E).

Etymology: Named after Richard A. Roeper for his work connecting ambrosia beetles, mycangia and their fungal symbionts.

Specimens examined: USA. GEORGIA: Charlton County, Winokur, dried culture of *CBS 135864*, isolated from *Xylosandrus crassiusculus*, beetle collected 12 Mar 2007, *S. Fraedrich, BPI892693* (HOLOTYPE), *ISC450860* (ISOTYPE).

Cultures examined: USA. GEORGIA: Charlton County, Winokur, isolated from *Xylosandrus crassiusculus*, 20 Mar 2007, *S. Fraedrich, CBS 135864 (C2448); CBS 135863 (C2415)*; Clyo, *Xylosandrus crassiusculus* removed from *Litsea aestivalis*, 18 Apr 2007, *S. Fraedrich, CBS 135865 (BPI892694, C2451)*; *C2453*. SOUTH CAROLINA: Hunting Island State Park, *Xylosandrus crassiusculus*, 18 Apr 2007, *S. Fraedrich*, C2454: MISSOURI: Boone County, *Xylosandrus crassiusculus*, May 2013, *S. Reed, C3041; C3042; C3043; C3044*. OHIO: Eaton, *Xylosandrus crassiusculus*, Jun 2013, *R. Roeper, C3129*.

Notes: The symbiont of *X. crassiusculus* produces only one spore type in culture, that is, terminal spores that appear to be homologous to the enteroblastic aleurioconidia produced by many *Ceratocystis* spp. (Paulin-Mahady et al. 2002). Deep-seated phialides producing basipetal chains of conidia, as illustrated for *A. beaveri* (Six et al. 2009), were not seen in our cultures of *A. roeperi* or in our sporulating culture (C2455) of *A. xylebori*. No culture of *A. trypodendri* (Harrington et al. 2010) was available, and our cultures of *A. hartigii* (CBS 403.82) and *A. ferruginea* (CBS 408.68) no longer were sporulating well. However, Batra's (1967) illustrations suggest that these three species produce conidia in basipetal chains from conidiophores similar to those of *A. beaveri* and distinct from the terminal aleurioconidia of *A. roeperi* and *A. xylebori*. Terminal aleurioconidia on conidiophores with swollen cells were seen in a culture (CBS 110.61) of *A. xylebori* from the type material in the Ivory Coast and in our isolate (C2455) from *X. compactus* in Georgia. In contrast to *A. roeperi*, *A. xylebori* isolates, including C2455, also produce terminal aleurioconidia on a second type of conidiophore with narrow, hyphal-like branches (von Arx and Hennebert 1964, Batra 1967, Gebhardt et al. 2005), and it is assumed that these hyphal-like conidiophores do not disarticulate.

DISCUSSION

Ambrosiella roeperi was the only filamentous fungus recovered consistently in high numbers of CFU from *X. crassiusculus* females. Isolation attempts generally yielded several hundred to thousands CFU per beetle, and the fungus was recovered from surface-sterilized females, suggesting that it was growing within the mycangium and not just a superficial contaminant on the exoskeleton. Beetles collected in four states yielded the same *Ambrosiella* sp. based on LSU

sequences, which did not vary among the tested isolates, and the ITS rDNA sequences were nearly identical. Microscopic examinations consistently revealed a homogeneous mass of fungal spores in mycangia of *X. crassiusculus*, and PCR and DNA sequencing confirmed that the spores were those of a single species, *A. roeperi*.

Ambrosiella roeperi is probably the unidentified *Ambrosiella* (sp. 2) isolated from mycangia of *X. crassiusculus* in Japan (Kinuura 1995). Gebhardt et al. (2005) illustrated conidiogenesis of an *Ambrosiella* sp. (identified as *A. xylebori*) from *X. crassiusculus* in Taiwan, showing terminal conidia from phialides with small collarettes and swollen conidiophore cells, identical to the terminal aleurioconidia and conidiophores of *A. roeperi*. Brader (1964) noted the similarity of the *X. crassiusculus* symbiont in Africa to *A. xylebori* but considered the two fungi distinct. The association of *A. roeperi* and *X. crassiusculus* appears to be consistent and specific, and it is likely that this economically important Asian and African beetle brought its fungal symbiont with it to USA, where it is now well established (Rabaglia et al. 2006).

In microscope slides prepared from cultures of both *A. roeperi* and *A. xylebori*, individual aleurioconidia were not found free but instead the aleurioconidia were attached to one or two swollen conidiophore cells. The disarticulating conidiophore cells may be an adaptation for beetle grazing, wherein the conidiophore breaks apart at the junction of the swollen conidiophore cells. The conidiophore may regrow to produce more swollen cells and new terminal aleurioconidia. The conidiophore cells and the aleurioconidia were filled by what appeared to be lipid bodies, which may be of nutritional benefit to feeding larvae and adults. Ergosterol is thought to be of important nutritional benefit to ambrosia beetles (Norris 1976, Kok 1979), and ergosterol precursors and enzymes associated with sterol metabolism are associated with lipid bodies in yeast (Müllner and Daum 2004).

The disarticulating conidiophore cells with terminal aleurioconidia also may be inoculum for entry into the mesonotal mycangium. After spreading over MYEA, both the aleurioconidia and the subtending conidiophore cells germinated. Kaneko (1967) illustrated loose aleurioconidia with attached conidiophore cells in galleries of *X. compactus* and *X. germanus*. He also observed that when young females of these species moved their heads, it caused the mycangium to invert and protrude from the body. Spores of the *Ambrosiella* spp. stuck to the protruding mycangium and were brought into the mycangium as it was brought back into the mesonotum. The mycangium of *X. crassiusculus* similarly appears to have a dorsal opening where the mycangium is attached to the pronotum and mesonotum (scutellum), which may allow for its inversion (protrusion) for spore acquisition. Fungal sporulation in the galleries of *X. crassiusculus* and other ambrosia beetles is typically a mixture of different microbes (Batra 1967, Kinnuura 1995), but selective growth of *Ambrosiella* spp. in the mycangium may proceed due to excretions by gland cells near the mycangial opening (Batra 1963, Francke-Grosmann 1967, Beaver 1989). Kinuura (1995) isolated *Ambrosiella* sp. 2 from mycangia of few callow (immature) *X. crassiusculus* females, but mature and dispersing females yielded only this fungus. The mycangial contents of trapped females of both *X. crassiusculus* and *X. germanus* were easily squeezed out of filled mycangia through the dorsal opening, and it is assumed that sporulation in the mycangium leads to an overflow of spores and continual inoculation of gallery walls by boring females. The *X. crassiusculus* mycangia were densely and uniformly packed with tightly adhering masses of fungal spores of similar morphology, with the spores in *X. germanus* mycangia similar in shape but slightly larger. The number of propagules in the mycangium appeared to be greater than the CFU of *A. roeperi* recovered from ground beetles, perhaps because the spores adhere tightly together and do not easily separate by grinding.

Microscopic examinations and isolations from *X. crassiusculus* females suggest that the cells within the mycangium are exclusively those of *A. roeperi*. Amplification and sequencing of ITS rDNA of the mycangial contents with general fungal or *Ceratocystis*-specific primers produce only a single DNA sequence; that of *A. roeperi*. Yeasts were isolated in significant numbers from some *X. crassiusculus* females, but these yeasts were likely in the gut of the insects (Harrington and Fraedrich 2010). No *Fusarium* sp. was recovered from *X. crassiusculus* in our isolations, in contrast to the report by Hulcr and Dunn (2011). Hulcr et al. (2012) reported little bacterial DNA associated with the mycangia of *X. crassiusculus* using amplification of bacterial 16S rDNA extracted from whole mycangia, but no 16S rDNA was amplified from *X. germanus* mycangia. Our microscopic examinations did not reveal bacteria cells among the contents of the mycangia of *X. crassiusculus* or *X. germanus*.

Six species are now recognized in *Ambrosiella*. No culture of *A. trypodendri* is available (Harrington et al. 2011), but phylogenetic analyses place the other species within the genus *Ceratocystis*. However, the *Ambrosiella* species may not be a monophyletic group based on sequences of LSU-rDNA, beta-tubulin and translation elongation factor 1α (Paulin-Mahady et al. 2002, Harrington 2009, Kolarik and Hulcr 2009, Massoumi Alamouti et al. 2009, Six et al. 2009). The

Trypodendron (tribe Xyloterini) symbiont *A. ferruginea* consistently groups separate from the other *Ambrosiella* spp. It is assumed that *A. trypodendri* is closely aligned with *A. ferruginea* because it is a symbiont of *Trypodendron scabricollis* (LeConte) (Batra 1967).

The other species (*A. hartigii, A. xylebori, A. beaveri, A. roeperi*) appear to be a monophyletic group close to *C. adiposa*, which is not a known associate of ambrosia beetles (Harrington 2009). The four *Ambrosiella* spp. are symbionts of *Xylosandrus, Anisandrus* and *Cnestus* spp., a monophyletic group of genera within the Xyleborini with large, mesonotal mycangia (Dole et al. 2010, Dole and Cognato 2010, Hulcr and Cognato 2010). The *X. compactus* associate, *A. xylebori*, is also the reported symbiont of *Corthylus columbianus* and *C. punctatissimus* (Zimmermann) (tribe Corthylini) (Batra 1967, Roeper 1995).

Thus far only a single *Ambrosiella* sp. has been isolated from the mycangium of a given ambrosia beetle species, so the relatively large and developed mycangia found in these genera (Roeper et al. 1980, Roeper 1995) appear to be selective and the symbiosis a tight association. More detailed study might find that there are many cryptic species in *Ambrosiella* that are associated with different species of *Xylosandrus, Anisandrus, Cnestus, Corthylus* and *Trypodendron*. However, the ITS sequence obtained from the fungal growth in a *X. amputatus* gallery in Georgia was identical to that of *A. beaveri*, which is the symbiont of *Cnestus mutilatus* (Six et al. 2009). Like *C. mutilatus, X. amputatus* is an Asian species that has been recently introduced into southeastern USA (Cognato et al. 2011).

The large and apparently selective mycangia found in the above genera, and their consistent relationship with a single, primary ambrosia fungus, is in contrast to the much looser association of *Raffaelea* spp. with ambrosia beetles that have smaller, less-developed mycangia in the tribes Xyleborini, Xyloterini and Corthylini and the subfamily Platypodinae (Coleoptera: Curculionidae). *Raffaelea canadensis* LR Batra has been associated with both Platypodinae and Corthylini (Harrington et al. 2010) and *R. montetyi* Morelet with both Platypodinae and Xyleborini (Gebhardt et al. 2004). *Raffaelea* spp. produce small, yeast-like cells in the mycangia of their ambrosia beetle symbionts (Fraedrich et al. 2008), and numerous species apparently are capable of growth in the mycangium of an individual beetle species. In the well studied redbay ambrosia beetle (*Xyleborus glabratus*), nine species of *Raffaelea* have been isolated in high numbers of CFU, with up to four *Raffaelea* spp. isolated from an individual female beetle (Harrington and Fraedrich 2010, Harrington et al. 2011). Some ambrosia beetle species in genera that would be expected to be associated with *Raffaelea* spp. also may have symbiotic relationships with *Fusarium ambrosium* (Gadd & Loos) Agnihothr. & Nirenberg (syn. *Microsporium ambrosium* Gadd & Loos) and related *Fusarium* spp. (Batra 1967, Kasson et al. 2013).

There has been concern that ambrosia beetles other than *Xyleborus glabratus* could serve as vectors of the aggressive vascular wilt pathogen, *Raffaelea lauricola* (Harrington et al. 2008, Harrington and Fraedrich 2010, Carillo et al. 2013). In southeastern USA, *X. crassiusculus* is one of the most common ambrosia beetles attacking trees with laurel wilt (Fraedrich et al. 2008), but our isolation attempts from many genera of ambrosia beetles and the literature indicate that ambrosia beetles with *Ambrosiella* symbionts do not carry *Raffaelea* spp. in their mycangia (Harrington et al. 2010, Harrington and Fraedrich 2010). When a whole female *X. crassiusculus* from a gallery in a sassafras tree with laurel wilt was plated directly on MEA, *R. lauricola* was isolated (Harrington et al. 2010) but no *Raffaelea* species has been isolated in our dilution platings of ground *X. crassiusculus* females, even females excavated or reared from trees with laurel wilt (Fraedrich et al. 2011, Fraedrich and Harrington unpubl). Carillo et al. (2013) reported isolation of *R. lauricola* in low CFU from a single female *X. crassiusculus* (out of 39 females tested) reared from a swamp bay (*Persea palustris*) with laurel wilt. However, it seems unlikely that *X. crassiusculus* could serve as an important vector of *R. lauricola* relative to the efficiency of *Xyleborus glabratus* (Harrington and Fraedrich 2010) because the mycangium of *X. crassiusculus* seems to be highly selective for its *Ambrosiella* symbiont.

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