

# First report of a sexual state in an ambrosia fungus: *Ambrosiella cleistominuta* sp. nov. associated with the ambrosia beetle *Anisandrus maiche*

Chase G. Mayers, Thomas C. Harrington, and Christopher M. Ranger

**Abstract:** Genera of ambrosia beetles in the tribe Xyleborini with large, mesonotal mycangia host unique fungal symbionts in the genus *Ambrosiella*. The symbiont of a recent invasive to the USA from Asia, *Anisandrus maiche* Stark, had not been previously characterized. We found the mycangium anatomy of *An. maiche* collected in Ohio to be similar to that of *Anisandrus dispar* and consistently isolated a novel fungus, *Ambrosiella cleistominuta* sp. nov., from *An. maiche* mycangia and galleries. The fungus was distinguished from other named *Ambrosiella* by morphological characters and DNA sequences (ITS rDNA and *tef-1 $\alpha$* ). The mycangial symbionts of ambrosia beetles had been assumed to be strictly asexual, but *A. cleistominuta* produces cleistothecious ascomata with ascospores in beetle galleries and in culture. In contrast to ascomata of other *Ceratocystidaceae*, the relatively small ascomata of *A. cleistominuta* are neckless and without ostioles. The ascospores are relatively large, and single-ascospore colonies produced ascomata and ascospores in culture, showing that *A. cleistominuta* is homothallic.

**Key words:** ambrosia, *Ceratocystidaceae*, Xyleborini.

**Résumé :** Les genres de scolytes du bois de la tribu des Xyleborini possédant de larges mycangia mésonotales hébergent les symbiotes fongiques uniques du genre *Ambrosiella*. Les symbiotes d'une espèce qui a récemment envahi les USA en provenance d'Asie, *Anisandrus maiche*, n'ont pas été caractérisés jusqu'à présent. Les auteurs ont trouvé que l'anatomie du mycangium de *An. maiche* récolté en Ohio était similaire à celle de *Anisandrus dispar* et ont systématiquement isolé un nouveau champignon, *Ambrosiella cleistominuta* sp. nov., des mycangia et des galeries de *An. maiche*. Le champignon était distinct d'autres identifiés *Ambrosiella* selon des caractères morphologiques et les séquences d'ADN (ITS de l'ADNr et *tef-1 $\alpha$* ). Les symbiotes des mycangia des scolytes du bois ont été présumés strictement asexués, mais *A. cleistominuta* produit des ascomes à cleistothèce comprenant des ascospores dans les galeries du scolyte et en culture. Contrairement aux ascomes d'autres *Ceratocystidaceae*, les ascomes relativement petits de *A. cleistominuta* sont sans col et sans ostioles. Les ascospores sont relativement grandes, et les colonies d'une seule ascospore produisaient des ascomes et des ascospores en culture, montrant que *A. cleistominuta* est homothallic. [Traduit par la Rédaction]

**Mots-clés :** scolyte, *Ceratocystidaceae*, Xyleborini.

## Introduction

*Anisandrus maiche* Stark (Coleoptera: Curculionidae: Scolytinae) is an Asian ambrosia beetle that has recently invaded the USA and Europe (Rabaglia et al. 2009; Terekhova and Skrylnik 2012). Adult female *A. maiche* have been recovered from flood-stressed *Cornus florida* L. trees attacked in Ohio, USA (Ranger et al. 2015), and could present a similar risk to ornamental and horticultural trees as other invasive Xyleborini (Ranger et al. 2016). Based on studies of other *Anisandrus* spp. (Mayers et al. 2015), a species of

*Ambrosiella* Arx & Hennebert emend. T.C. Harr. (Microascales: Ceratocystidaceae) would be expected to serve as a mycangial symbiont of *An. maiche*.

Ambrosia beetles cultivate fungal gardens along the walls of galleries tunneled in sapwood, and larvae and adults feed on crops of conidia and conidiophores as their food source (Batra 1967; Harrington et al. 2010). Ambrosia beetles are polyphyletic (Kirkendall et al. 2015), and tribes of ambrosia beetles have independently evolved special organs (mycangia) to transport their symbionts to

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new trees (Batra 1967; Francke-Grosman 1967; Beaver 1989; Six 2012). The adult beetles secrete nutrients into the mycangium to support active growth of the symbiont, and the overflow of fungal propagules leads to inoculation of newly excavated galleries (Beaver 1989). Mycangia of various genera and tribes of ambrosia beetles vary considerably in size, shape, and location on the body (Francke-Grosman 1967; Beaver 1989), and their primary fungal symbionts also vary (Harrington et al. 2010, 2014; Mayers et al. 2015).

The Xyleborini genera *Anisandrus*, *Cnestus*, *Eccoptopterus*, *Hadrodemius*, and *Xylosandrus* form a monophyletic group with relatively large mesonotal (mesothoracic) mycangia (Hulcr and Cognato 2010). These mycangia are formed by a deep invagination of the intersegmental membrane between the scutellum and pronotum (Francke-Grosman 1956; Happ et al. 1976; Stone et al. 2007). Species of *Ambrosiella* have proven to be the primary fungal symbionts of Xyleborini with large mesonotal mycangia, such as *Anisandrus dispar* F., *Anisandrus sayi* Hopkins, *Cnestus mutilatus* (Blandford), *Eccoptopterus spinosus* (Oliver), *Xylosandrus compactus* (Eichhoff), *Xylosandrus crassiusculus* (Motschulsky), and *Xylosandrus germanus* (Blandford) (Six et al. 2009; Harrington et al. 2010, 2014; Mayers et al. 2015).

The goals of this study were to characterize the mycangium and identify the mycangial symbiont of an invasive population of *An. maiche* established in Ohio, USA. We hypothesized that *An. maiche* would have a mesonotal mycangium similar to that of other *Anisandrus* (Francke-Grosman 1956, 1967; Happ et al. 1976; Hulcr et al. 2007). Further, we expected the mycangium to harbor budding spores of *Ambrosiella*, which would serve as the primary food source of the larvae (Mayers et al. 2015).

## Materials and methods

### Beetle collection

Live *An. maiche* females were collected using ethanol-baited bottle traps deployed at four locations in Wayne County, Ohio: Barnard Road Site, lat. 40°45'41.43"N, long. 81°51'16.88"W; Davey Farm Site, lat. 40°51'53.41"N, long. 82°3'8.80"W; Badger Farm Site, lat. 40°46'38.62"N, long. 81°51'9.34"W; Metz Road Site, lat. 40°52'19.87"N, long. 81°56'26.06"W. Bottle traps were assembled according to Ranger et al. (2010), but moist paper towels were placed in the lower collection bottle rather than low-toxicity antifreeze to maintain beetle and fungal viability (Ranger et al. 2015). Female adults were stored refrigerated in parafilm-sealed Petri dishes with moist filter paper, then killed by crushing the exoskeleton. They were then shipped overnight in glass vials with or without sterile moist filter paper. Male *An. maiche* are flightless and were not collected.

Additional adult females were excavated from naturally infested *Gleditsia triacanthos* L. trees growing in a commercial ornamental nursery in Ohio (lat. 41°49'35.41"N; long. 81°2'27.40"W). Stem sections were refrigerated and

then split using a sterilized hand pruner. Adult female *An. maiche* collected from their host galleries were stored, killed, and shipped as described above.

### Mycangia

Intact mycangia were dissected from beetles and separated from the scutellum with sterile needles, forceps, and razors on glass slides in a manner similar to that described by Batra (1985). Intact mycangia and spore masses teased from intact mycangia were mounted in cotton blue on a microscope slide and observed with Nomarski interference contrast (BH-2 compound microscope, Olympus, Melville, NY) and digitally photographed (Leica DFC295 camera and Leica Application Suite V3.6; Leica Camera Inc., Allendale, New Jersey, USA). For some images, composites of several images taken at the same magnification and focus level were stitched together with the Photomerge function in Photoshop CS6 (Adobe, San José, California, USA) in "reposition" mode with blending enabled.

### Fungal isolations

Intact *An. maiche* females were first surface-sterilized by submerging in 75% ethanol for 10 s, then submerging in two successive baths of sterile deionized water and allowed to dry on paper towels. The beetles were then pulled apart with sterile forceps and the portions containing the prothorax/mesothorax, scutellum, and mycangium were separately plated directly on malt extract agar with streptomycin (SMA; 1% malt extract, Difco Laboratories, Detroit, Michigan, USA; 1.5% agar, Sigma-Aldrich, St. Louis, Missouri, USA; and 100 ppm streptomycin sulfate added after autoclaving). The fungal colonies were subcultured to malt yeast extract agar (MYEA; 2% Difco malt extract, 0.2% Difco yeast extract, 1.5% agar).

### Hyphal tip and single spore cultures

Round, pigmented structures resembling cleistothecia were observed in galleries with ambrosia growth and in one of the cultures on MYEA. Individual spherical structures were removed from the galleries, cleaned by dragging across the surface of sterile malt extract agar (MEA; 1.5% malt extract, 1.5% agar), and then DNA was extracted from these cleaned structures using PrepMan Ultra (Applied Biosystems, Foster City, Calif.).

Single hyphal tip and single-ascospore colonies were obtained from the culture (C3843) that produced spherical bodies on MYEA. Isolated hyphal tips beyond the advancing margin of growth were identified on MYEA using a dissecting scope (at 25× to 40× magnification and substage lighting), excised, and transferred to MYEA. Single-ascospore cultures were obtained by crushing a single, spherical structure in a drop of sterile water on a flame-sterilized glass slide under a sterile coverslip, confirming the presence of the putative ascospores but absence of conidia at 500×, carefully raising the coverslip, and transferring the liquid containing spores to MEA with a micropipette. Individual spores were separated at

25× to 40× using a sterile needle. The isolated spores were allowed to germinate, and spores with a single germ tube were transferred to fresh MYEA plates.

#### Artificially infested stem segments

Live *An. maiche* trapped in-flight at the Badger Farm and Davey Farm locations were allowed to infest *C. florida* stem sections under laboratory conditions that were 1.0–2.5 cm in diameter and 9–10 cm long. Stem sections taken from live trees were soaked in distilled/deionized water for ~18 h, blotted and air-dried for 5 min, and placed in closed plastic containers (13 cm in diameter, 9 cm tall) with moist paper towels. Approximately 12 punctures were placed in the lid for ventilation. Stems were held at room temperature for 14 days at 23 °C and stored refrigerated until dissection. The infested stems were then split open and ambrosia growth within galleries was removed with sterile needles and plated on SMA for isolation or mounted in cotton blue for microscopic observation.

#### Culture description

Isolates from *An. maiche* and *C. florida* were grown at room temperature on MYEA. Agar plugs cut with a #1 cork borer (approximately 3 mm in diameter) were transferred from the leading margin of growth to three MYEA plates, grown at 25 °C for 7 days in the dark, and then the diameter of the colonies was measured and averaged for each isolate. Culture pigmentation/colors were in accordance with Rayner (1970).

#### DNA sequencing and analysis

Extractions of mycelia and spores were as previously described (Mayers et al. 2015), but extractions from some *Ambrosiella* cultures with excessive pigment were performed with the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, Norcross, Georgia, USA). The internal transcribed spacer (ITS) region of the ribosomal DNA, small subunit rDNA (SSU; 18S rDNA), and translation elongation factor 1-alpha (*tef-1α*) were amplified and sequenced as per Mayers et al. (2015). Forward and reverse reads were compared using Sequence Navigator version 1.0.1 (Applied Biosystems). The SSU and *tef-1α* sequences were used as queries in the National Center for Biotechnology Information (NCBI) BLASTn tool.

The ITS sequences of the new species were aligned with those of eight other putative and named *Ambrosiella* (Mayers et al. 2015) in PAUP 4.0bb10 (Swofford 2002). The outgroup taxon was *Ceratocystis adiposa* (E.J. Butler) C. Moreau (DQ318195), a close relative to *Ambrosiella* within the *Ceratocystidaceae* (de Beer et al. 2014; Mayers et al. 2015). The dataset had 557 aligned characters, including gaps, and 106 of the characters were parsimony-informative. Gaps were treated as a fifth state. The analyses used stepwise addition and the tree bisection and reconnection branch-swapping algorithm. Bootstrap support values were obtained by a full heuristic, maximum parsimony, 10 000-replicate bootstrap analysis in PAUP.

## Results

### Mycangium observations

Each of the seven examined female *An. maiche* (three trapped in-flight, and four infesting *G. triacanthos*) had a mesonotal mycangium that opened between the scutellum and pronotum. The prominent dual lobes described for the mycangia of related genera, such as *Cnestus*, *Eccoptyterus*, and *Xylosandrus* (Stone et al. 2007; Harrington et al. 2014; Mayers et al. 2015), were not observed in the *An. maiche* mycangia. Instead, spores were observed in a small, unlobed pouch below the scutellum (Fig. 1), as illustrated in *An. dispar* by Francke-Grosmann (1956, 1958, 1967) and Happ et al. (1976). The scutellum curves ventrally on its anterior side, as in *An. dispar* (Francke-Grosmann 1958), and the dorsal surface is covered with pits, each ornamented on its rim with a single seta (Figs. 1d–1f). The pit setae posterior to the hinge of the scutellum point anteriorly and medially, whereas the pit setae anterior to the scutellum hinge are not as uniform in their direction. The pits often contained one or more fungal propagules (Fig. 1g). A tuft of hairs at the base of the pronotum, often associated with mesonotal mycangia (Hulcr et al. 2007), was present in all females of *An. maiche*, as illustrated by Rabaglia et al. (2009).

The material teased from inside the mycangium was a dense, homogenous mass of fungal propagules (Figs. 1h and 1i), similar in appearance to that of other *Ambrosiella* (Francke-Grosmann 1956; Kaneko and Takagi 1966; Harrington et al. 2014; Mayers et al. 2015). The propagules appeared to proliferate by schizogenous, arthrospore-like growth. Some beetles had external masses of spores associated with the tuft of hairs on the posterior edge of the pronotum (Fig. 1a). The external mass appeared to be composed of germinating propagules with branching hyphae (Fig. 1b).

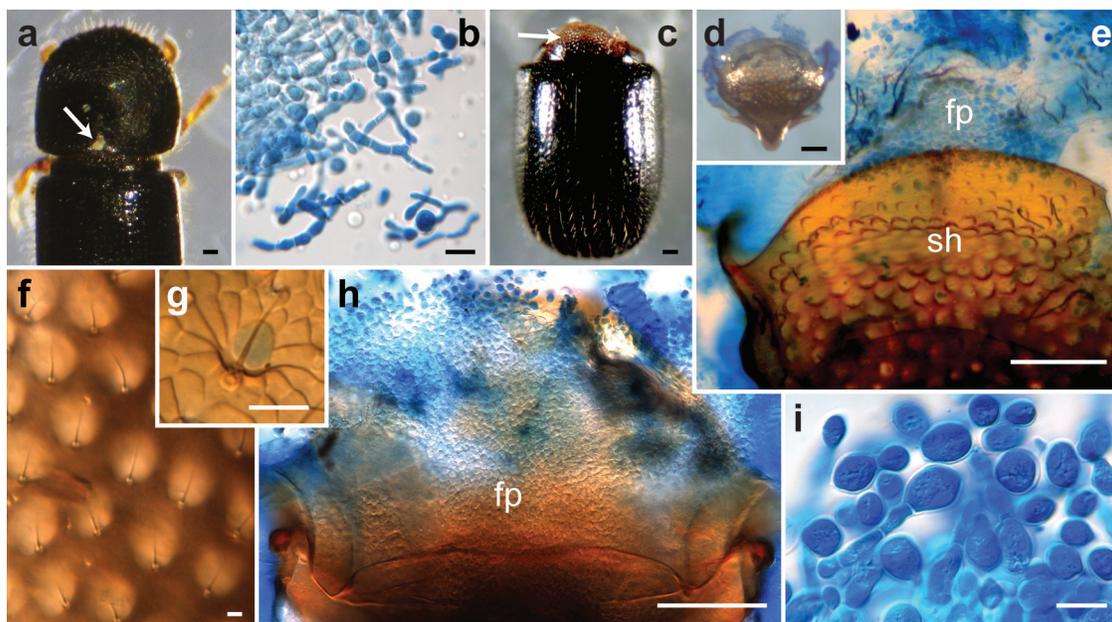
### Fungal isolation and identification

Isolations from dissected mycangia of surface-sterilized *An. maiche* trapped in-flight or taken from infested *G. triacanthos* stems consistently yielded cultures of a fast-growing fungus that produced red-brown aerial hyphae with rust-colored liquid drops. The cultures had a sweet, fruity-ester smell and only rarely sporulated on MYEA.

Twelve of the 13 crushed beetles caught in-flight and shipped with moist filter paper yielded the new fungal species, but only one of the nine beetles shipped without moist filter paper yielded the fungus. Only the new species grew from the mycangia of beetles shipped with moist filter paper, although in some cases other fungi grew from other plated parts of the beetle, such as unidentified yeasts from pieces of the gut. Three of the four plated beetles excavated from *G. triacanthos*, all shipped with moist filter paper, yielded the new fungal species.

Each of the 12 sequenced isolates from *An. maiche* adults and three from galleries in *C. cornus* yielded the same ITS sequence (GenBank KX909940), which differed from *Ambrosiella hartigii* L.R. Batra, the symbiont of *An. dispar*,

**Fig. 1.** Mycangium of *Anisandrus maiche* and mycangial propagules of *Ambrosiella cleistominuta* sp. nov. (a) Exterior spore mass on the posterior margin of the pronotum (arrow). (b) Exterior spore mass with germinating propagules. (c) Female with pronotum removed revealing the light brown scutellum (arrow) protruding from below the mesonotum. (d) Dorsal aspect of excised scutellum. (e) Pits covering scutellum, scutellum hinge (sh), and fungal spores (fp) exiting from the mycangium below. (f) Detail of scutellum pits with rim setae. (g) Fungal propagule in shallow pit with setae above. (h) Ventral aspect of scutellum showing mycangium pouch full of fungal spores (fp). (i) Detail of fungal propagules growing in mycangium. Photos a, c, and d taken through a dissecting microscope. All other photos were taken by Nomarski interference microscopy of material stained with cotton blue. Scale bar = 10  $\mu\text{m}$  in b, f, g, and i. For all other photos, the scale bar = 100  $\mu\text{m}$ . [Colour online.]



by an additional T near the end and a repeated AATT at the very end of ITS2. The new species formed a strongly supported clade with *A. hartigii* separate from other *Ambrosiella* in phylogenetic analysis (Fig. 2).

Isolates C3843 and C3924 from *An. maiche* had identical *tef-1 $\alpha$*  and SSU sequences. Sequences for both gene regions confirmed placement of the new species within *Ambrosiella*. The trimmed *tef-1 $\alpha$*  sequence (KX925309) was 1168 bases long and included a 107-bp intron; it was most similar (1154/1167 bp matching) to the *tef-1 $\alpha$*  sequence of *A. hartigii* (KT318383.1). The trimmed SSU sequence of the new species (KX925304) was 1657 bases long and was most similar to the SSU sequence of *Ambrosiella grosmanniae* C. Mayers, McNew, & T.C. Harr. (KR673884, 1655/1655 bp matching) and *A. hartigii* (KR673885, 1653/1655 bp matching).

#### Culture morphology

Conidiophores (Figs. 3a–3h) of the new species were rare in culture but were morphologically similar to those of *A. hartigii* and *Ambrosiella batrae* C. Mayers, McNew, & T.C. Harr. (Mayers et al. 2015). Two types of conidiophores were observed, but intermediate forms were seen. Phialoconidiophores (Figs. 3a and 3b) were usually composed of multibranched, moniloid hyphae and moderately seated phialides that produced cylindrical to barrel-shaped phialoconidia in chains. Aleurioconidiophores (Figs. 3c–3h) produced globose, thick-walled aleurioconidia from what appeared to be very shallow phialides, often with inconspicuous collarettes. Aleurioconidiophores

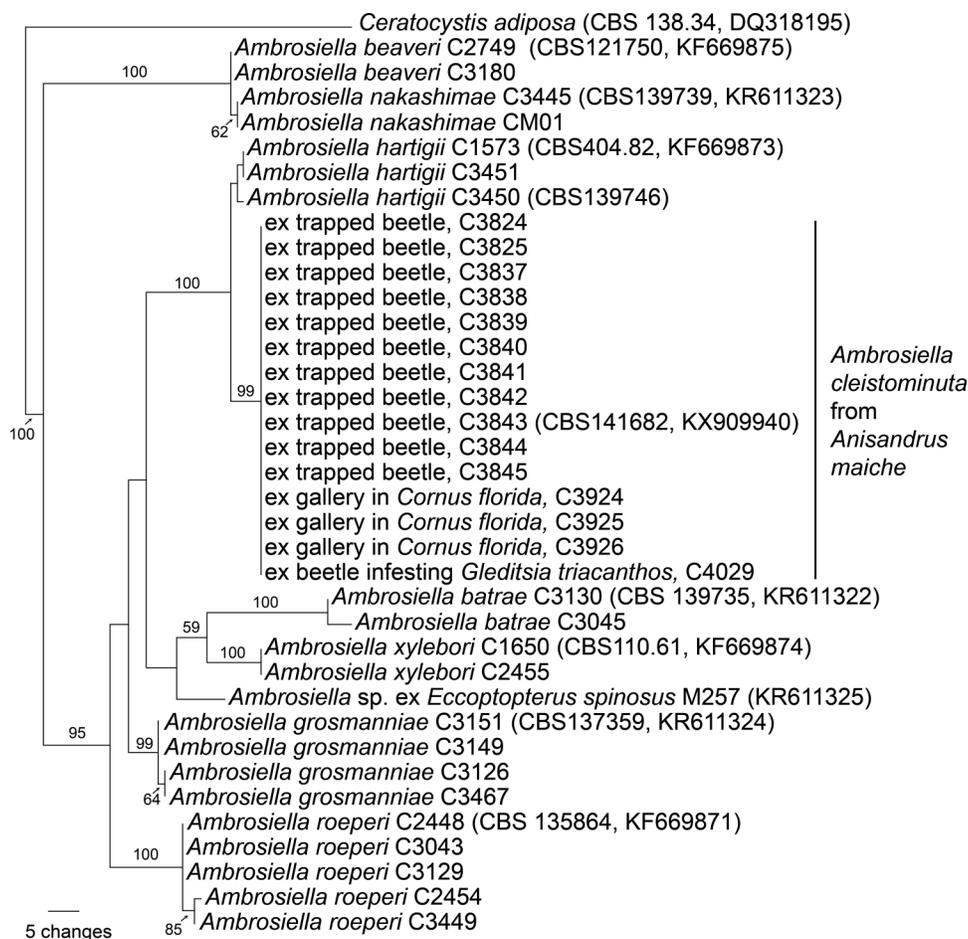
usually produced a single terminal aleurioconidium (Figs. 3c–3e), but occasionally chains of lightly pigmented aleurioconidia surrounded by a membranous sheath (Fig. 3f) and (or) red-brown pigment (Figs. 3g and 3h) were seen.

#### Ascomata

None of the studied isolates of the new species initially produced ascomata. However, after several serial transfers of isolate C3843, a sector produced thick, white, fluffy aerial mycelia with many small, brown spherical structures in the aerial mycelium (Fig. 4). The fluffy white phenotype and the production of the spherical structures persisted through several serial transfers when grown on MYEA, but not on MEA. The spherical structures were small (40–80  $\mu\text{m}$  diameter) and lacked ostioles or necks, and were first assumed to be protoperithecia, as reported in *Ambrosiella nakashimae* McNew, C. Mayers & T.C. Harr. (Mayers et al. 2015). However, microscopic examination of crushed spheres revealed reniform spores of uniform size and shape (Figs. 4h–4m), sometimes found in pairs (Fig. 4k), as has been found with ascospores of other *Ceratocystidaceae* (Van Wyk et al. 1993).

Five hyphal tip colonies from C3843 and five colonies derived from single spores teased from the spherical structures each produced white, fluffy mycelia and the spherical fruiting bodies with reniform spores. The ITS sequence obtained from two cleaned ascomata, a hyphal tip colony, and a single-ascospore colony were identical

**Fig. 2.** One of 12 most parsimonious trees of *Ambrosiella* spp. produced by unweighted maximum parsimony analysis of an ITS rDNA dataset of 557 aligned characters, 106 of which were parsimony-informative. Branch support values are from 1000 bootstrap replications. The outgroup was *Ceratocystis adiposa*. Species names or sources are followed by isolate numbers from the Iowa State University collection. Accession numbers for the Centraalbureau voor Schimmelcultures and GenBank are given in parentheses, where available.



to the original C3843 culture and to the other isolates of the new species.

#### Gallery growth

Some of the *C. florida* stem segments infested by *An. maiche* had only short, abandoned galleries, which contained neither brood nor ambrosia growth. However, three stem segments had one or more galleries that ran along the pith, and each gallery had larvae and (or) pupae and luxurious, white ambrosia growth (Fig. 4a). Phialoconidiophores and aleurioconidiophores, identical to those in culture, dominated the galleries, though there was also limited sporulation of unidentified contaminating molds. Isolates obtained from the ambrosia growth in each of the three stems had the ITS sequence and culture morphology of the isolates from individual beetles (Fig. 2).

Buried in the ambrosia growth of the three stem segments were spherical fruiting structures without necks or ostioles, identical to those seen in culture but slightly larger, bearing reniform spores (Figs. 4a–4c). Where the ambrosial growth had been grazed by larvae, the spheres were open, irregular hemispheres, with edges of the

dark, pigmented outer walls flush with the surrounding grazed mycelium. Pale yellow-brown spore masses were visible inside the cup-like remains of the spheres (Fig. 4a). It appeared that the spheres were broken or chewed open by the grazing of larvae because the white, ungrazed growth had only intact spheres, which were buried in the ambrosia growth (Fig. 4a).

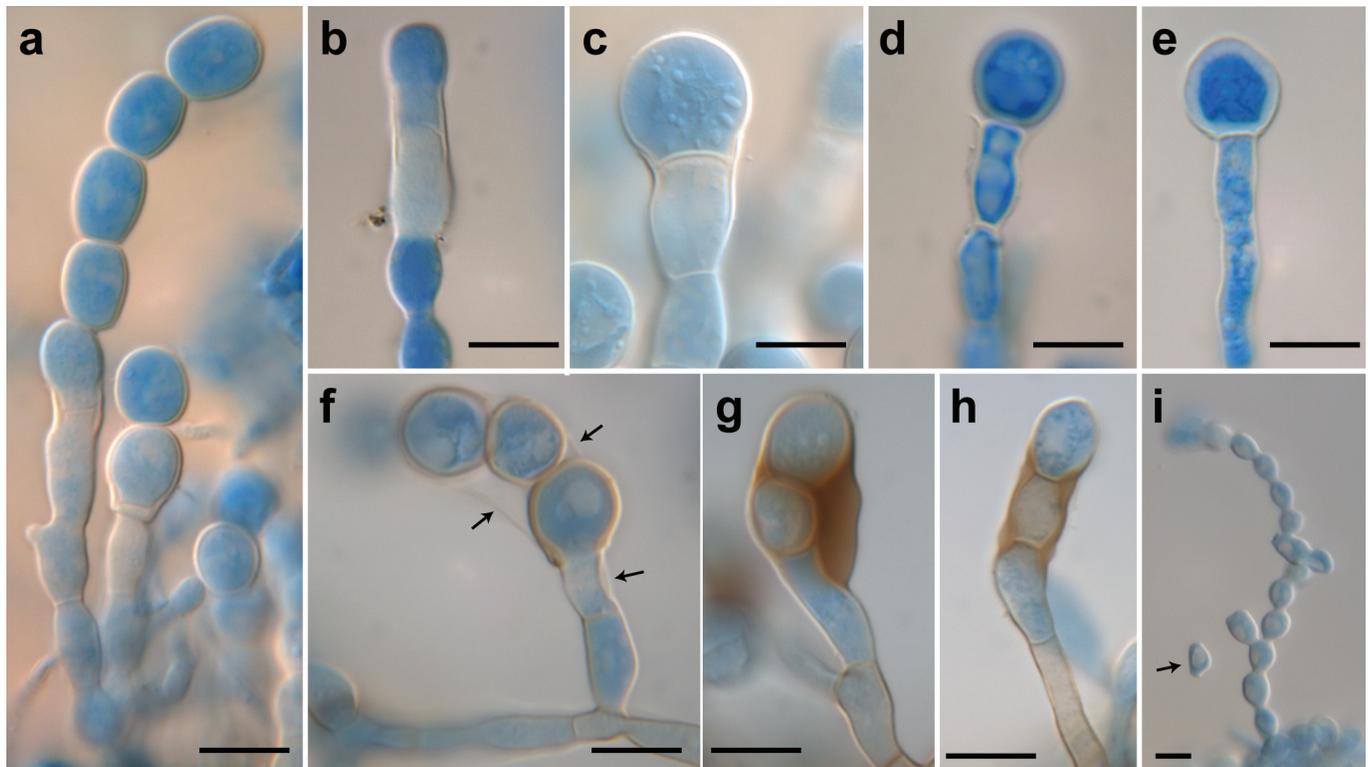
#### Taxonomy

Morphological characters and DNA sequence analyses supported the recognition of the symbiont of *An. maiche* as a new species of *Ambrosiella*.

***Ambrosiella cleistominuta*** C. Mayers & T.C. Harr. sp. nov. (Figs. 1, 3, and 4)  
MycoBank #: 819507.

TYPE: United States of America. Ohio: Wayne County, near Barnard Rd, a dried culture isolated from an *An. maiche* female caught in-flight, 40°45'41.43"N, 81°51'16.88"W, 8 July 2015, coll. C. Ranger (BPI 910177, holotype; CBS 141682 = C3843, extype culture). GenBank ITS rDNA sequence accession No. KX909940.

**Fig. 3.** Conidiophores and conidia of *Ambrosiella cleistominuta* sp. nov. (a, b). Phialoconidiophores. (a) Bearing chained phialoconidia. (b) Deeply seated phialide on monillioid hyphae. (c–h) Aleurioconidiophores. (e) On simple hyphae. (f) With membranous sheath (arrows). (g and h) With pigment. (i) Arthrospore (arrow) disarticulating from monillioid chain. All photos by Nomarski interference microscopy of material stained with cotton blue of ex-holotype isolate C3843 (CBS 141682). Scale bar = 10  $\mu\text{m}$ . [Colour online.]



**ETYMOLOGY:** (L.) *cleistominuta*, in reference to its small cleistothecia.

**DESCRIPTION:** Colonies on MYEA 45–75 mm diameter after 4 days at 25 °C; odor sweet at 3–5 days, fading by 7 days; surface growth aerial, white to buff, dense and matted or sparse and in tufts with small, wet, rust-colored clumps sometimes suspended on aerial hyphae, older cultures producing amber- to rust-colored liquid drops, margin hyaline, submerged, coloring the agar medium deep rust to chestnut. Ascomata dark brown, spherical, texture intricata, suspended in aerial hyphae, 40–80  $\mu\text{m}$  in diameter at maturity (Figs. 4d–4g), lacking necks or any apparent opening. Asci not observed. Ascospores 9.0–12.0  $\mu\text{m}$   $\times$  4.5–7.0  $\mu\text{m}$  in side view, 7.0–12.0  $\mu\text{m}$   $\times$  4.5–6.5  $\mu\text{m}$  in top view, thick-walled, reniform, occasionally in pairs or groups (Figs. 4h–4m). Sporodochia rare in cultures, white, spherical, superficial, bearing conidiophores. Conidiophores (Figs. 3a–3h) often branching, scattered in tufts on media surface, in clusters near plate edges, or on sporodochia, single- or many-celled, of two types: phialoconidiophores (Figs. 3a, 3b) hyaline, bearing single or chained phialoconidia from moderately to deeply seated phialides; aleurioconidiophores (Figs. 3c–3h) hyaline to dark red-brown, bearing single or chained aleurioconidia, apparently from shallow phialides with inconspicuous collarettes. Phialoconidia cylindrical, aseptate, smooth,

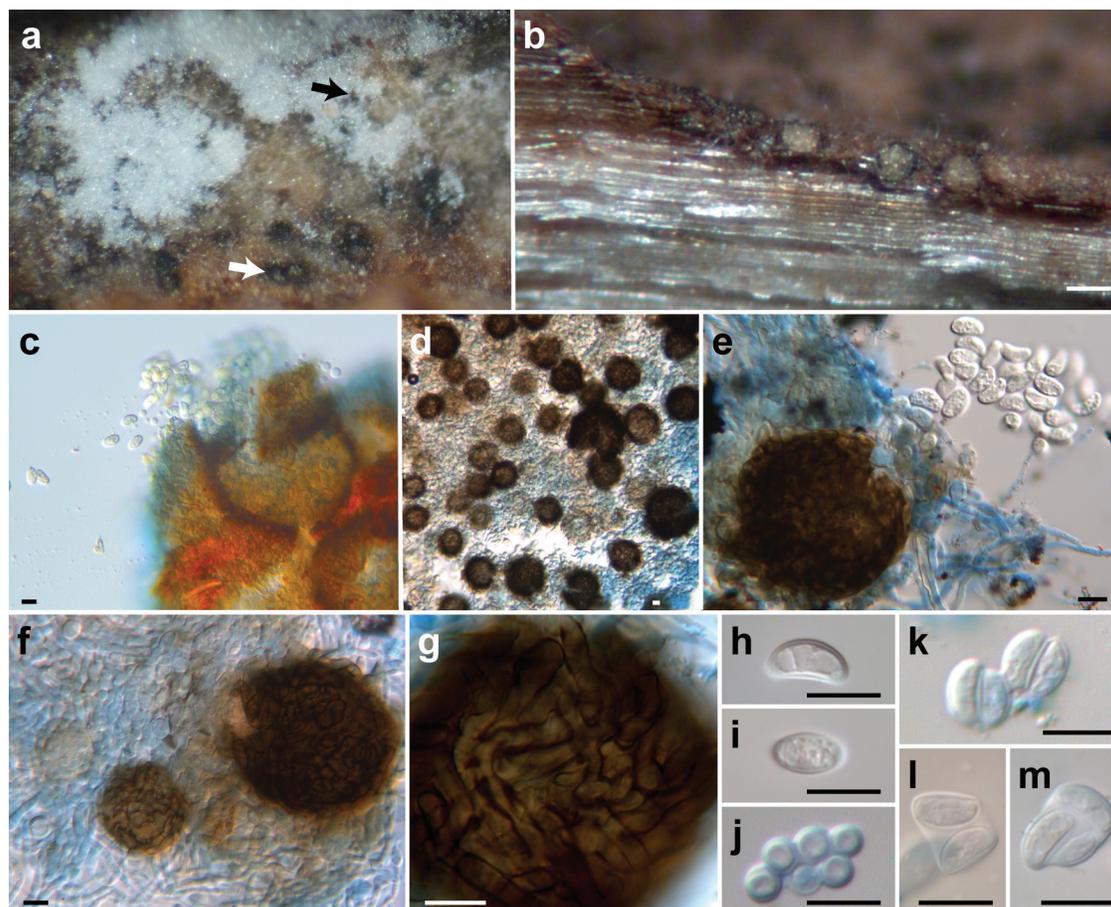
hyaline, 8.0–14.0  $\mu\text{m}$   $\times$  6.0–14.0  $\mu\text{m}$ , usually longer than wide (Figs. 3a, 3b), detaching singly or in chains. Aleurioconidia globose to ellipsoidal, generally thick-walled, 7.0–10.5  $\mu\text{m}$   $\times$  8.0–12.0  $\mu\text{m}$ , not detaching easily, hyaline to red-brown, borne singly and (or) a red-brown pigment (Figs. 3f–3h). Arthrospores (Fig. 3i) rare in culture, exogenous, derived from disarticulating chains of monillioid cells, globose to ellipsoidal, 8.5–10.0  $\mu\text{m}$   $\times$  6.5–8.0  $\mu\text{m}$ . Mycangial growth (Figs. 1c and 1d) composed of irregular to globose, thick-walled cells 4.5–10.5  $\mu\text{m}$   $\times$  5.5–14.0  $\mu\text{m}$ , with polar growth and dividing schizogenously, germinating with short, branching hyphae upon exiting the mycangium (Figs. 1a and 1b). Gallery growth as in cultures, but cleistothecia somewhat larger, 70.0–110.0  $\mu\text{m}$  diam. (Fig. 4c).

**ECOLOGY AND DISTRIBUTION:** In the galleries and mycangia of *An. maiche*.

**OTHER SPECIMENS EXAMINED:** USA, Ohio, Wayne County. Ambrosia growth in *C. florida* artificially infested by *An. maiche* that were caught in-flight in Wayne County, August 2015, C. Ranger, OHAnma1-3 gal1, BPI 910176.

**OTHER CULTURES EXAMINED:** USA, Ohio, Lake County. Isolated from *An. maiche* infesting saplings of *Gleditsia triacanthos*, 15 August 2015, C. Ranger, C4029. Wayne County, culture isolated from gallery with ambrosia growth (OHAnma1-3

**Fig. 4.** Gallery growth and sexual state of *Ambrosiella cleistominuta* sp. nov. (a–c) In gallery of *Anisandrus maiche*. (a) Opened cleistothecia (white arrow) in grazed area of the ambrosia growth and unopened cleistothecia (black arrow) in ungrazed areas. (b) Longitudinal sections of three cleistothecia embedded in ambrosia growth showing lighter ascospore masses inside. (c) Crushed cleistothecium and ascospores. (d–m) From cultures of the ex-holotype, isolate C3843 (CBS 141682). (d) Dense cluster of cleistothecia in white, aerial mycelium. (e) Ascospores from a cracked cleistothecium. (f) Three developmental stages of cleistothecia. (g) Outer texture of cleistothecium. (h–m) Ascospores. (h) Side view. (i) Top view. (j) End view. (k) Paired ascospores. (l and m) Ascospores in membranous material. All of the photos were taken by Nomarski interference microscopy of material stained with cotton blue. Scale bar = 100  $\mu\text{m}$  in b; 10  $\mu\text{m}$  in c–m. [Colour online.]



gal1) in *C. florida* artificially infested by *An. maiche* caught in-flight in Wayne County, August 2015, *C. Ranger*, C3926.

**COMMENTS:** Based on DNA analyses, the mycangial symbiont of the Asian species *An. maiche* is most closely related to *A. hartigii*, which is the mycangial symbiont of the related European species, *An. dispar* (Mayers et al. 2015). Although *A. cleistominuta* produces two types of conidiophores that could be classified as phialoconidiophores or aleurioconidiophores, there was a gradient of conidiophore morphologies, similar to what has been found in *A. hartigii* (Mayers et al. 2015). Cultures of *A. cleistominuta* are a darker red-brown, lacking the white, chalky surface growth sometimes seen in cultures of *A. hartigii*. Of all known *Ambrosiella*, only *A. cleistominuta* is known to produce ascomata and ascospores.

## Discussion

As hypothesized, *An. maiche* has a mesonotal mycangium like other *Anisandrus*, and the mycangium har-

bors budding spores of *Ambrosiella*. Although *Ambrosiella* spp. can be difficult to isolate because the mycangium and gallery propagules are intolerant of desiccation (Zimmermann and Butin 1973; Beaver 1989), *A. cleistominuta* was consistently isolated from beetles shipped with moist filter paper. *A. cleistominuta* was the only ambrosia fungus isolated from the mycangium of *An. maiche*, supporting the conclusion that *Ambrosiella* is tightly associated with *Xyleborini* species with mesonotal pouch mycangia (Harrington et al. 2014; Mayers et al. 2015). Surprisingly, *A. cleistominuta* produced ascomata in cultures and in galleries. Aside from associated yeasts (Batra 1963), a sexual state has never been reported from a mycangial symbiont of an ambrosia beetle.

## Mycangium

The large mesonotal mycangia of *Anisandrus*, *Cnestus*, *Eccoptopterus*, *Hadrodemius*, and *Xylosandrus* are formed by an invagination of the intersegmental membrane be-

tween the scutellum and posterior base of the pronotum (Francke-Grosmann 1956, 1967; Happ et al. 1976; Stone et al. 2007). Francke-Grosmann (1956, 1958) noted morphological differences in the mycangia of *X. germanus* and *An. dispar*. *Xylosandrus germanus* has two large bilateral lobes forming the left and right sides of the mycangium, as illustrated by Mayers et al. (2015), and its posterior membrane is attached to the anterior edge of the scutellum. The mycangium of *An. dispar* lacks the large bilateral lobes, is somewhat smaller, and its posterior membrane is attached further back on the ventral scutellum. Like *An. dispar*, the mycangium of *An. maiche* is relatively small and lacks the dual lobes reported for *Cnestus*, *Eccoptypterus*, and *Xylosandrus* (Francke-Grosmann 1967; Stone et al. 2007; Harrington et al. 2014; Mayers et al. 2015).

The conspicuous pits on the dorsal side of the scutellum are ornamented with setae on their rims, and sometimes hold fungal spores. Stone et al. (2007) illustrated similar pits holding propagules of *Ambrosiella beaveri* Six, de Beer & W.D. Stone on the scutellum of *C. mutilatus*. We (unpublished data) have also noted scutellum pits with setae in *An. sayi*, *C. mutilatus*, and *E. spinosus*; setae with no pits in *Xylosandrus amputatus* (Blandford); and the absence of pits or setae on the completely smooth scutella of *X. compactus*, *X. crassiusculus*, and *X. germanus*. The biological significance of these pits and setae is unclear. In *An. maiche*, the setae that are posterior to the hinge of the scutellum generally point toward the opening of the mycangium, perhaps assisting movement of spores into the mycangium of a callow female, or setae may spread or filter the spore mass exiting the mycangium of a tunneling female.

#### Culture morphology

Like the symbionts of *An. dispar* and *An. sayi*, *A. cleistominuta* produces phialoconidiophores bearing chains of barrel-shaped conidia and aleurioconidiophores that generally produce larger, single, globose, thick-walled conidia. The former apparently produces conidia via ring wall building and the latter via replacement wall building, following the terminology of Nag Raj and Kendrick (1993). The delicate chains of phialoconidia may be better adapted to enter the mycangium of their beetle hosts, whereas the aleurioconidia may be better adapted as food for grazing by the larvae and adults. Associates of *Xylosandrus*, such as *Ambrosiella roeperi* T.C. Harr. & McNew and *A. grosmanniae*, only produce aleurioconidiophores (Harrington et al. 2014; Mayers et al. 2015). Females of *Xylosandrus* have been reported to evert their large, lobed mycangia to acquire aleurioconidia from the ambrosia growth along the gallery walls (Kaneko 1967), and these species may not need phialoconidiophores for sowing of their mycangia.

Aleurioconidia are common in the family, but the unusual membranous sheaths around chains of aleurioconidia of *A. cleistominuta* have been described for only one other species in the *Ceratocystidaceae*, *C. adiposa*

(Hawes and Beckett 1977a, 1977b), a close relative of *Ambrosiella* (Mayers et al. 2015). In *C. adiposa*, the sheath surrounding the first aleurioconidium is formed within the phialide neck from the inner wall of the conidiogenous cell, and the sheath elongates along with the developing spore chain (Hawes and Beckett 1977b).

#### Sexual state of *Ambrosiella*

This is the first report of a sexual stage in a mycangial symbiont of an ambrosia beetle, but spherical structures assumed to be protoperithecia were previously seen in cultures and galleries of *A. nakashimae* associated with *X. amputatus* (Mayers et al. 2015). Beauverie (1910) also illustrated spherical structures with textured, darkened walls that may have been ascocarps of *A. hartigii* embedded in ambrosia growth in galleries of *An. dispar*. Recently, Musvuugwa et al. (2015) reported sexual states for some species of *Raffaelea*, and *Raffaelea* are generally associates of ambrosia beetles (Harrington et al. 2010). However, the three reported *Raffaelea* spp. with sexual states may not be ambrosia beetle symbionts. *Raffaelea vaginata* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer, & F. Roets was isolated from a *Lanurgus* sp. (Coleoptera: Curculionidae) (Musvuugwa et al. 2015), but beetle species in this genus are herbivorous or phloeophagous scolytids (Kirkendall et al. 2015) and do not have mycangia (Hulcr et al. 2015). *Raffaelea deltoideospora* (Olchow. & J. Reid) Z.W. de Beer & T.A. Duong was isolated from wood and from pupal chambers of cerambycid beetles, not ambrosia beetles (Musvuugwa et al. 2015). *Raffaelea seticollis* (R.W. Davidson) Z.W. de Beer & T.A. Duong was reported from an abandoned beetle gallery in a hemlock stump (Davidson 1966). Cryptic sex was hypothesized for *Raffaelea lauricicola* T.C. Harr., Fraedrich, & Aghayeva, the mycangial symbiont of *Xyleborus glabratus* Eichhoff., but ascomata were not identified (Wuest et al. 2017).

Ambrosia fungi have been assumed to be strictly asexual, clonal lineages (Farrell et al. 2001; Normark et al. 2003; Harrington 2005; Harrington et al. 2010) because of the yeast-like reproduction in the mycangium during dispersal. Additionally, vertical transmission from mothers to daughters in the haplodiploid lifestyle of the *Xyleborini* (Cognato et al. 2011) limits opportunities for effective heterothallic mating. Although asexual species are widespread in ascomycetes, it may be a transient or unstable state in nature, even in ancient lineages (Taylor et al. 1999). Truly strict asexual lineages are hypothesized to accumulate deleterious mutations over time, leading to evolutionary dead-ends (Haigh 1978; Taylor et al. 1999). *Ambrosiella cleistominuta* formed fertile ascomata in nature and in culture, and all single-ascospore and hyphal tip colonies also produced ascomata and ascospores, indicating homothallism. The discovery of a sexual state in *A. cleistominuta* and ascocarp initials in *A. nakashimae* (Mayers et al. 2015) suggests that other ambrosia fungi may maintain cryptic sexual states despite the nature of their obligate mutualisms. Male *Xyleborine* beetles, which

are flightless, travel to other galleries in heavily infested trees, which may allow for contact between fungal strains and genetic recombination. Homothallism may facilitate sexual reproduction in spite of limited contact between thalli, but it may also limit outcrossing.

Cain (1956) argues that adaptations from ostiolate to cleistothecious ascomata are common in the ascomycetes and not taxonomically informative, but the unique cleistothecia of *A. cleistominuta* are noteworthy. Ascomata of other *Ceratocystidaceae* have spherical bases within which ascospores are produced, and the spores travel through long necks, exiting from ostioles and forming a wet mass of ascospores at the tip, which is an adaptation for contamination of the exoskeleton for insect-based dispersal (Malloch and Blackwell 1993; Harrington 2005, 2009). Ascomata of other families of *Microascales* also are typically ostiolate, although some species of *Kernia*, *Pithoascus*, and *Pseudallescheria* in the *Microascaceae* are known to be cleistothecious (Malloch 1970; von Arx 1978; von Arx et al. 1988; Barr 1990).

The ascomata of *A. cleistominuta* have no apparent opening, and the only exposed ascospore masses were observed in grazed galleries. The ascomata appeared to be broken open following grazing by the larvae or adults, despite the fact that pigmented ascomata are thought to be resistant to grazing by insects (Malloch and Blackwell 1993). The ascospores may be eaten by the beetles as a supplemental food source, or dispersed on the beetle exoskeleton or passed through the gut. The ascospores produced by *A. cleistominuta* are relatively large and are most similar in shape to those produced by its close relative, *C. adiposa* (Van Wyk and Wingfield 1990), which also produces reniform ascospores but has perithecia with very long necks (Malloch and Blackwell 1993).

Other lineages of mycangial symbionts of ambrosia fungus may harbor cryptic sexual states. Fruiting bodies buried in gallery growth may have been missed, overlooked as contaminants, or ignored due to a lack of evidence that they were produced by the fungal symbionts. Sexual fruiting structures in ambrosia beetle galleries may be rare, as in the fungal cultivars of some attine ants (Taylor et al. 1999). Future studies should take special note of spherical bodies found in ambrosial growth.

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