A new endoconidial black meristematic genus, *Atramixtia*, associated with declining white spruce and phylogenetically allied to a lineage of dothidealean conifer pathogens

A. Tsuneda, M.L. Davey, and R.S. Currah

Abstract: An endoconidial, black meristematic taxon ***Atramixtia arboricola*** gen. et. sp. nov. (Dothideales) from the black subicula found on twigs of declining white spruce, *Picea glauca* (Moench) Voss, in Alberta is described. It is morphologically distinguishable from other endoconidial taxa by the conidioma composed of clumps of endoconidial conidiogenous cells, scattered meristematically dividing cells, dematiaceous hyphae, abundant brown, granular matrix materials, and sometimes plant tissue. Endoconidia also occur in conidiogenous cellular clumps that are not organized into a conidioma but develop directly from stromatic cells on the bark. In culture, it forms similar endoconidial conidiomata and also a mycelial, blastic synanamorph that superficially resembles *Hormonema*. *Atramixtia arboricola* is a member of the Dothideales and shows phylogenetic affinities to a clade of conifer-stem and -needle pathogens, including *Sydowia* and *Delphinella*, although no teleomorph was found either on the natural substrate or in culture. It has not been determined whether *A. arboricola* is pathogenic to its host, but the occurrence of abundant intracellular hyphae in the host periderm suggests that the fungus is at least parasitic.

Key words: black yeasts, Dothideomycetes, fungal taxonomy, *Atramixtia*, *Hormonema*, *Sclerophoma*, *Sydowia*, *Delphinella*.

Résumé : Les auteurs décrivent un taxon méristématique noir endoconidien, l'***Atramixtia arboricola*** gen. et sp. nov. (Dothideales) à partir de subiculums noirs venant sur rameaux en déclin d'épinette blanche, *Picea glauca* (Moench) Voss, en Alberta. On le distingue morphologiquement des autres taxons endoconidiens par les conidiomes composés de touffes de cellules conidiogènes endoconidiales, des cellules à divisions méristématiques éparses, des hyphes dématiés et une abondance de matériaux matriciels granulaires bruns avec, ou sans tissus végétaux. On retrouve également des endoconidies dans les touffes cellulaires conidiogènes, n'étant pas organisées en conidiomes mais se développant directement à partir de cellules stromatiques dans l'écorce. En culture, il forme des conidiomes endoconidiens semblables ainsi que des synamorphes blastiques mycéliens ressemblant superficiellement aux *Hormonema*. L'*Atramixtia arboricola* appartient aux Dothideales et montre des affinités phylogénétiques avec un clade pathogène des tiges et des aiguilles de conifères, incluant les *Sydowia* et les *Delphinella*, bien qu'on ne trouve pas de téléomorphe dans les substrats naturels ainsi qu'en culture. Les auteurs n'ont pas déterminé si l'*A. arboricola* est pathogène pour son hôte, mais la présence de nombreux hyphes intracellulaires dans le périderme de l'hôte suggère que ce champignon est au moins parasite.

Mots-clés : levure noire, Dothideomycètes, taxonomie fongique, *Atramixtia*, *Hormonema*, *Sclerophoma*, *Sydowia*, *Delphinella*.

[Traduit par la Rédaction]

Introduction

Black meristematic fungi (BMFs) are a phylogenetically diverse, arbitrarily defined group of asexual fungi that are characterized by darkly pigmented, slowly expanding colonies that increase in size through the more or less isodiametric enlargement and subdivision of cells, rather than through hyphal tip growth, during at least a part of their life cycle (de Hoog et al. 1999; Sterflinger et al. 1999; Tsuneda and Currah 2006). Many BMFs also show yeast-like growth and, therefore, may also be called black yeasts. Most tree-inhabiting BMFs found in Alberta form endoconidia (Sigler et al. 1981; Tsuneda and Currah 2006; Tsuneda et al. 2010), which are defined as endogenously produced asexual spores that have no wall layers in continuum with the conidiogenous cell (Hennebert and Sutton 1994). Endoconidial BMF genera are morphologically differentiated from each other primarily based on conidioma structure (Tsuneda and Currah 2006; Tsuneda et al. 2010). Phylogenetically, endoconidial BMFs are diverse and are distributed among at least five orders in the Ascomycota (sensu Hibbett et al. 2007): Capnodiales,

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Figs. 1–7. *Atramixtia arboricola* on a twig of *Picea glauca* (white spruce). Figs. 1, 2, and 7, scanning electron microscopy; Figs. 3–6, light microscopy (sectional views). Fig. 1. Subiculum on bark. Scale bar: 20 μm. Fig. 2. Young conidiomata, more or less flask shaped in side view (arrows). Scale bar: 30 μm. Fig. 3. Conidioma consisting of aggregated and scattered clumps of meristematic conidiogenous cells and abundant matrix materials with plant tissue (arrowhead). The arrow indicates a detached clump (probably during sectioning) containing endoconidia. Scale bar: 20 μm. Fig. 4. Enlarged view of an internal portion of a conidioma showing hyphae and meristematic cells (arrowheads), embedded in granular matrix materials (asterisks). Scale bar: 10 μm. Fig. 5. Conidiogenous cells containing endoconidia (arrowheads) developed directly from stromatic cells on bark. Scale bar: 15 μm. Figs. 6 and 7. Host peridermal cells containing hyphal (arrowheads) and meristematic cells (arrows). Scale bars: Fig. 6, 30 μm; Fig. 7, 10 μm.

Figs. 8–16. *Atramixtia arboricola* in culture. Figs. 8–10 and 13, malt extract agar; Figs. 11 and 12, cornmeal agar; Figs. 14 and 15, potato dextrose agar. Figs. 8 and 10–14, light microscopy; Figs. 9, 15, and 16, scanning electron microscopy. Figs. 8 and 9. Tubular structures (aggregated cellular clumps) (arrows), and submerged and aerial hyphae. Scale bars: Fig. 8, 0.8 mm; Fig. 9, 2 mm. Fig. 10. Early developmental stage of a tubular structure consisting of dematiaceous hyphae and numerous meristematically dividing cells (arrow). The arrowheads indicate arthroconidia-like fragments of hyphae. Scale bar: 50 μm. Fig. 11. Unicellular, hyaline blastic conidia. Scale bar: 10 μm. Fig. 12. Developing (left arrowhead) and near mature, darkly pigmented, two-celled blastic conidia (right arrowhead). Scale bar: 10 μm. Fig. 13. Two-celled, blastic conidia. Those pointed by arrowheads are dislocated but still attached to the hypha. The one pointed by the arrow has become larger and darker after detaching from the conidiogenous cell. Scale bar: 10 μm. Figs. 14 and 15. Aerially formed conidiomata (arrows). Scale bars: Fig. 14, 30 μm; Fig. 15, 50 μm. Fig. 16. Aerially formed conidioma, artificially broken into halves, showing released endoconidia (arrows), scant hyphae, and clumps of conidiogenous cells. Scale bar: 20 μm.

Figs. 17–25. Developmental stages of *Atramixtia arboricola* conidiomata on agar surface (malt extract agar). Figs. 17–21 and 24, scanning electron microscopy; Figs. 22 and 23, light microscopy (LM) (sectional views); and 25, differential interference contrast LM. Fig. 17. Hypha initiating meristematic growth (arrowhead) and aggregated cellular clumps (arrow). Scale bar: 30 μm. Fig. 18. Early stage of conidioma formation. Scale bar: 50 μm. Fig. 19. Cerebriform structure resulting from continuous aggregations of conidiomata. Scale bar: 100 μm. Fig. 20. Transversely sectioned conidioma composed of aggregated cellular clumps (arrow) and matrix materials (asterisks), resembling the one formed on bark (Fig. 3). Scale bar: 50 μm. Fig. 21. Enlarged view of a part of Fig. 20 showing a conidiogenous cell containing endoconidia (arrow). Scale bar: 15 μm. Fig. 22 Endoconidia in a conidiogenous cell (arrow). Scale bar: 10 μm. Fig. 23. Endoconidia (arrows) surrounded by conidiogenous cells in which endoconidiogenesis has not initiated. Scale bar: 10 μm. Fig. 24. Numerous endoconidia being released from conidiogenous cells whose walls have degenerated but cell outlines are still identifiable (arrows). Released endoconidia often give rise to secondary conidia by budding (arrowheads). Scale bar: 50 μm. Fig. 25. Released endoconidia. Scale bar: 10 μm.

Chaetothyriales, Dothideales, Myriangiales, and Pleosporales (Hambleton et al. 2003; Tsuneda et al. 2004*a*; Tsuneda and Currah 2006; Crous et al. 2007; Tsuneda et al. 2008). Some endoconidial BMF species are pathogenic to plants (Tsuneda et al. 2001, 2008) or humans (Palencarova et al. 1995; de Hoog et al. 2000; Chabasse 2002).

In our 2007–2009 survey for tree-inhabiting BMFs in Larch Valley, Banff National Park, Alberta, we found that the bark of many trees of subalpine larch (*Larix lyallii* Parl) and white spruce (*Picea glauca* (Moench) Voss) had black subicula of BMFs. Most of these trees appeared weakened or dead, although it was uncertain whether the BMFs were the primary cause of their decline. Among the BMFs on larch, two endoconidial BMF genera were described previously as *Celosporium larixicolum* gen. et sp. nov. (Dothideales) and *Hispidoconidioma alpina* gen. et sp. nov. (Capnodiales) (Tsuneda et al. 2010). We report herein another endoconidial BMF genus, ***Atramixtia arboricola*** gen. et. sp. nov. (Dothideales), from white spruce.

Materials and methods

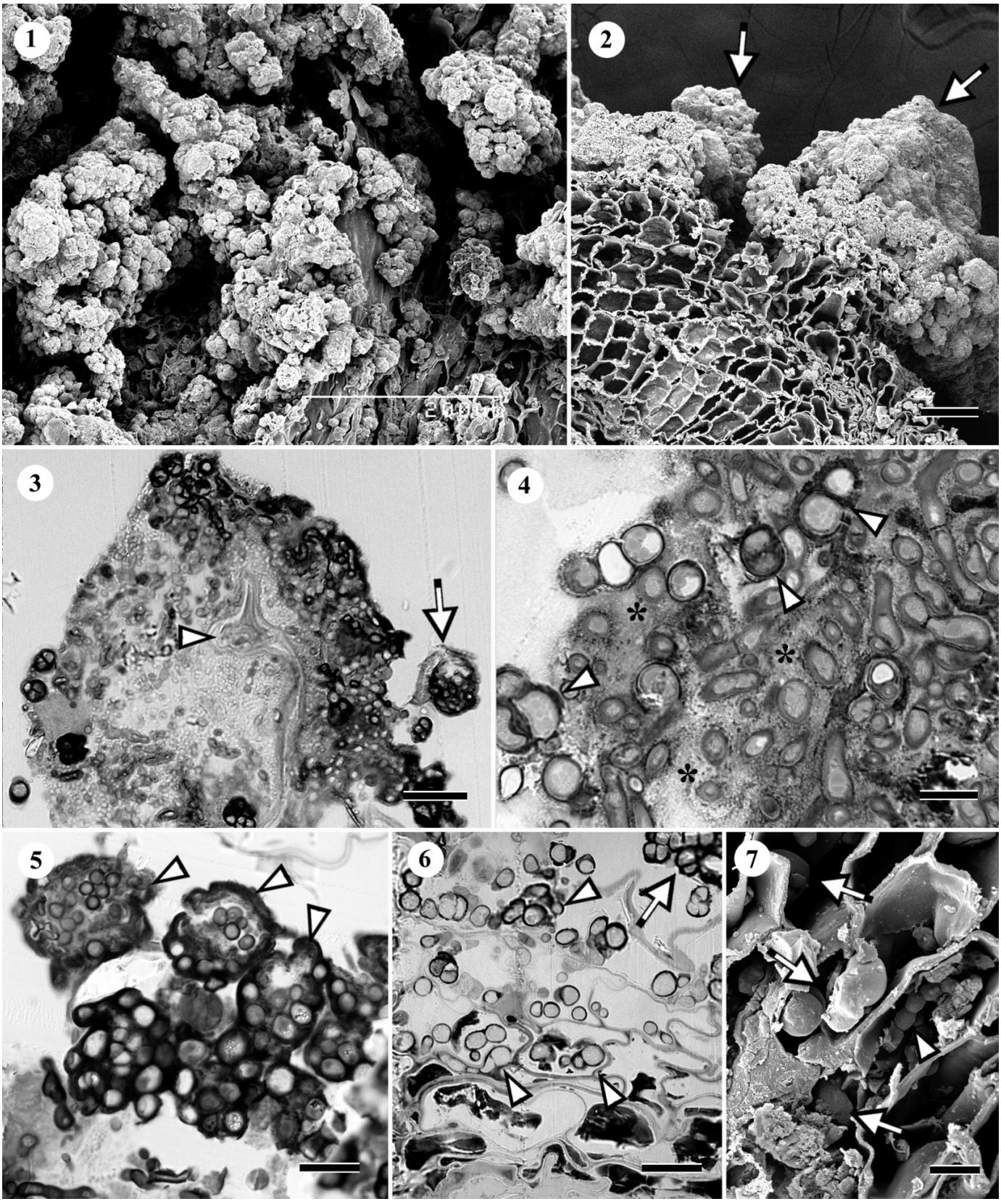
Isolation and microscopy

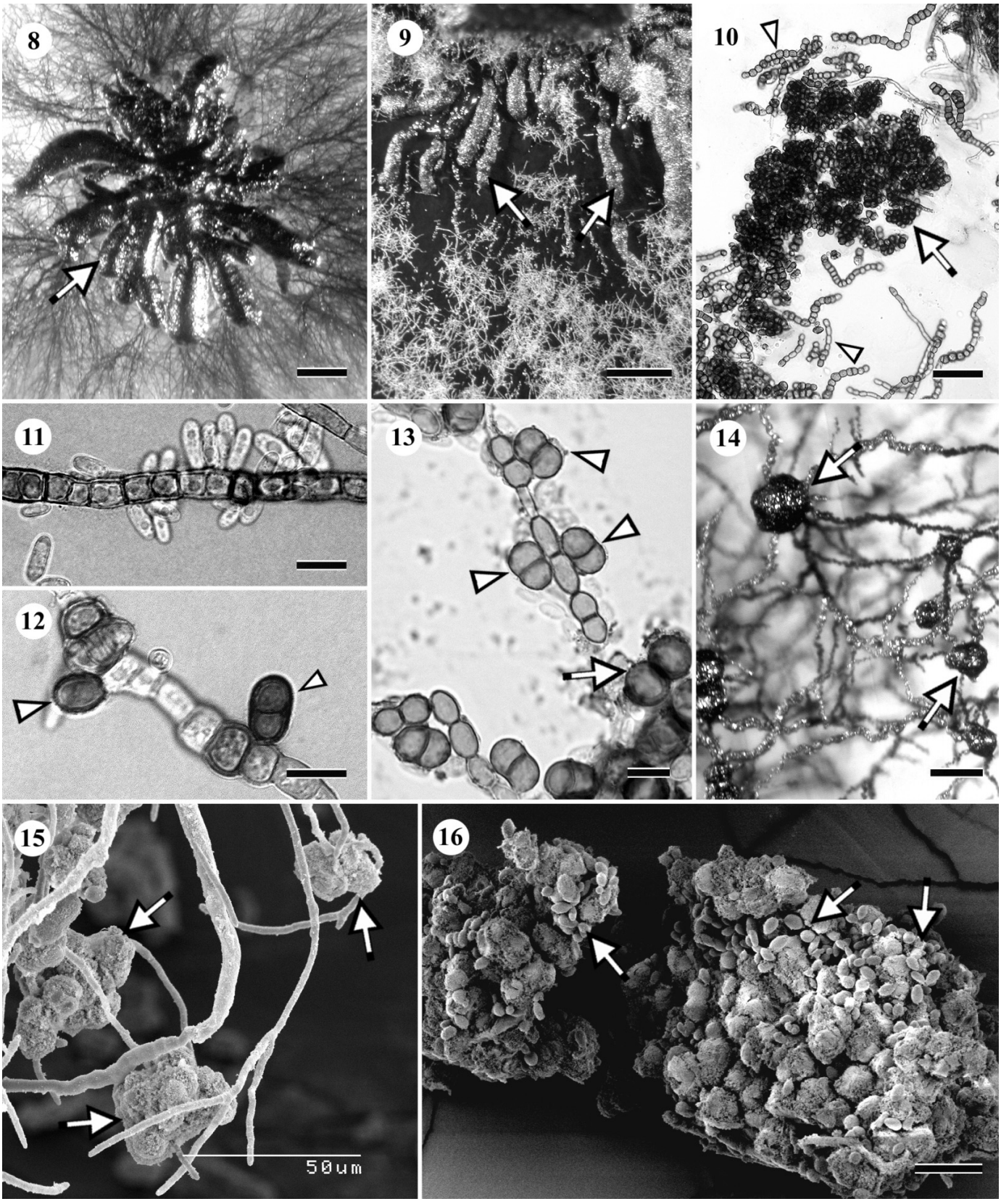
Small twigs bearing black fungal subicula were cut from 20 randomly selected trees (two to three twigs from each tree) of *P. glauca* in the Larch Valley and were examined by light microscopy and scanning electron microscopy (LM and SEM, respectively). The fungus was isolated using the method described in Tsuneda et al. (2000), and cultures were deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH 11212 and 11213). Colony and microscopic descriptions were made using subcultures grown on 2% malt extract agar (MEA; Difco Laboratories, Detroit, Michigan), potato dextrose agar (PDA, Difco Laboratories), or cornmeal agar (CMA, Difco Laboratories) at 20 °C in the dark. Specimens for thin-section observations by LM were prepared according to Meek (1976) and those for SEM (20 bark specimens; a piece of a twig, ca. 5 mm × 5 mm) were prepared according to Tsuneda et al. (2000). SEM specimens were examined and photographed using a Hitachi S-510 electron microscope at 5, 10, or 15 kV (Hitachi, Tokyo, Japan).

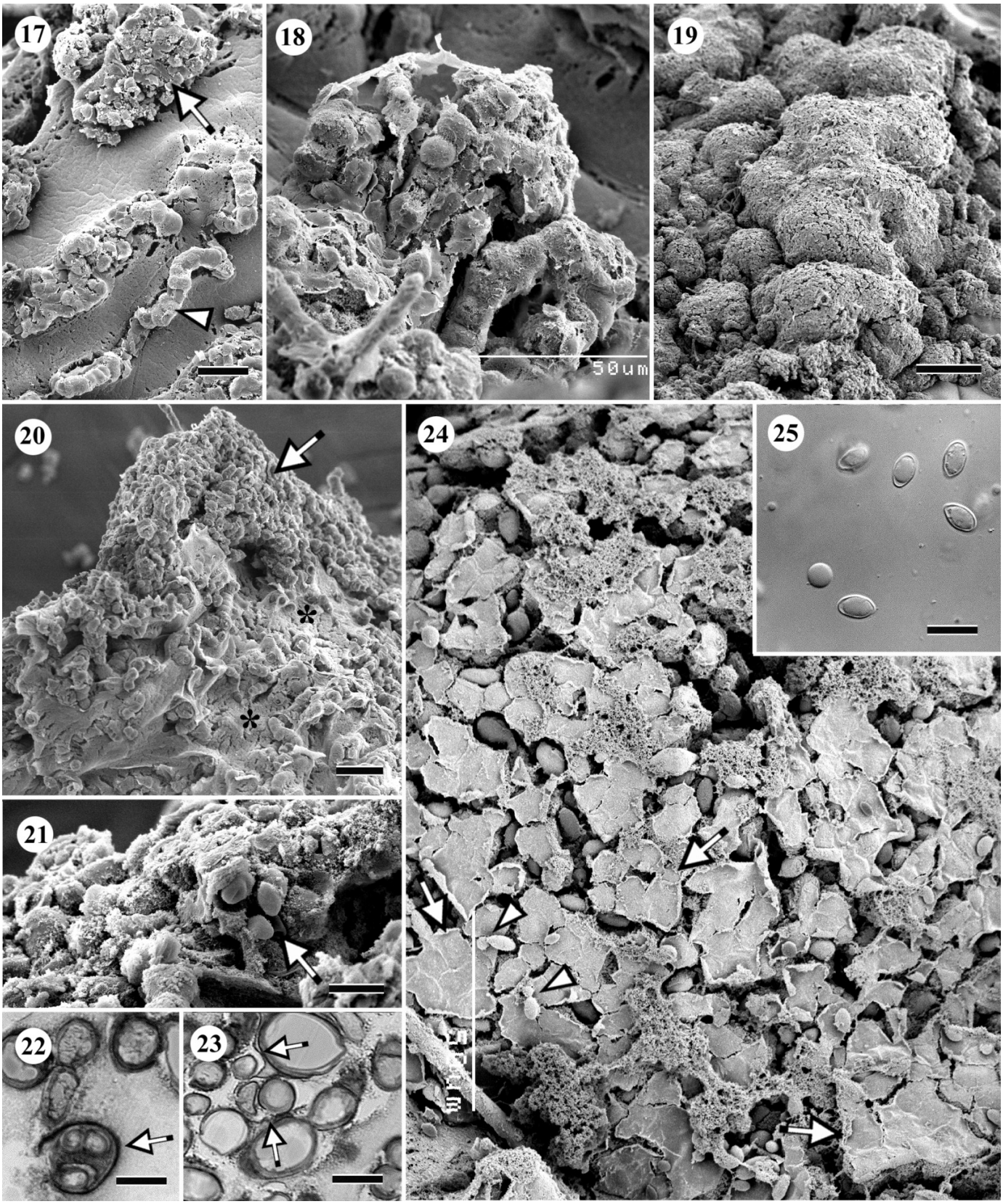
DNA sequencing and phylogenetic analysis

The ex-type strain of the fungus (UAMH 11211) was grown on PDA for 30 d at ambient light and temperature, and genomic DNA was extracted from the mycelium using a cetyltrimethylammonium bromide – chloroform extraction protocol (Murray and Thompson 1980; Gardes and Bruns 1993). The large subunit (LSU) and internal transcribed spacer (ITS) regions of the genomic rRNA gene complex were amplified as described in Davey and Currah (2007) using the forward and reverse primer sets LROR and LR7 (Vilgalys and Hester 1990), and ITS4 and ITS5 (White et al. 1990), respectively. Amplicons were sequenced with an ABI 3100 automated sequencer (Applied Biosystems Inc., Foster City, California), the primers ITS4 and ITS5 (White et al. 1990), and LROR, LR1, LR3R, LR5, and LR7 (Vilgalys and Hester 1990).

Data matrices were assembled from LSU and ITS sequences of *A. arboricola* and other members of the Dothideales, as well as outgroup taxa from the Myriangiales, and aligned using MAFFT version 6.717 (Katoh and Toh 2008), and the







subsequent alignments were manually verified. Gblocks v. 0.91b (Castresana 2000) was used to remove ambiguously aligned bases from the ITS alignment, and the resulting LSU and ITS matrices were subjected to maximum parsimony, maximum likelihood, and Bayesian analyses. Maximum parsimony analyses were conducted using PAUP v4.0d106 (Swofford 2003) with Fitch parsimony, random simple step-wise addition of taxa, and tree bisection-reconnection (TBR) branch swapping, and gaps were treated as missing data. Support for branching topologies was evaluated using 1000 resamplings of the data by bootstrapping analysis using the same criterion described above (Felsenstein 1985). All trees were scored for length in steps, consistency index (CI), retention index (RI), and homoplasy index (HI). The Bayesian information criterion in jModelTest v0.1.1 (Guindon and Gascuel 2003; Posada 2008) was used to determine the best-fit model of evolution for both maximum likelihood and Bayesian analyses. Maximum likelihood analyses to determine the most likely tree and maximum likelihood bootstrap support for each dataset were conducted using GARLI v. 1.0 (Zwickl 2006) with the selected models of evolution implemented. Bayesian analyses were conducted using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003) with two independent runs of four Markov-chain Monte Carlo chains with 1.0×10^7 generations each, sampling trees every 1000th generation. A final standard deviation of <0.01 for the split frequency was taken as an indication that convergence had been achieved. The first 10% of sampled trees were discarded as burn-in and posterior probabilities for each node of the 50% majority rule consensus tree were recorded.

Results

Taxonomy

Atramixtia Tsuneda, Davey & Currah, gen. nov.

TYPUS GENERIS: *Atramixtia arboricola*

ETYMOLOGY: Atramixtia — "dark mixture" in reference to the conidioma that contains cellular, hyphal, and sometimes plant elements. Arboricola — tree inhabitant.

Conidiomata constans ex cellulis conidiogenosis sparsis, aggregatis, fasciculis, nigellis, hyphis dematiaceis et materiis matricibus brunneis, granulatis cum vel sine materia planta. Endoconidia unicellularis, hyalina, ovales ad obverse-ovata, et dissolutione tunicae cellulae conidiogenosae exsoluta.

Conidiomata consisting of darkly pigmented, aggregated, and scattered clumps of conidiogenous cells, dematiaceaceous hyphae, and brown, granular matrix materials with or without plant tissue. Endoconidia unicellular, hyaline, oval to obovate, and released by cell-wall dissolution of the conidiogenous cells.

Atramixtia arboricola Tsuneda, Davey & Currah sp. nov.

Figs. 1–25.

Subicula in ramis Picea glauca*, nigra, magnitudine varia, constans ex conidiomatibus aggregatis. Conidiomata nigra, superficiales, semiorbiculata, demum confluentibus et irregularibus, et ex globis cellullis aggregatis et hyphis dematiaceis. Cellulae conidiogenosae ovales vel ovoidea, brunnea. Endoconidia unicellularis, hyalina, ovales ad obverse-ovata. Coloniae in MEA subglobosae constans ex hyphis memnoniis submersis et hyphis spadiceis aeriis. Conidia in CMA formantia blastice, ex lateribus hypharum dematiacearum, e 1–2 cellulis: hyphae unicellularis hyalinae, fusiformes ad ellipsoidei; hyphae bicellularis spadiceae, ovales cum septis constrictis.*

ON TWIGS OF *PICEA GLAUCA*: subicula black, irregular in shape, consisting of aggregated conidiomata. Conidiomata black, superficial, separate when young, hemispherical (often flask shaped in side view), becoming confluent and irregular in shape, composed of aggregated cellular clumps, scattered meristematically dividing cells, scant dematiaceous hyphae, and brown granular matrix materials with or without plant tissue. Cellular clumps prior to merging with adjacent ones, subglobose, dark brown, 20–34 μm × 18–30 μm. Conidiogenous cells, oval or ovoid, dark brown, 6–10.5 μm × 5–7 μm. Endoconidia unicellular, hyaline, subglobose, oval, or obovate, 3.5–7.5 μm × 3–4 μm.

ON MEA: vegetative hyphae light to dark brown, straight or moniliform, with markedly swollen cells at irregular intervals, 7–22 μm in diameter. Colonies subspherical, consisting of brownish black submerged and light brown aerial hyphae, and elevated tubular structures composed of cellular clumps of thick-walled meristematically dividing cells, scant dematiaceous hyphae, and brown granular matrix materials. Endoconidia forming in cellular clumps in discontinuous areas of tubular structures, hyaline, subglobose, oval, ovoid, or ellipsoid, 3–9.5 μm × 3–6 μm.

ON CMA: blastic conidia abundant, either unicellular (hyaline, 6–10.5 μm × 2.5–4 μm, fusiform to ellipsoidal) or two celled (light brown, 10.4–13.8 μm × 2.7–8.8 μm, mostly oval with a slight constriction at the septum), arising from sides of dematiaceous hyphae.

HOLOTYPE: Dried culture prepared from an isolate (UAMH 11211) obtained from a black subiculum formed on a twig of *Picea glauca* collected at the Larch Valley (collection permit, LL-2008-1753; 51°19′N, 116°12′W), 5 August 2008 by A. Tsuneda.

Microscopic observations

At least three different BMFs were isolated from black subicula on the bark specimens of white spruce, and *A. arboricola* was the most frequent. Examination of longitudinal sections of subicula was essential to confirm the presence of *A. arboricola* on the natural substrate, because black subicula of BMFs superficially looked very similar under both dissecting LM and SEM (Fig. 1). Conidiomata of *A. arboricola* were superficial and when young, they often appeared more or less flask shaped in side view (Fig. 2), but this distinguishing characteristic became obscured when adjacent ones merged in older subicula. Thin-section observations revealed that conidiomata were composed of clumps of thick-walled, meristematically dividing, darkly pigmented cells (cellular clumps); dematiaceous hyphae; and brown, granular matrix materials (Figs. 3 and 4) with or without host tissue (Fig. 3, arrowhead). The cellular clumps in a conidioma were either densely aggregated, particularly in the surface areas, or scattered and embedded in the matrix materials (Figs. 3 and 4). Endoconidiogenesis was not synchronous and often occurred only in some cellular clumps of the conidioma. Endoconidia sometimes occurred in cellular clumps that were not organized into a conidioma but developed directly from stromatic

cells on the bark (Fig. 5). Host peridermal cells underneath the conidiomata or stromatic cells contained abundant meristematically dividing cells and hyphae of *A. arboricola* (Figs. 6 and 7).

Two isolates of *A. arboricola*, UAMH 11211 and 11212, were examined in culture. They were similar in morphology but both varied in colony morphology, growth rate, and conidiogenesis depending on the agar medium. On MEA, colonies were ca. 12.5 mm in diameter after 15 d and composed of (*i*) submerged brown hyphae, (*ii*) light brown, cottony, aerial hyphae, and (*iii*) black, elevated, and often tubular structures consisting of meristematically dividing cells, brown granular matrix materials, and scant thick-walled hyphae (Figs. 8–10, 19, and 20). Blastic conidia were absent or scarce but one to several-celled, arthroconidium-like hyphal fragments were often present (Fig. 10, arrowheads). On PDA, colonies were about 7.8 mm in diameter after 15 d and initially resembled those of white yeasts owing to abundant, hyaline blastic conidia, but later became black and cerebriform. Mature colonies on PDA were too dense to observe blastic conidiogenesis. Colonies on CMA were approximately 14.7 mm in diameter after 15 d and consisted of sparse, light brown hyphae bearing blastic conidia that were either one or two celled; the former were hyaline and fusiform to ellipsoid (Fig. 11), while the latter were light brown and mostly oval with a slight constriction at the septum (Figs. 12 and 13). After secession, the two-celled conidia enlarged (Fig. 13, arrow), became darker, and often merged with adjacent ones to form multicellular bodies. Both one- and two-celled blastic conidia frequently gave rise to secondary conidia. No annellides or phialides were found.

Conidiomata developed on MEA and PDA, but not on CMA, and formed among the aerial mycelium or on the agar surface. Aerial conidiomata (Figs. 14 and 15) were shiny black, subglobose to irregular in shape, and developed from hyphal cells that underwent meristematic growth. When mature, they contained liberated endoconidia, scant hyphae, and conidiogenous cellular clumps (Fig. 16). Conidiomata forming in contact with agar surface were black, raised, tubular or cerebriform (Figs. 8, 9, and 17–19), and morphologically similar to the flask-shaped conidiomata on the natural substrate (Figs. 2–4); hyphae first underwent meristematic growth to form cellular clumps that subsequently merged with adjacent ones (Figs. 17 and 18) while producing granular matrix materials. Black, elevated, subglobose conidiomata thus formed further merged and developed into tubular or cerebriform structures (Figs. 19 and 20, sectional view). Endoconidiogenesis was evident after prolonged incubation (3–5 months at 20–22 °C), although the formation was irregular, i.e., endoconidia were largely absent and occurred only in some cellular clumps (Figs. 21–23) or were abundant in some discontinuous areas of the tubular or cerebriform structure (Fig. 24). Endoconidia were released by the degeneration of conidiogenous cell walls (Figs. 24 and 25). The released endoconidia often gave rise to secondary conidia by budding (Fig. 24, arrowheads).

Phylogenetic analyses

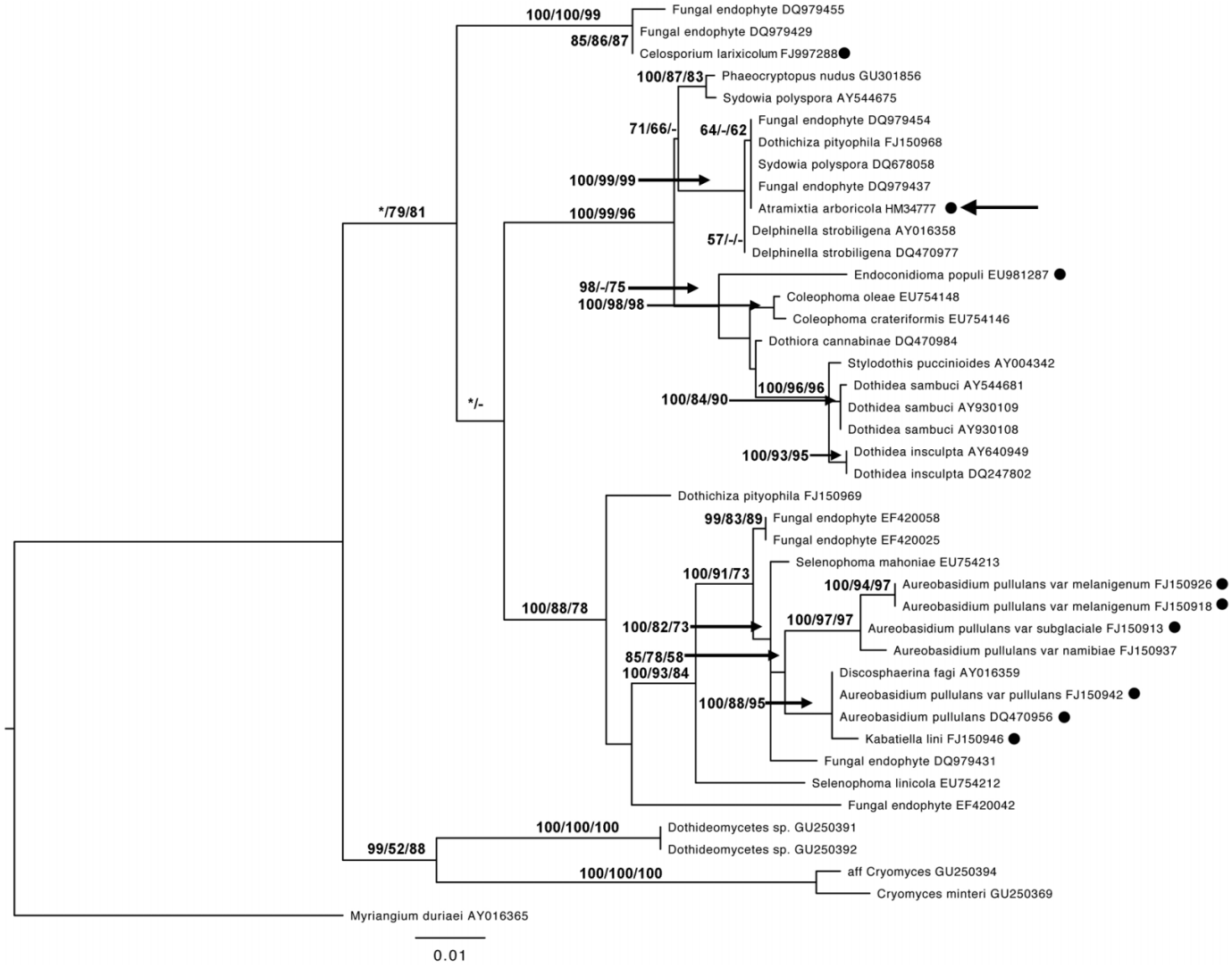
The aligned matrix of LSU sequences included 1324 characters, of which 10 120 were constant, 46 were parsimony uninformative, and 158 were parsimony informative. Maximum parsimony analysis generated 8292 most parsimonious trees of 442 steps (CI = 0.638, RI = 0.862, and HI = 0.362). The TIM1+I+G model was selected by jModelTest as the best-fit model of evolution for the data and was implemented in both maximum likelihood and Bayesian inference analyses. Results of the maximum parsimony and maximum likelihood bootstrap analyses and the Bayesian inference are shown on the maximum likelihood tree ($-\ln L$ 4195.48) (Fig. 26). *Atramixtia arboricola,* isolates of *Sydowia polyspora* (Bref. & Tavel) E. Müll. and its anamorph *Dothichiza pityophila* (Corda) Petr., and two endophytes of *Picea mariana* (Müll.) Britton et al. needles form an unresolved subgroup (64% Bayesian posterior probability (BPP) and 62% maxium likelihood bootstrap proportion (MBP)) within a strongly supported clade (100% BPP, 99% parsimony bootstrap proportion (PBP), and 99% MBP) that also includes isolates of the conifer needle pathogen *Delphinella strobiligena* (Desm.) Sacc. ex. E. Müll & Arx. This clade is well resolved and excludes other Dothidealean taxa known to produce endoconidia (i.e., *Celosporium larixicolum* Tsuneda & Davey, *Endoconidioma populi* Tsuneda et al., and *Aureobasidium* species).

The aligned matrix of ITS sequences included 494 characters, of which 282 were constant, 79 were parsimony uninformative, and 133 were parsimony informative. The 288 most parsimonious trees of 476 steps (CI = 0.641, RI = 0.888, and HI = 0.359) were generated by maximum parsimony analysis. The TIM2ef+G model was selected by jModelTest as the best-fit model of evolution for the data and was implemented in both maximum likelihood and Bayesian inference analyses. Results of the maximum parsimony and maximum likelihood bootstrap analyses and the Bayesian inference are shown on the maximum likelihood tree ($-\ln L$ 2973.52) (Fig. 27). *Atramixtia arboricola* is nested within a large, strongly supported clade (100 BPP, 99% MBP, and 99% MBP) that includes representatives of *Sydowia polyspora* and its hyphomycetous anamorph, *Hormonema dematioides* Lagerb. & Melin, and representatives of the conifer needle pathogens *Delphinella abietis* (Rostr.) E. Müll. and *Rhizosphaera kalkhoffii* Bubák. Pairwise sequence similarity among members of this clade is >98%. The clade containing *A. arboricola* is well resolved from those clades containing other dothidealean taxa known to produce endoconidia.

Discussion

Morphologically, *A. arboricola* is unique in its conidioma structure that consists of a mixture of conidiogenous cellular clumps, dematiaceous hyphae, and abundant brown, granular matrix materials. This conidomatal structure distinguishes the species from other previously reported endoconidial BMFs that form conidiomata, viz., *Phaeotheca* (Sigler et al. 1981; Tsuneda et al. 2004*b*), *Scleroconidioma* (Tsuneda et al. 2000), *Endoconidioma* (Tsuneda et al. 2004*a*, b), *Endosporium* (Tsuneda et al. 2008), *Celosporium* (Tsuneda et al. 2010), and *Hispidoconidioma* (Tsuneda et al. 2010). This morphological distinction from other dothidealean endoconidial taxa is further supported by our phylogenetic analysis of the LSU rDNA region, which places *A. arboricola* in a well-resolved clade that is distinct from other lineages that include endoconidial taxa (*Scleroconidioma* and *Endoconidioma*)

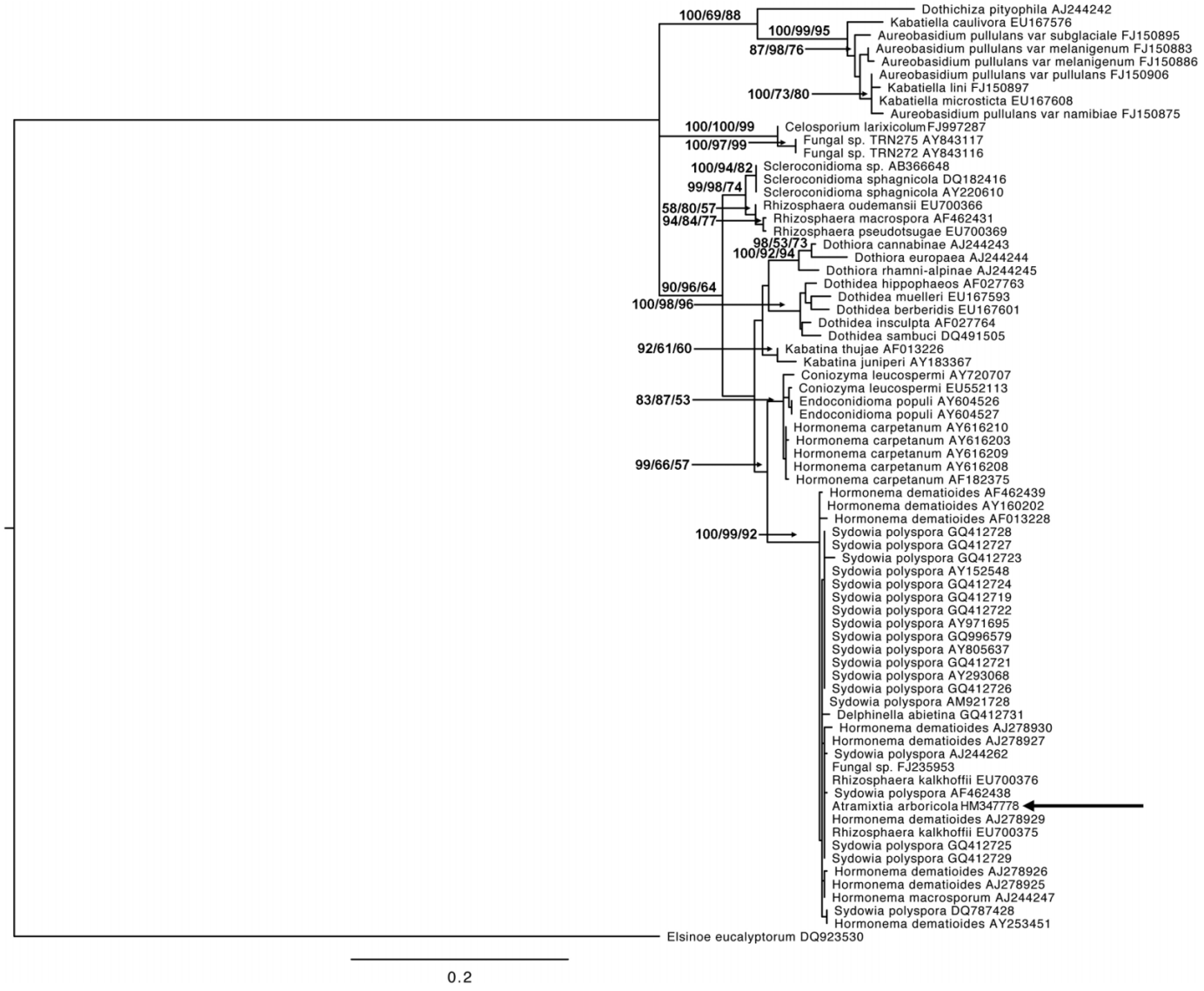
Fig. 26. Maximum likelihood tree (–ln *L* 4195.48) inferred from a maximum likelihood analysis of large subunit rDNA sequences showing the placement of *Atramixtia arboricola* and other endoconidial taxa among the Dothideales. Support values are given above the branches as Bayesian posterior probability / parsimony bootstrap proportion / maximum likelihood bootstrap proportion. Gaps (-) indicate a collapsed node and asterisks (*) indicate a node was resolved differently in the analysis. *Myriangium duriaei* serves as the outgroup taxon. Accession numbers for sequences retrieved from or deposited in GenBank are indicated following the taxon name, and source information can be found in Table 1. *Atramixtia arboricola* is indicated by an arrow. Taxa producing endoconidia are indicated by a solid circle (•) following the name.



(Fig. 26), as well as some taxa reported to occasionally form endoconidia (*Aureobasidium* and *Kabatiella*).

Phylogenetically, *A. arboricola* is closely allied to a group of dothidealean conifer-stem and -needle pathogens, including *Delphinella abietis*, *Rhizosphaera kalkhoffii*, and *Sydowia polyspora* and its anamorphs *Hormonema dematioides* and *Sclerophoma pythiophila* (Cda) Höhn (= *Dothichiza pythiophila*). While these taxa form a well-supported clade within the Dothideales in analyses of both the LSU and ITS regions, relationships between members of the clade are poorly resolved, even in ITS analyses. Although the ITS region is used as a barcode for distinguishing fungal species, it is widely recognized that the region lacks a barcode gap for some species complexes, and it is not useful for distinguishing between members of these complexes (Chase and Fay 2009; Seifert 2009; Eberhardt 2010). Further phylogenetic analyses using other gene regions (i.e., β-tubulin and actin) and multigene analyses are needed to elucidate the relationship between *A. arboricola* and other members of this group of conifer-stem and -needle pathogens. Although our phylogenetic analyses do not demonstrate unequivocally that *A. arboricola* represents a distinct evolutionary lineage within the Dothideales, we feel *A. arboricola* cannot be adequately taxonomically accommodated within the existing anamorph genera represented in the conifer pathogen clade. *Atramixtia arboricola* produces distinct coelomycetous conidiomata that are inconsistent with the pycnidial taxa *Rhizosphaera kalkhoffii*, *Sclerophoma pythiophila* (teleomorph = *Sydowia polyspora*), and *Dothiorella* sp. (teleomorph = *Delphinella abietis*) (Butin 1964; Sutton and Waterston 1970; Barr 1972; Sutton 1980), as the species undergoes endoconidiogenesis rather than blastic conidiogenesis. *Hormonema dematioides*, a cultural, mycelial anamorph of *S. polyspora*, forms blastic conidia of similar shape and size to those of *A. arboricola*

Fig. 27. Maximum likelihood tree (–ln *L* 2973,52) inferred from a maximum likelihood analysis of internal transcribed spacer rDNA sequences showing the placement of *Atramixtia arboricola* and other endoconidial taxa among the Dothideales. Support values are given above the branches as Bayesian posterior probability / parsimony bootstrap proportion / maximum likelihood bootstrap proportion. *Elsinoë eucalyptorum* serves as the outgroup taxon. Accession numbers for sequences retrieved from or deposited in GenBank are indicated following the taxon name, and source information can be found in Table 1. *Atramixtia arboricola* is indicated by an arrow.



and also occasionally forms endoconidia within intercalary cells of its hyphae (Hermanides-Nijhof 1977; Sutton 1980; Yurlova et al. 1999). The blastic synanamorph of *A. arboricola*, however, lacks both annellated conidiogenous cells and endoconidia formed within its hyphae. Because *A. arboricola* is distinguishable from other closely related taxa, and its morphological characters are stable both in culture and on natural substrates (including where one would expect to find other members of the conifer pathogen clade), we feel it is unlikely that *A. arboricola* represents an example of phenotypic plasticity within currently existing taxa and that the erection of a new genus is warranted.

The description of *A. arboricola* within this clade of conifer pathogens represents an additional morphological form within the lineage, making it a diverse group that includes pseudothecial teleomorph taxa with septate spores and anamorphic taxa that are pycnidial, annellidic, endoconidial, or forming occasional endoconidia within intercalary cells of the hyphae. However, without additional taxon sampling of this lineage and further phylogenetic analysis, it is unclear whether this morphological diversity is reflective of divergence among species to occupy different microniches on the host or is reflective of a lineage composed of species with complex life histories and multiple life stages. *Atramixtia* shares 98%–100% pairwise sequence similarity across the ITS region of rDNA with other members of the conifer pathogen clade it clusters in, including the teleomorphic genera *Sydowia* and *Delphinella*. Given that *S. polyspora* shares 97%–100% pairwise sequence similarity across this region with its synanamorphs and is known to have a high degree of intraspecific genetic variability (Bills et al. 2004; Kraj 2009; Kraj et al. 2009), we can speculate that *A. arboricola*

Table 1. Sources and accession numbers of the isolates and sequences used in this study.

Taxon	Source[a]	Substrate	Locality	Large subunit	Internal transcribed spacer	References
Atramixtia arboricola	UAMH 11211	*Picea glauca*	Alberta, Canada	HM347777	HM347778	This study
Aureobasidium pullulans var. *namibiae*	CBS 147.97	Dolomitic marble	Namibia	FJ150937	FJ150875	Zalar et al. 2008
Aureobasidium pullulans var. *melanigenum*	CBS 105.22	Not Known	Not known	FJ150926	FJ150886	Zalar et al. 2008
	Not known	Ponds on sea ice	Svalbard, Norway	FJ150918	FJ150883	Zalar et al. 2008
Aureobasidium pullulans var. *pullulans*	CBS 584.75	*Vitis vinifera*	Beaujolais, France	FJ150942	FJ150906	Zalar et al. 2008; Spatafora et al. 2006
				DQ470956		
Aureobasidium pullulans var. *subglaciale*	Not known	Subglacial ice from seawater	Svalbard, Norway	FJ150913	FJ150895	Zalar et al. 2008
Celosporium larixicolum	UAMH 11008	*Larix laricina*	Alberta, Canada	FJ997288	FJ997287	Tsuneda et al. 2010
Coleophoma crateriformis	CBS 473.69	*Phillyrea angustifolia*	Spain	EU754146	—	de Gruyter et al. 2009
Coleophoma oleae	CBS 615.72	*Olea europaea*	Greece	EU754148	—	de Gruyter et al. 2009
Coniozyma leucospermi	CBS 114035	*Protea repens*	South Africa	—	AY720707	Lennox et al. 2004
	CBS 11289	*Leucospermum conocarpodendron*	South Africa	—	EU552113	Marincowitz et al. 2008
Cryomyces minteri	Not known	Rock	Antarctica	GU250369	—	Selbmann et al. 2005
Delphinella abietis	Not known	*Abies* sp.	Norway	—	GQ412731	
Delphinella strobiligena	CBS 735.71	*Pinus halepensis*	Greece	AY016358, DQ470977	—	Lumbsch and Lindemuth 2001; Spatafora et al. 2006
Discosphaerina fagi	CBS 171.93	*Populus* sp.	UK	AY016359	—	Lumbsch and Lindemuth 2001
Dothichiza pityophila	CBS 215.50	*Abies concolor*	Norway	FJ150968	AJ244242	Zalar et al. 2008; de Hoog et al. 1999
Dothidea berberidis	CBS 186.58	*Berberis vulgaris*	Switzerland	—	EU167601	Simon et al. 2009
Dothidea hippophaës	DAOM 231303	Not known	Not known	—	AF027763	Jacobs and Rehner 1998
Dothidea insculpta	CBS 189.58	*Clematis vitalba*	France	DQ247802, AY640949	AF027764	Schoch et al. 2006; Reeb et al. 2004; Jacobs and Rehner 1998
Dothidea muelleri	CBS 191.58	*Daphne striata*	Albulapass, Switzerland	—	EU167593	Simon et al. 2009
Dothidea sambuci	CBS 198.58	*Acer pseudoplatanus*	Switzerland	AY930109	DQ491505	Shoemaker and Hambleton 2005; Jeewon et al. 2002
	CBS 197.58	*Sambucus nigra*	Switzerland	AY930108	—	Shoemaker and Hambleton 2005
	DAOM 231303	Not Known	Not known	AY544681	—	Unpublished
Dothideomycetes sp.	Not known	Rock	Alps	GU250391	—	Selbmann et al. 2005
	Not known	Rock	Alps	GU250392	—	Selbmann et al. 2005
Dothiora cannabinae	CBS 737.71	*Daphne cannabina*	India	DQ470984	AJ244243	Spatafora et al. 2006; de Hoog et al. 1999
Dothiora europaea	CBS 739.71	*Salix helvetica*	Switzerland	—	AJ244244	de Hoog et al. 1999
Dothiora rhamni-alpinae	CBS 745.71	*Rhamnus alpina*	Italy	—	AJ244245	de Hoog et al. 1999
Elsinoë eucalyptorum	CBS 120084	*Eucalyptus propinqua*	Australia	—	DQ923530	Summerell et al. 2006
Endoconidioma populi	UAMH 10297	*Populus tremuloides*	Edmonton, Alberta, Canada	EU981287	AY604526	Tsuneda et al. 2004*a*
	UAMH 10298	*Populus tremuloides*	Edmonton, Alberta, Canada	—	AY604527	Tsuneda et al. 2004*a*
Fungal endophyte	Not known	*Picea mariana*	Quebec, Canada	DQ979455	—	Higgins et al. 2007
	Not known	*Platycladus orientalis*	Not known	EF420058	—	Hoffman and Arnold 2008
	Not known	*Platycladus orientalis*	Not known	EF420025	—	Hoffman and Arnold 2008
	Not known	*Platycladus orientalis*	Not known	EF420042	—	Hoffman and Arnold 2008
	Not known	*Picea mariana*	Quebec, Canada	DQ979429	—	Higgins et al. 2007
	Not known	*Picea mariana*	Quebec, Canada	DQ979437	—	Higgins et al. 2007
	Not known	*Picea mariana*	Quebec, Canada	DQ979454	—	Higgins et al. 2007
Fungal sp.	Not known	Wood	Antarctica	—	FJ235953	Arenz and Blanchette 2009
	Not known	Slate	Atazar, Spain	—	AY843116	Ruibal et al. 2008
	Not known	Slate	Atazar, Spain	—	AY843117	Ruibal et al. 2008
Hormonema carpetanum	Not known	*Juniperus communis*	Avila, Spain	—	Ay616210	Bills et al. 2004
	Not known	*Juniperus communis*	Madrid, Spain	—	AY616209	Bills et al. 2004

Table 1 (*concluded*).

Taxon	Source[a]	Substrate	Locality	Large subunit	Internal transcribed spacer	References
	Not known	*Juniperus sabina*	Albacete, Spain	—	AY616208	Bills et al. 2004
	Not known	*Juniperus communis*	Avila, Spain	—	AY616203	Bills et al. 2004
	ATCC 74360	*Juniperus communis*	Madrid, Spain	—	AF182375	Bills et al. 2004
Hormonema dematioides	Not known	*Pinus sylvestris*	Brandenburg, German	—	AJ278927	Unpublished
	Not known	*Pinus sylvestris*	Lower Saxonia, Germany	—	AJ278930	Unpublished
	Not Known	*Picea mariana*	Quebec, Canada	—	AY971695	Sokolski et al. 2007
	Not known	*Pinus nigra*	Lexington, Kentucky, USA	—	AY160202	Flowers et al. 2003
	Not known	*Pinus sylvestris*	Brandenburg, Germany	—	AJ278925	Unpublished
	Not known	*Pinus* sp.	Germany	—	AY253451	Filip et al. 2003
	Not known	*Pinus sylvestris*	Michigan, USA	—	AF013228	Unpublished
	Not known	*Pinus sylvestris*	Lower Saxonia, Germany	—	AJ278929	Unpublished
	Not known	*Pinus sylvestris*	Brandenburg, Germany	—	AJ278926	Unpublished
	Not known	*Pseudotsuga menziessii*	Michigan, USA	—	AF462439	Unpublished
Hormonema macrosporum	CBS 536.94	*Rutilus rutilus* gills	Vologda region, Russia	—	AJ244247	de Hoog et al. 1999
Kabatiella caulivora	CBS 242.64	*Trifolium incarnatum*	USA	—	EU167576	Simon et al. 2009
Kabatiella lini	CBS 125.21	*Linum usitatissimum*	UK	FJ150946	FJ150897	Zalar et al. 2008
Kabatiella microsticta	CBS 342.66	*Convallaria majalis*	Germany	—	EU167608	Simon et al. 2009
Kabatina juniperi	Not Known	*Juniper chinensis*		—	AY183367	Unpublished
Kabatina thujae	CBS 238.66	*Thuja occidentalis*	Berlin, Germany	—	AF013226	Unpublished
Phaeocryptopus nudus	CBS 268.37	*Abies balsamea*	Germany	GU301856	—	Schoch et al. 2009
Rhizosphaera kalkhoffii	CBS 114656	*Picea pungens*	Germany	—	EU700376	Unpublished
	CBS 280.38	*Picea pungens*	Eberswalde, Germany	—	EU700375	Unpublished
Rhizosphaera macrospora	ATCC 4636	*Abies alba*	France	—	AF462431	Unpublished
Rhizosphaera oudemansii	CBS 226.83	*Abies pinsapo*	Spain	—	EU700366	Park et al. 2006
Rhizosphaera pseudotsugae	CBS 101222	*Pseudotsuga menziesii*	Not known	—	EU700369	Unpublished
Scleroconidioma sp.	Not known	*Gaultheria shallon*	Canada	—	AB366648	Osono et al. 2008
Scleroconidioma sphagnicola	UAMH 9731	*Sphagnum fuscum*	Alberta, Canada	—	AY220610	Hambleton et al. 2003
	Not known	Spruce Litter	Czech Republic	—	DQ182416	Koukol et al. 2006
Sclerophoma pityophila	Not known	*Pinus sylvestris*	Michigan, USA	—	AF462438	Unpublished
Selenophoma lini	CBS 468.48	*Linum usitatissimum*	Canada	EU754212	—	de Gruyter et al. 2009
Selenophoma mahoniae	CBS 388.92	*Mahonia repens*	USA	EU754213	—	de Gruyter et al. 2009
Sterile mycelium	Not known	*Ammophila arenaria*	Galicia, Spain	—	AM921728	Sánchez-Márquez et al. 2008
Stylodothis puccinioides	CBS 193.58	*Viburnum lantana*	Switzerland	AY004342	—	Lumbsch et al. 2000
Sydowia polyspora	Not known	*Abies nordmanniana*	Slovakia	—	GQ412728	Unpublished
	Not known	*Abies nordmanniana*	Denmark	—	GQ412722	Unpublished
	Not known	*Abies nordmanniana*	Norway	—	GQ412726	Unpublished
	Not known	*Abies nordmanniana*	Norway	—	GQ412727	Unpublished
	CBS 248.93	*Abies nordmanniana*	Germany	—	GQ412724	Unpublished
	Not known	*Abies nordmanniana*	USA	—	GQ412729	Unpublished
	Not known	*Abies nordmanniana*	Germany	—	GQ412723	Unpublished
	Not known	*Abies nordmanniana*	Denmark	—	GQ412721	Unpublished
	Not known	*Abies nordmanniana*	Austria	—	GQ412719	Unpublished
	Not known	*Abies sp.*	Norway	—	GQ996579	Unpublished
	Not known	*Abies nordmanniana*	Norway	—	GQ412725	Unpublished
	CBS 128.64	*Pinus sylvestris*	Wageningen, Netherlands	—	AJ244262	de Hoog et al. 1999
	CBS 544.95	*Larix decidua*	Putten, Netherlands	—	AY152548	Verkley et al. 2004
					AY293068	Crous et al. 2003
	Not known	*Picea abies*	Sweden	—	AY805637	Menkis et al. 2004
	CBS 116.29	*Pseudotsuga menziesii*		AY544675	—	Schoch et al. 2006

[a]Cultures are deposited in the following collections: ATCC, American Type Culture Collection, Manassasø CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; DAOM, National Mycological Herbarium, Ottawa, Ontario; and UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta.

may represent an additional anamorph of *S. polyspora*, but further research is needed to properly elucidate the life histories of members of this lineage of conifer pathogens. We consider that *A. arboricola* is a potential causal agent for the white spruce decline observed in our study area in Larch Valley because it develops numerous hyphal and meristematically dividing cells in the peridermal region of white spruce bark beneath the conidiomata, and it shows strong phylogenetic affinities to a clade of fungi known to be conifer pathogens: *Sydowia polyspora* (Smerlis 1970; Kraj 2009; Kraj et al. 2009), *Delphinella abietina* (Solheim and Skage 2002), and *Rhizosphaera kalkhoffii* (Juzwik 1993). However, additional infection and re-isolation experiments are required to confirm this supposition.

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