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Bulgariella pulla, a Leotiomycete of uncertain placement, with an uncommon type of ascus opening

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ABSTRACT

Bulgariella pulla (Leotiomycetes) is redescribed with the addition of characters of the ascus, spores, and habitat that were previously unconsidered. The ascus dehiscence mechanism in *Bulgariella* is unusual among Leotiomycetes. In this genus, asci lack a pore and open by splitting to form valves. Phylogenetic analyses of partial sequences of translation elongation factor 1-α (*TEF1-α*), the second largest subunit of RNA polymerase II (*RPB2*), and the 18S and 28S nuc rRNA genes determined that *Bulgariella* belongs within Leotiomycetes but without conclusive assignment to an order or family. A comparison of the nuc rDNA internal transcribed spacers 1 and 2 plus the 5.8S gene (ITS) determined that *Bulgariella* isolates from the USA, Norway, and Sweden had 100% sequence similarity, and an isolate from Chile had 99.3% similarity with these isolates. These results support the proposition that these collections represent a single species, *B. pulla*. *Bulgariella sphaerospora*, a more recently described species, is confirmed as conspecific with *B. pulla*.

ARTICLE HISTORY
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KEYWORDS
Ascus dehiscence; Helotiales; Pezizomycotina; phylogeny; taxonomy

INTRODUCTION

Identification of a dark apothecial ascomycete with unicellular brown to olivaceous ascospores proved challenging. The collection in question was identified as belonging to the genus *Bulgariella*, but further study led to questions about species concepts and placement of the genus within Pezizomycotina. To resolve these questions, we undertook a detailed study. Here, we summarize the taxonomic history of *Bulgariella* and provide an emended description. The phylogenetic position of *Bulgariella* within Pezizomycotina was examined through phylogenetic analyses of DNA sequences of the translation elongation factor 1-α (*TEF1-α*), the second largest subunit of RNA polymerase II (*RPB2*) genes, and the 18S and 28S nuc rDNA. Sequence similarity of the nuc rDNA internal transcribed spacers 1 and 2 plus the 5.8S gene (ITS) among five isolates of *Bulgariella* from the USA, Europe, and Chile were compared to assess the level of diversity within this genus.

Taxonomic history

***Bulgariella pulla* (Fr.) P. Karst.**—Fries described *Patellaria pulla* Fr. (1822, p. 160) and included it in tribe subgelatinosa. He later divided *Patellaria* into two parts and typified Tremellei with *P. pulla* (1828, p. 15). Subsequently, when providing a new classification of Discomycetes (1849, p. 364), Fries placed *P. pulla* in *Bulgaria* (1849, p. 358) and transferred it from Patellariacei (persistent and coriaceous fungi) to Bulgariacei, together with fungi with highly gelatinized tissues. Karsten (1885, p. 142) erected the genus *Bulgariella* P. Karst. with one species, *B. pulla*, to accommodate what he considered a smaller version of *Bulgaria inquinans* (Pers.) Fr. He indicated that the two genera differed in several characters. *Bulgaria inquinans*, a species from both northern and southern temperate zones, occurs on dead wood, particularly *Quercus*. It has large, turbinate, fleshy-gelatinous ascomata and asci with eight ascospores, of which four are dark brown and four are hyaline. Its dark brown ascospores are ellipsoidal, aseptate, and have a long germ slit. An asexual morph assigned to the anamorph genus *Endomelanconium* was described (Fenwick 1992). In comparison, *Bulgariella* has a sessile, convex discoid apothecium with eight brown spores per ascus.

Karsten (1885) assigned *Bulgariella* and *Bulgaria* to subfamily Bulgarieae Karst. He included two other subfamilies in the family Bulgariaceae, Leotiaeae Karst. and Ombrophileae Karst. For much of its history, *Bulgariella* was paired with *Bulgaria* in tribes or families, e.g., Bulgarieae (Phillips 1887; Boudier 1907)

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or Bulgariaceae (Massee 1895). Nannfeldt (1932) included *Bulgariella* (with *Ombrophila, Neobulgaria*, and *Phaeobulgaria*) in subfamily Ombrophiloideae (Helotiaceae). This subtribe includes soft-fleshed species that are either completely gelatinous or have highly gelatinized tissues or a gelatinous layer in the apothecia. Nannfeldt (1932) and Dennis (1956, 1978) considered the presence of gel to be a taxonomically informative character. Emphasizing this character, Korf (1973) grouped *Bulgaria* and *Bulgariella* together with *Leotia*, in Leotiaceae, subfamily Leotioideae, tribe Leotieae. Eriksson (2006) considered *Bulgaria* the sole genus in Bulgariaceae (Helotiales). Earlier, Korf (1957) stabilized the use of the name *Bulgaria* for a member of Leotiomycetes. Prior to his work, *Bulgaria* was used for species with either operculate and inoperculate asci (Seaver 1942).

Bulgariella sphaerospora *(Berk. & M.A. Curtis) Gamundí.*—*Patellaria sphaerospora* was proposed by Berkeley and Curtis (in Phillips 1887). Subsequently, *Lagerheima* Sacc. was described by Saccardo (1892) with six species, including *Lagerheima sphaerospora* (Berk. & M.A. Curtis) Sacc. Clements and Shear (1931) treated *Lagerheima* in family Patellariaceae and designated *L. sphaerospora* the type species, a designation accepted by Nannfeldt (1932), who knew *L. sphaerospora* only from Massee's (1895) description and drawings and placed it in Helotiales. Gamundí (1981) studied the six species originally included in *Lagerheima* and concluded that, with the exception of *L. sphaerospora*, they belonged to *Catinella* Boud. She considered *L. carteri* (Berk.) Sacc. a conspecific synonym of *Catinella olivacea* (Batsch) Boud.; *Lagerheima carbonicola* Torrend to be *C. olivacea* f. *carbonicola* (Torrend) Gamundí; *Lagerheima melanochlora* Boedijn to be a distinct species, *Catinella melanochlora* (Boedijn) Gamundí; and *Lagerheima pilosa* Sydow to be *C. olivacea* f. *pilosa* (Sydow) Gamundí and suggested that *Lagerheima dermatoidea* Rehm was near *Catinella*. In 1981, Gamundí transferred the type species of *Lagerheima, L. sphaerospora*, to *Bulgariella* as *B. sphaerospora*. In this way, *Lagerheima* became a synonym of *Bulgariella. Bulgariella argentinensis* Speg. was considered a synonym of *C. olivacea* by Dennis (1954) and verified as such by Gamundí (1981). *Catinella* has been treated among the Leotiomycetes, but the molecular and morphological analyses by Greif et al. (2007) placed it among Dothideomycetes. Whether all the *Lagerheima* species mentioned above and examined by Gamundí (1981) belong in *Catinella* has not been independently verified. Here, we use molecular and morphological analyses to examine the phylogenetic position of *Bulgariella*. This is the first time that *Bulgariella* has been included in a phylogenetic analysis.

MATERIALS AND METHODS

Collections.—Fresh material and herbarium specimens of material identified as both *B. pulla* and *B. sphaerospora* were examined and sampled for DNA. Collection, preservation, mounting, and morphological analyses used standard mycological methods (Iturriaga et al. 1999; Iturriaga and Pfister 2006). Measurements of structures and photographs were made with an Olympus SZX-1LLK100 stereoscope microscope (SM) (Olympus Optical, Center Valley, Pennsylvania, USA), an Olympus BX40F4 light microscope (Olympus Optical) with an Olympus digital camera (Olympus U-TVO.5XC), and MicroSuite Special Edition software 3.1 (Olympus Soft Imaging Solutions, model XC50; Germany). Dried herbarium specimens were rehydrated in distilled water for 1–2 h, and sections of ascomata were made with a freezing microtome. These sections were then mounted in water or one of the following: 3% KOH (KOH), lactophenol cotton blue (CBL), Congo red (CR; particularly for the study of ascus walls), or Melzer's reagent. India ink in water was also used to determine the presence of gel. Samples were pretreated in KOH to determine if there was variation in reaction of the ascus to iodine (Kohn and Korf 1975). Images of microscopic structures were taken under bright-field (BF) or phase-contrast (PC) microscopy, unless otherwise stated. Differential interference contrast (DIC) optics photography was done at the Harvard Center for Biological Imaging using a Zeiss AxioImager microscope (Carl Zeiss Microscopy, LLC, Thornwood, New York, USA) and ZEN software (Jena, Germany).

Vouchers and collections studied are deposited as stated. Microscopic preparations are deposited with the specimens. All the collections used in the molecular study are deposited in the Farlow Herbarium of Harvard University (FH) (TABLE 1).

Cultures.—Ascospores ejected consistently in groups of eight from inverted ascomata attached to a small piece of wet filter paper to the lid of Petri dishes containing 2% malt extract agar (MEA) and incubated at room temperature (RT). No attempts were made to make single ascospore isolations.

DNA extraction, PCR, and sequencing.—DNA was extracted from five *Bulgariella* specimens collected by Donald Pfister (DHP 06-607, DHP 15-215), T. Læssøe

Table 1. *Bulgariella* isolates examined, sequenced, and used in the molecular phylogenetic study.

		GenBank accession numbers				
Isolate number	Collector, geographic origin	18S	28S	*TEF1-α*	*RPB2*	ITS
DHP 06-607	Donald Pfister, USA	KJ704850	KJ704849	KU845542	KU845544	KJ704848
DHP 15-215	Donald Pfister, Chile	KU845533	KU845536	KU845543	KU845546	KU845540
TL 2011	Thomas Læssøe, Norway	KU845534	KU845535	KU845541	KU845545	KU845537
IM 2003-212	Sven-Åke Hanson, Sweden	NA	NA	NA	NA	KU845538
IM 2008-217	Sven-Åke Hanson, Sweden	NA	NA	NA	NA	KU845539

Note. All isolates are deposited in the Farlow Herbarium.

(TL 2011), and Sven-Åke Hanson (IM 2003-212, IM 2008-217) (TABLE 1), using the DNeasy Plant Mini Kit (Qiagen, Germantown, Maryland; cat. no. 69104). Either 1/10 or 1/100 dilutions of the DNA were used for polymerase chain reaction (PCR) amplifications of (i) a 900-bp region of the *TEF1-α* gene using primers EF1-983F and EF1-1567R (Rehner and Buckley 2005); (ii) the 6–7 region of *RPB2* (Denton et al. 1998) using primers RPB2-P6Fa and RPB2-P7Ra (Hansen et al. 2005); (iii) 18S using primers NS1, NS2, NS4, and NS8 (White et al. 1990) and SL1, SL122, and SL344 (Landvik 1996); (iv) 28S using primers LROR and LR5 (Moncalvo et al. 2000); and ITS using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). All PCRs were done in a Peltier Thermal cycler PTC-200 (MJ Research, Watertown, Massachusetts) and used Econo Taq DNA polymerase (Lucigen, Middleton, Wisconsin). The primers listed above were also used for DNA sequencing. PCR amplifications, purifications, and sequencing were as previously described by Hansen et al. (2005). Sequencher 4.6 (GeneCodes, Ann Arbor, Michigan) was used to edit DNA sequences.

Sequence alignment and phylogenetic analyses.—Alignment of DNA sequences was done using MUSCLE 3.7 through the CIPRES Science Gateway (Miller et al. 2009) and Se-Al 2.0a11 (Rambaut 2002). The data matrix included DNA sequences of the *TEF1-α, RPB2*, 18S, and 28S genes (5731 characters) from three isolates of *Bulgariella* (TABLE 1) and 147 taxa from Spatafora et al. (2006) (TreeBASE S1983), which represent taxa from nine classes of Pezizomycotina. Outgroups were *Neolecta vitellina* and *Neolecta irregularis*. The alignment for this paper is available from TreeBASE (http://www.treebase.org/treebase/) as reference S20785.

For phylogenetic analyses, maximum parsimony (MP) was run using PAUP 4.0b10 (MP; Swofford 2002). Maximum likelihood (ML) used the default GTRGAMMA model of rate heterogeneity and was run on RAxML-HPC2 through the CIPRES Science Gateway (Miller et al. 2009). Bayesian inference (BI) posterior probabilities (PPs) were determined using MrBayes 3.1 with the GTR+I+I substitution model (Ronquist et al. 2005). Four Markov chain Monte Carlo (MCMC) chains were run from random trees for 1 million generations and sampled every 100 generations. The first 1000 trees were discarded as the burn-in phase of each analysis, and PP values were determined from the 50% majority rule consensus of the remaining trees (10 000). Branch supports for MP and ML analyses were determined by 1000 bootstrap replicates. The degree of sequence similarity among the ITS rDNA region of five isolates of *Bulgariella* (TABLE 1) was assessed from the distance matrix calculated by PAUP 4.0b10 (Swofford 2002). Phylogenetic placement of *Bulgariella* within Leotiomycetes was attempted through phylogenetic analyses of 18S, 28S, and 5.8S sequences for 90 representative Leotiomycetes taxa, including *B. pulla* (data not shown).

RESULTS

DNA sequence and phylogenetic analyses.—A comparison of the ITS among the five specimens of *Bulgariella* examined determined that the *Bulgariella* DNA isolated from DHP 06-607, TL 2011, IM 2003-212, and IM 2008-217 had 100% sequence similarity. The collection from Chile, DHP 15-215, had 99.3% similarity to the collections from USA, Norway, and Sweden. DNA sequence obtained for *TEF1-α, RPB2*, 18S, and 28S gene regions also showed a high degree of sequence similarity among the three *Bulgariella* isolates examined (DHP 06-607/ME, DHP 15-215/Chile, and TL 2011/Norway). The DNA sequence similarity was 98.3–99.5% for *TEF1-α*; 98.1–99.9% for *RPB2*; 100% for 18S; and 99.9–100% for 28S. The highest similarity in a BLAST analyses of the *Bulgariella* ITS was only 88% similarity with *Coleophoma ericicola* and *Hymenoscyphus scutula*. BI, MP, and ML analyses of the *TEF1-α, RPB2*, 18S, and 28S sequences positioned *B. pulla* within Leotiomycetes (FIG. 1) with strong support. BI, MP, and ML results did not resolve a statistically supported phylogenetic position for *Bulgariella* among Leotiomycetes taxa sampled (data not shown).

Morphological analyses.—Morphological examination of holotype and authentic material showed that *B. pulla* and *B. sphaerospora* are conspecific. Diagnostic characters

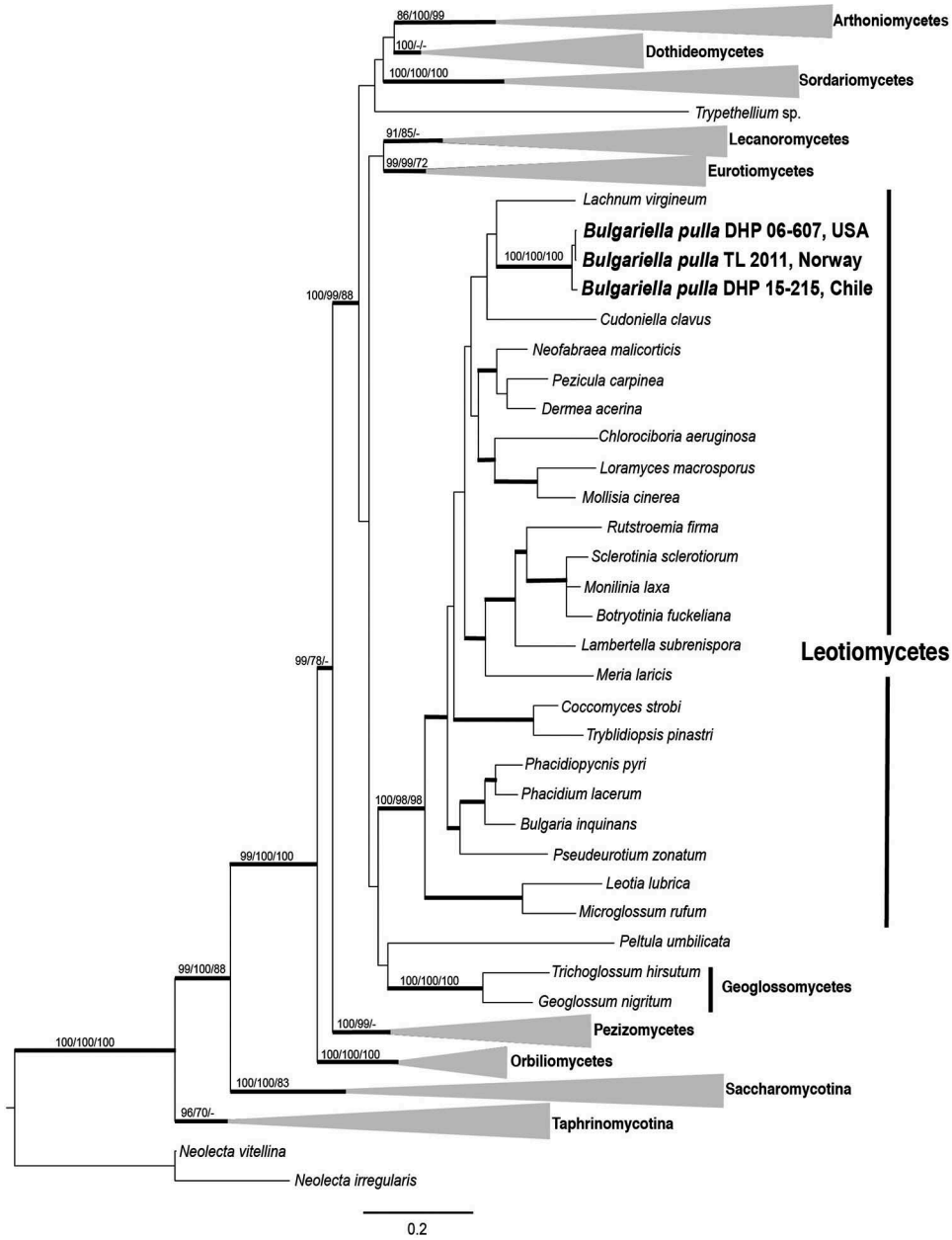
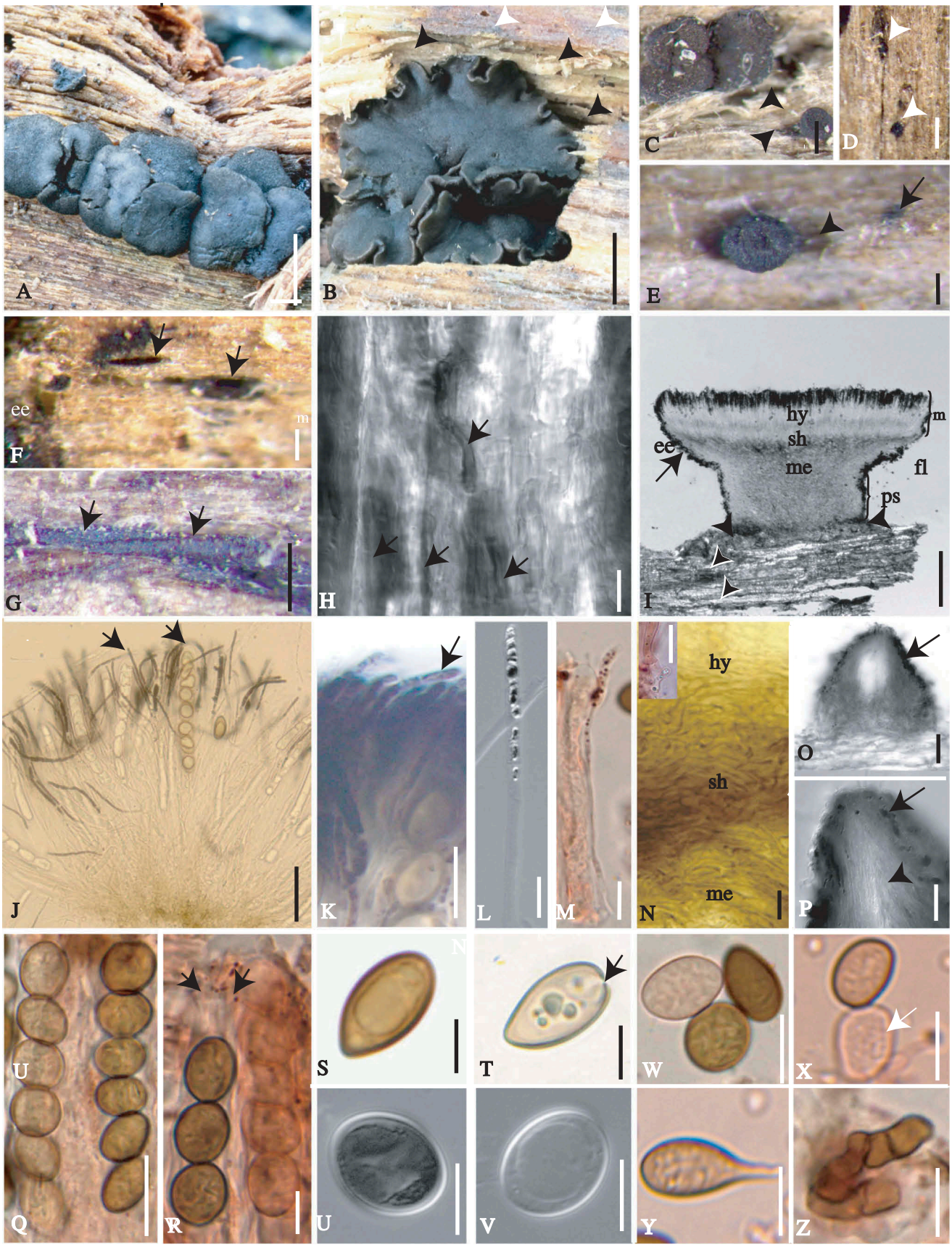


Figure 1. Phylogenetic placement of *Bulgariella pulla* in Pezizomycotina. The phylogeny presented is the maximum likelihood tree from the combined *TEF1-a, RPB2*, 18S, and 28S sequence data (5731 characters and 150 taxa). Branches in bold with numbers above represent clades that have Bayesian posterior probability (PP) values ≥95% and bootstrap values ≥70% for maximum likelihood (ML) and maximum parsimony (MP) analyses (PP/ML/MP, dashes indicate low support values). Branches in bold without numbers represent clades that have Bayesian PP values ≥95% and or ML bootstrap values ≥70%.

for this species are (i) the black gregarious to confluent ascomata with black stromal lines; (ii) erumpent ascomata breaking through the substrate from black immersed stromal masses (FIG. 2A–E); (iii) thick-walled bivalvate asci (FIGS. 3 and 4); (iv) dark brown, variously shaped ascospores, i.e., round, ellipsoidal, obovoid to pyriform; and (v) paraphyses and tissues with granular purple to violet to brown contents.

Ionomidotic reaction.—Our specimens responded variously to KOH treatment. The color of the leachate varied from purple to purple-brown, chestnut-brown, brown, or yellow-orange; the intensity of the color varied. A fresh collection from Chile (DHP 15-215) exuded a purple-to-violet pigment in water, and a reddish-brown pigment in 2% KOH.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Gelatinization.—We found that the amount of tissue gelatinization varied among collections. The ectal excipulum appeared to be composed of interwoven hyphae (at the flanks) or parallel hyphae (at the margin) that were densely agglutinated by gel, as seen by negative staining of sections with India ink. The medullary excipulum lacked gel in the spaces between the hyphae. Hymenial elements likewise were usually agglutinated to some extent (FIG. 2H, I).

Ascospores.—Spores were variable in shape, including in the holotype of *B. sphaerospora* (FIG. 2H, I, Q–T, V), ranging from ellipsoidal, broadly ellipsoidal, irregularly ovoid, obovoid, to subglobose. Ascospores germinated to produce brown, thick-walled, branching hyphae. We were able to induce germination of ascospores from a specimen (DHP 15-215) dehydrated using silica gel and subsequently rehydrated with distilled water. All ascospores germinated after 1 wk, although cultures subsequently became contaminated with *Penicillium* sp.

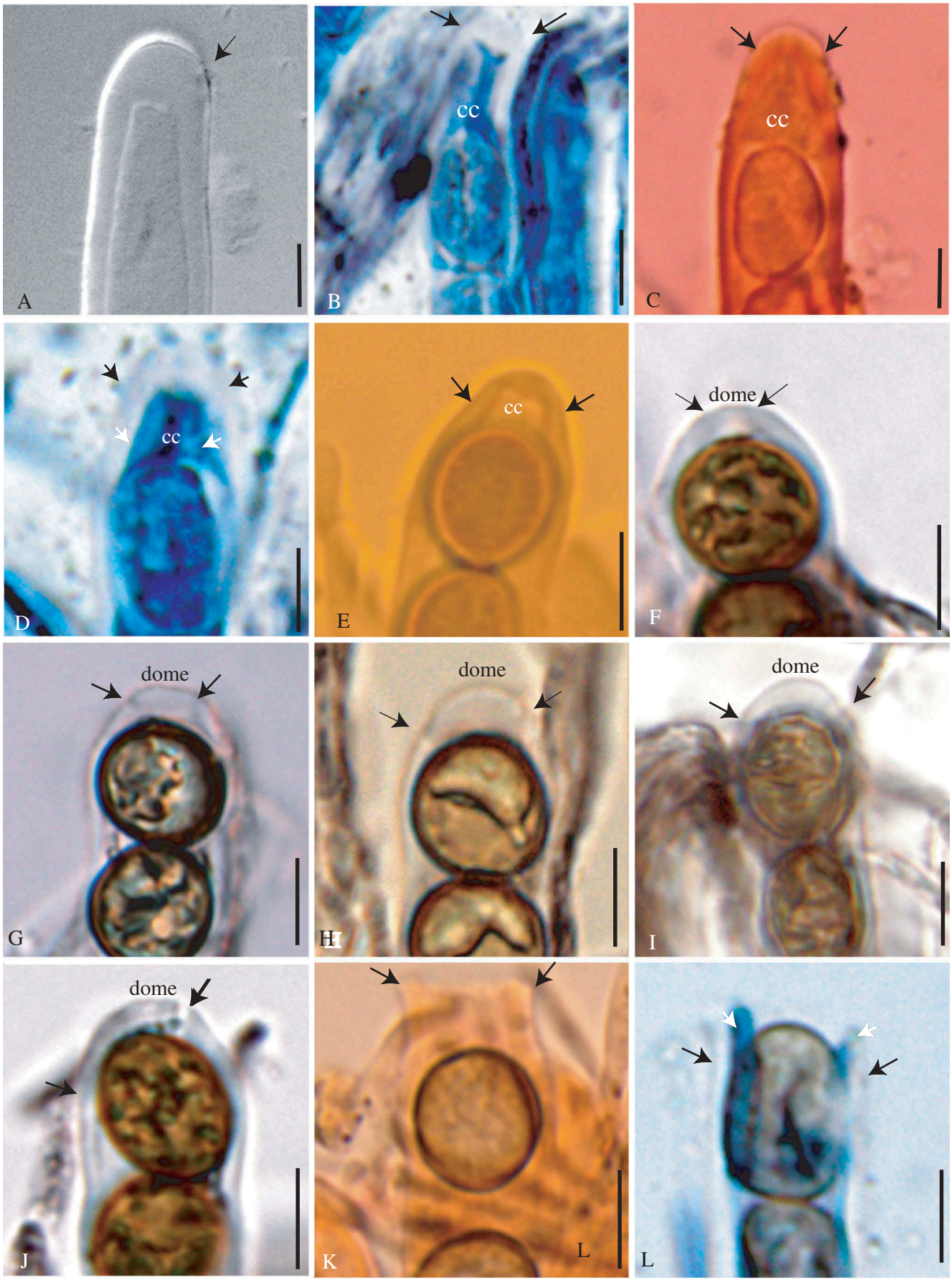
Ascus morphology.—At the earliest stages, the ascus had a thick-walled apex (FIGS. 3A, 4A), and the apical ascoplasm was compressed into a "cytoplasmic cavity" with a "viper tongue" shape; a flattened area occurred at the ascus apex (FIGS. 3B, 4B). Later, the ascus apical cytoplasmic cavity expanded (FIGS. 3C, 4C). As ascospores matured and moved towards the ascus apex, the ascus apical cytoplasmic cavity became smaller (FIG. 3D, E). The walls stained orange-gray with Congo red, whereas the ascoplasm did not stain (FIG. 3E). Eventually, the ascus apical cytoplasmic cavity disappeared as the ascospores matured and enlarged. As the ascus developed, the ascus wall developed "shoulders" around the ascus tip (FIGS. 3E–G, 4B–E). When the asci reached maturity and their wall expanded fully, a thin-walled apical region developed (dome initial) between the thick lateral walls of the ascus (FIGS. 3F, G, 4D, E). This thin-walled apical region, the apical dome, continued expanding at the ascus tip as a result of inner wall extension (FIGS. 3G–I, 4E–G); thus, the thick, lateral ascus wall spread apart at the ascus tip (FIGS. 3G, H, 4E, F). At the place where the thickened wall layer ends and the dome starts, an ascus rim was delimited (FIGS. 3H, 4F). The apical dome was present in mature asci above the ascus rim (FIGS. 3G–I, 4E, F). Eventually the ascus apical dome ruptured at the rim (FIGS. 3J, 4H, I). In other cases, the dome ruptured by the formation of a vertical slit (FIGS. 3K, 4J, K). When mature, the ascus apex had an apical opening within the unstained parallel wall (FIG. 3K, L). At this stage, the only structures seen at the apex of the ascus were lateral walls or valves (FIGS. 3L, 4L).

Ascus histochemistry.—No region of the ascus became blue in Melzer's reagent, with or without KOH pretreatment. We observed no positive iodine reactions of asci in any of the material studied.

Ascomata.—In the youngest condition, apothecia were round masses 0.25–0.5 mm diam with central openings (FIG. 2O, P), superficial or partially to totally immersed in the substrate (FIG. 2C–E). As they mature, ascomata become erumpent.

Stromata.—Ascomata arose from a thick hyphal mat or stroma. In early stages, as seen in most collections, the fungus formed a mat of loose brown hyphae on the wood

Figure 2. Morphological features of *Bulgariella pulla*. I–Z micrographs are bright-field microscopy penicillin G potassiumin water mounts except where noted. A, B. Ascomata (black arrowheads), stromal lines (white arrowheads). C. Erumpent ascomata from stromal masses (black arrowheads) (SM). D. Ascomata primordia (white arrowheads) (SM). E. Young ascoma from stromal masses (black arrowhead), primordium of ascoma (arrow) (SM). F. Immersed ascomata (arrows) (SM). G. Stromal lines (arrows) on host (SM). H. Hyphae (arrows) immersed in substrate (PC). I. Median section of mature apothecium; ectal excipulum (ee) with external granulose cell layer (arrow), medullary excipulum (me), subhymenium (sh), hymenium (hy), pseudostipe (ps), flanks (fl), and margin (ma). Stroma immersed in substrate (black arrowheads) (DIC). J. Mature asci with apical thinning of the wall (arrows), immature asci, and paraphyses, with brown granules at upper region. K. Paraphyses purple color reaction in KOH. L, M. Paraphyses granular contents: left water (PC), right CR. N. From top to bottom: hy repeating croziers (insert); sh of intertwined compact dark-walled hyphae; me of agglutinated hyphae. O, P. Sequential sections of immature ascomata on substrate, granules on the receptacle (arrows); paraphyses filling young ascomata cavity (arrowhead) (PC). Q. Asci with ascospores in CR. R. Asci in CR: left, mature ascus with a "V"-shaped apical rupture or bivalvate slit (arrows pointing at each side of the "V"), and ascospore; right, immature ascus and ascospores. S–V. Ascospores at different developmental stages. T. With germ pore (arrow). U, V. PC images. W–Z. Ascospores in CR. W. Mature ascospores. X. Perispore shed from ascospore. Y, Z. Germinating ascospores. Photos A–V from DHP 06-607 and W–Z from holotype of *L. sphaerospora*. Abbreviations of mounting media and optics: CR, Congo red; DIC, differential interference contrast; PC, phase contrast; SM, stereo microscopy. Bars: A, B = 1 mm; C, D, G = 0.25 mm; E = 200 µm; F = 100 µm; H = 5 µm; I, J = 50 µm; N = 10 µm, N (left insert) = 2 µm; K, L, M, O, P, Q, R, U, V = 20 µm; S, T = 5 µm; W, X, Y, Z = 10 µm.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substrate. Similar hyphae were present immersed in the wood substrate and as heavily colonized dark areas (FIG. 2B–E). Dark hyphae aggregated into dense tightly packed masses (stromata), or into stromatal lines (FIG. 1G) of thick-walled, encrusted, brown hyphae, irregular, brown cells, and round, dark brown granules. Stromatized areas were visible inside the substrate (FIG. 2C–D, F), under and/or around the apothecial base (FIG. 2G, E).

TAXONOMY

Bulgariella pulla (Fr.) P. Karst., Acta Soc. Fauna Flora Fenn 2(6):142. 1885. FIGS. 2, 3, and 4

≡ *Patellaria pulla* Fr., Syst Mycol ii:160. 1823.

= *Bulgariella sphaerospora* (Berk. & M.A. Curtis) Gamundí, Sydowia 34:92. 1981.

≡ *Patellaria sphaerospora* Berk. & M.A. Curtis in Phillips, Grevillea 88:85. 1890.

≡ *Lagerheima sphaerospora* (Berk. & M.A. Curtis) Sacc., Syll Fung (Abellini) 10:55. 1892.

Typification: Patellaria sphaerospora B. & C. USA. SOUTH CAROLINA: Monte Nigro, Car. Sept. [Carolina Septentrionalis], ad ligno carioso, Sep 1854, *Curtis 4460*, folder 391 (holotype!, FH). Isotype (K). [This specimen has drawings by Curtis of asci, ascospores, paraphyses, and a microscopic slide showing 4-spored asci, ellipsoidal brown ascospores with a germ slit, ascomata arising from densely stromatized areas.]

Ascoma an apothecium, dark brown to olivaceous brown, superficial or erumpent through the surface of the wood; turbinate, pulvinate to discoid, (0.5–)1–3 mm diam (FIG. 2A–D), sometimes extruding a brown to purple pigment in KOH. Mature apothecia with short stipes 130–225 µm high and 225–250 µm diam, rarely sessile, or broadly attached, the attachment point 800–1000 µm diam and 200–300 µm high. Frequently gregarious in groups up to 7 mm diam, apothecia at different stages of development present (FIG. 2A); individual apothecia may become distorted because of mutual pressure (FIG. 2B). Apothecia arise from stromata located beneath the bark (FIG. 2C–E). The stromata appear as darkened areas of the substrate (FIG. 2F, G) and are composed of dark brown, densely aggregated hyphae that are superficial or penetrate the wood forming a loose subiculum (FIG. 2H). Receptacle black, with an undulate-irregular surface caused by the aggregation of the outer cells of the excipulum (FIG. 2I); these cells have brown walls and brown granular contents. Margin brown-olivaceous to black, with vertical furrows particularly evident in dry specimens, furfuraceous; margin rising noticeably above the hymenium in dried specimens, and in rehydrated material, the margin sometimes inrolled or undulating; in mature ascomata, margin equal to or below the level of the hymenium (FIG. 2E). Disc concave in young apothecia, plane or convex when mature, pruinose from protruding mature asci, dark brown to brown-olivaceous when fresh or rehydrated, entirely black when dry. Hymenium 125–140 µm thick in section, dark in the top 70–75 µm because of the dark contents of paraphyses and ascospores (FIG. 2I, J). Subhymenium a dark brown layer 2–4 µm thick, visible as a dark, thick line in vertical sections under the dissecting microscope, consisting of densely interwoven hyphae that are 1–3 µm diam, cell walls dark, and the cells filled with abundant dark granules (FIG. 2E). Medullary excipulum of loose textura intricata, mixed with irregular to round cells, all encrusted with dark, spiny granules; more or less globose granules present within hyphae and cells, either structure of variable diam 0.5–2 µm (FIG. 2N). Ectal excipulum of dense textura intricata throughout, sometimes turning deep purple in KOH; the margin of thin parallel agglutinated hyphae, walls thick, brown, sometimes with a wider, terminal cell that contains dark brown granules (FIG. 2I); at the flanks composed of agglutinated long-celled

Figure 3. Ascus apex development of *Bulgariella pulla*. All micrographs are bright-field microscopy in water mounts except where noted. A. Young ascus with thick-walled apex (arrow) in water (DIC). B. Thick apical wall of ascus (arrows), apical ascoplasm deposited in a fork-shaped cytoplasmic cavity (cc), and young ascospore; ascoplasm blue in CBL. C. cc expands, and wall extension at apex continues (arrows), walls red in CR. D. cc becomes smaller as maturation process continues, ascospores move towards ascus apex, and apical wall extension continues (black arrows at lateral ascus walls); ascoplasm at the apex following pattern of apical wall separation (white arrows), in CBL. E. cc progressively becomes smaller, ascospores continue maturation process; ascus wall as "shoulders" (arrows); walls orange-gray in CR, ascoplasm cc does not stain. F. cc disappears; ascospores further into ascus apex; mature asci with apical thin area between thick lateral wall (arrows), which will further develop into a dome, in water. G–I. Further development of dome region. J–L. Ascus apical rupture under different mounting agents. J. Rupture through the rind of the dome. K. Ascus apex after breakage, showing valves (arrows), walls stained in CR. L. Ascus apex showing apical opening within unstained parallel wall or valves (arrows), blue-stained ascoplasm (white arrows), and mature ascospores at release, CBL. All photos from DHP 06-607. Abbreviations of mounting media and optics: CBL, cotton blue in lactophenol; CR, Congo red; DIC, differential interference contrast. Bars: A = 5 µm; B = 10 µm; C and D = 5 µm; E–L = 10 µm.

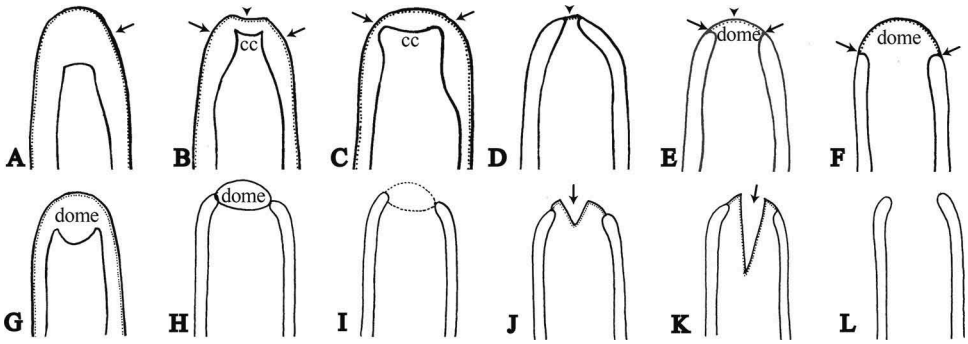

Figure 4. A–L. Schematic drawings of ascus apical development in *Bulgariella pulla* following FIG. 3. A. Young ascus with thick-walled apex (arrow). B. Thick apical wall of ascus (arrows), apical ascoplasm deposited in a fork-shaped cytoplasmic cavity (cc). C. cc expands, and wall extension at apex continues (arrows). D–H. Apical wall extension. D. Start (arrowhead). E, F. Further development of apical dome region delimited by ascus wall (arrows). G. Median view of F. H–L. Ascus apical rupture. H, I. Rupture through the rind of the dome. J, K. Rupture as valves (arrows). L. Ascus apex showing opening.

interwoven hyphae with brown walls; cells (8–)10–25(–30) × 1.5–6 µm, becoming narrower closer to the medullary excipulum; the outermost layer of round cells, dark-walled, containing very dark brown granules. Stipe short or ascomata attached broadly at the base, composed of tissues continuing from the ectal excipulum at the flanks and from the medullary excipulum. The inner core of the stipe composed of a mixture of hyphae 2–6.5 µm diam and rounded cells 12–25 × 7–15 µm, all with dark brown walls and dark granular contents. Asci mostly 8-spored but occasionally 4–6-spored (FIG. 2Q), spores uniseriate to obliquely 1-seriate, asci cylindrical in the upper three-quarters, narrowing progressively toward the base, arising from repeating croziers (FIG. 2N, insert), 100–170 × (6.5–)10.5–16 µm; mature asci protrude above the hymenium; ascus apex hemispherical to truncate (FIGS. 3A–G, 4A–D), with thick walls and two lateral "shoulders" surrounding a central apical dome (FIGS. 3G–I, 4E–G), J–, with or without pretreatment in KOH; the apical dome, located between the "shoulders" of the ascus, disintegrates at the time of dehiscence (FIGS. 3J and 4H–I), the ascus apex opening through an initial "V"-shaped rupture (FIG. 4J), which progresses to a broad slit across the ascus apex, ultimately with two valves (FIGS. 3K, L, 4K, L). Ascospores variable in shape from ellipsoidal, broadly ellipsoidal, irregularly ovoid, obovoid, to broadly fusoid (FIG. 2Q–X), aseptate, with one or two central guttule(s) (FIG. 2S, T), 9–14 × 6.5–10 (–11) µm, walls olive-brown and smooth at first, then dark brown and slightly rugose at maturity, with a germ pore in one end (FIG. 2T). Ascospores germinate to produce brown, thick-walled hyphae (FIG. 2Y). Paraphyses curved or straight; in young apothecia, bending over the asci and completely covering them, when mature, filiform to clavate, straight, undulating, hooked at the apex, 1–2 µm wide below, slightly wider at the apex, <4 µm wide in some collections; simple or divided, septate, cells hyaline to highly pigmented, pigments contained in very distinct and abundant brown to purplish or violet cytoplasmic granules, in the apical and subapical cells, granules rounded, single or amalgamated. The walls and dark granules in the paraphyses become purple-violet when treated with KOH (FIG. 2J–M).

Specimens examined: CHILE. REGIÓN X, REGIÓN DE LOS LAGOS: Parque Katalapa, near Puerto Mont, 22 May 2015, *D.H. Pfister 15-215* (FH: 18S = KU845533; 28S = KU845536; *TEF1-α* = KU845543; *RPB2* = KU845546; ITS = KU845540); Punta Arenas, 4 Mar 1906, *R. Thaxter* [annotated by him "nice disco, phaeosporia"] (FH). NORWAY. MØRE OG ROMSDAL: Nesset, Eikesdalen, Ljåstranda, on hazel (*Corylus avellana*), 18 Sep 2011, *T. Læssøe TL 2011* [in this collection, ascomata arise from densely stromatized areas] (FH: 18S = KU845534; 28S = KU845535; *TEF1-α* = KU845541; *RPB2* = KU845545; ITS = KU845537). SWEDEN. SKÅNE [SCANIA]: Konga parish, north of Skogvångshus, Kvärkabäckens dalgång, 3C2hNO, biotope deciduous forest dominated by *Fagus sylvatica*, on standing, dead trunk of *F. sylvatica*, 4 Feb 2002, *S.-Å. Hanson 2002-027* (FH: ITS = KU845539); SKÅNE [SCANIA]: Hörja; Aggarp, NB, Västervägen, 307d SV, biotope deciduous forest, on *Carpinus*, 5 Sep 2003, *I Månsson*, det. *S.-Å. Hanson 2003-212* (FH: ITS = KU845538); SKÅNE [SCANIA]: Riseberga, Söderåsens National Park, Serpentinvägen, NB 124073, biotope deciduous forest dominated by *Fagus*, on *Fagus* wood, 9 Sep 2008, *I. Månsson*, det. *Læssøe* and *S.-Å. Hanson 2008-217* (FH: ITS = KU845538). USA. MAINE: Eagle Hill, Steuben, on wood, decorticated log, 23 Jun 2006, *D.H. Pfister 06-607* [ascomata arising from densely stromatized

areas] (FH: 18S = KJ704850; 28S = KJ704849; *TEF1-α* = KU845542; *RPB2* = KU845544; ITS = KJ704848); NEW HAMPSHIRE: Crawford's Ravine, 8(?) Aug 1901, *R. Thaxter* (FH); NORTH CAROLINA: Cranberry, elev 3250 ft, Jul–Aug 1887, *R. Thaxter* (FH).

Specimens not seen: Original materials identified as *Patellaria pulla* by Fries from Sweden exist at UPS but are unavailable for loan. These could be eligible for lectotype selection (UPS:BOT:F-127121, F-127123). We have followed Gamundi's (1981) synonymy and have included recent material from Sweden that agrees with *B. pulla* in morphology and genetics.

Geographical distribution: Found in northern temperate areas of Europe, Australia (Victoria) (Beaton and Weste 1976), New Zealand (Bell and Mahoney 1991), and North and South America.

Habitat and occurrence: On decayed wood of *Betula* sp. in the UK (Dennis 1978) and Wales (T. Læssøe, pers. comm.), *Corylus avellana* in Norway, *Fagus sylvatica* and *Carpinus betulus* in Sweden, *Nothofagus* sp. in New Zealand (Bell and Mohoney 1991), *N. betuloides* in Argentina (Gamundí 1981), and on *Drymis winteri* in Tierra del Fuego, Argentina (Gamundí 1981). In temperate North America and Europe, *Bulgariella* is found during late summer, autumn, and winter. In temperate South America, it occurs in Argentina in Feb–Mar and in Chile (Punta Arenas in March, Parque Katalapa in May, Región de Los Lagos and Región del Bio-Bio on plant debris, dates of collection unknown) (Mujica et al. 1980; Gamundí et al. 2004).

DISCUSSION

Gamundí's studies of both *B. pulla* and *L. sphaerospora* led her to conclude that the species were conspecific (Gamundí 1981). She described and illustrated what she considered minor differences between them: (i) in the case of *L. sphaerospora*, apothecia arising from a common black stroma immersed in the substrate, and outer ectal excipulum composed of hyphae with blackish incrusted unevenly thickened walls; and (ii) for *B. pulla*, absence of a stroma, and the hyphae of the outer ectal excipulum with black contents. We observed variation related to the degree of stromatization and/or dark granulation in the outer ectal excipulum. The basal stroma also varies because it is immersed in the substrate and becomes exposed only when the substrate breaks. Variation of basal stroma, and dark contents of outer hyphae of the excipular tissues, may vary among ascomata within single collections. We agree with Gamundí (1981) that *B. pulla* and *B. sphaerospora* are conspecific. This synonymy is also suggested by our comparison of the ITS sequences.

Our studies of *B. pulla* consistently place it in the class Leotiomycetes (FIG. 1). Phylogenetic analyses of members of Leotiomycetes did not resolve the phylogenetic position within this class of *Bulgariella*. From this partial sampling and without support, we noted that *Bulgariella* specimens were consistently positioned on a long branch with *Chlorovibrissea* and *Vibrissea* (data not shown).

We are able to make some observations on characters employed in the Leotiomycetes. Although dark ascospores have sometimes been used as a character to group taxa in Leotiomycetes, the possession of dark spores is not a trait that seems useful in assuming any phylogenetic relationships in this group. Korf (1973) considered gel an important character in the classification of taxa in Helotiales; he treated both *Bulgaria* and *Bulgariella* in Leotiaceae tribe Leotieae together with genera characterized by having tissue gelatinization such as *Ascocoryne, Ascotremella, Chloroscypha, Claussenomyces, Durandiella, Leotia, Neocudoniella, Neobulgaria, Ombrophila, Pezoloma, Phaeangellina*, and *Polydiscidium*. Moore (1965) studied the ontogeny of gel tissue in representatives of several families of Leotiomycetes and concluded that the presence or absence of gel alone is insufficient to delimit any of the groups she studied. Phylogenetic studies of species belonging to Leotiomycetes conducted by Wang et al. (2006) and Baral et al. (2013), among others, included genera and species from several families of Helotiales where gel occurs. Results from their studies indicate that gel probably has evolved in Leotiomycetes independently multiple times. Gel production may be related to ecology rather than phylogeny.

The release of dark pigments into aqueous potassium hydroxide (KOH+) is used as a taxonomic character in some dark-colored apothecia in Helotiales (Korf and Abawi 1971) and is called the ionomidotic reaction. The ionomidotic reaction of the ascomata is not mentioned in most descriptions of this species; Gamundí (1981) studied the type and stated that it exuded a chestnut-brown pigment in KOH. The fungus was reported as nonionomidotic (KOH−) in one collection (Gamundí 1981).

The reaction to iodine in this fungus was reported to be variable by Gamundí (1981). She indicated that there is some variation in the iodine reaction of the asci in *B. sphaerospora* (as *L. sphaerospora*). She describes all specimens studied as having a J− ascus apex, except LPS 39594, which she reported to be J+. In this study, we found asci from all specimens to have J− apices.

Ascus apical dehiscence is considered an important character in the classification of the Ascomycetes. Within Helotiales, at least 15 types of ascus apical

apparatus are recognized (Bellemère 1977;Verkley 1992, 1995; Pärtel and Raitviir 2005). Baral (1987) studied the ascal apex of several members of the Helotiales using light microscopy. He concluded that *Bulgaria inquinans* represents, together with several members of the family Helotiaceae, the Bulgaria-type of ascal apex. Verkley (1992, 1993, 1995) studied ascus dehiscence in species belonging to different genera of Helotiaceae, such as *Ombrophila, Neobulgaria, Chlorociboria, Crocicreas* (= *Cyathicula*), *Cudoniella, Discinella*, and *Pezizella*, in tribe Hymenoscyphoideae (Verkley 1993). Our studies of *Bulgariella* specimens show asci with unusual ascus apices and dehiscence mechanisms. The apical ascus mechanism in *B. pulla* resembles that described by Verkley (1993) for *Cudoniella acicularis*; the reaction in iodine is negative in both species. Verkley (1995) and Baral and Richter (1997) concluded that members of Helotiales have considerable variation in ultrastructure of the apical apparatus. *Bulgariella* has not been examined using transmission electron microscopy (TEM). Asci of *Bulgariella pulla* also resemble those of some members in the family Thelebolaceae (Leotiomycetes, Thelebolales), such as *Thelebolus* (Samuelson and Kimbrough 1978; Kimbrough 1981) and *Ascozonus*. They share a similar character of ascus dehiscence through an apical ring and/or an apical wall split (Brummelen 1985; Landvik et al. 1998; de Hoog et al. 2005).

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