Rugosporella, a new genus to accommodate the North American species *Peziza atrovinosa* (*Pezizaceae*) and its predicted ectomycorrhizal status

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Abstract: The genus *Rugosporella* is proposed to accommodate *Peziza atrovinosa* in the modern classification scheme of the *Pezizaceae*. We used DNA sequences of ITS, LSU and RPB2 to resolve the phylogenetic placement of this taxon. The species occupies a distinct position within a large and diverse clade of hypogeous and epigeous taxa, all of which are either known to be or presumed to be ectomycorrhizal. Although no DNA sequences of this species have been identified from ectomycorrhizal roots, we provide isotopic data supporting its ectomycorrhizal lifestyle. *Peziza atrovinosa* is distinctive in its moderately large vinaceous brown apothecia with thick flesh and the relatively small ascospores that are ornamented with a high reticulum and that become yellow-brown at maturity. This species is found across eastern North America.
Keywords: *Ascomycota*, ascospore pigmentation, isotopic analysis, molecular phylogeny, *Pezizales*.

Introduction

The operculate discomycete *Peziza atrovinosa* Cooke is commonly collected on soil in mixed woods in late summer and autumn in eastern North America. This species is moderately large (3–5 cm) and is distinctive in its relatively small ascospores, which have a high, coarse reticulum that at maturity becomes yellow-brown. This species has most often been confused with *Legaliana badia* (Pers.) Van Vooren (formerly *Peziza badia* Pers.). In *L. badia* the incomplete reticulum is finer and is not pigmented (Van Vooren, 2020).

Molecular phylogenetic studies have revealed relationships among *Pezizaceae*, both hypogeous and epigeous, that have led to major revision of the generic circumscriptions (Hansen *et al.*, 2001; Hansen *et al.*, 2005; Hansen & Pfister, 2006). The large, heterogeneous genus *Peziza* has been broken into several genera, some recognized by earlier workers. Other genera have been described anew as clades have been identified through sequence analyses (see Pfister, 2015; Van Vooren, 2020). There remain described species named in the genus *Peziza* that have not been yet placed in modern genera. One such species is *Peziza atrovinosa*.

Peziza atrovinosa was described from material provided to M. C. Cooke by William R. Gerard, a druggist in Poughkeepsie, New York. From our present study we know that the species is broadly distributed across eastern North America. It is frequently collected on soil on trail verges and banks. Its moderate size makes it a target for general collectors. Although distinctive in size, color and notable spore characteristics, the species has not been without controversy. In the European literature it was suggested that *P. atrovinosa* and *P. ostracoderma* Korf were synonyms, the older name being *P. atrovinosa*. This confusion was discussed and clarified by Hennebert & Korf (1975). These two species are quite different in both morphology and in habitat. Although both have ascospores with reticulations, those in *Peziza atrovinosa* are prominent and become pigmented at maturity. The finely reticulate ascospores of *P. ostracoderma* remain hyaline. *Peziza atrovinosa* is collected on soil in forests with no anamorph recorded whereas *P. ostracoderma* is generally found as both teleomorph and chromelosporium-like anamorph in greenhouses on steam-sterilized soil or where the substrate has been exposed to heat and is often noted in its anamorph form (Hennebert & Korf, 1975). These authors state that *P. atrovinosa* has not been reliably reported in Europe. Our findings agree with this statement.

Still other questions remain, including potential synonyms of this species. At least two species described from eastern North America have been suggested as synonyms of *P. atrovinosa* (Pfister, 1978a, 1978b). *Peziza retiderma* Cooke has been variously characterized but, aside from the type collection, it has not been reliably recollected. It has been reported from Australia (Rifai, 1968) and Madagascar (Le Gal, 1953), but these reports have been revised (Moravec & Spooner, 1988). That revisionary work resulted in the Austral-Asian material being named as a different species, *Peziza rifaii* J. Moravec & Spooner. Collections from Madagascar have been re-examined in our study. *Peziza chlamydospora* Ellis has also been considered a synonym of *P. atrovinosa* (Seaver, 1928; Pfister, 1978a). It too is known reliably only from type and authentic collections from the 1880s. In both cases the type material of these species was collected in eastern North America in regions and habitats where *P. atrovinosa* has been documented.

In this contribution we place *P. atrovinosa* in a new genus and resolve some of the questions surrounding synonymy.

Materials and methods

Morphology studies

The isotype of *Peziza atrovinosa* was borrowed from the Cornell University Plant Pathology Fungarium (CUP). Collections studied morphologically are cited with depositories in the specimens examined section These specimens were examined microscopically using free hand sections mounted in tap water, 3% KOH, and in Melzer's reagent. Examination was carried out on an Olympus BX-40, or a Zeiss Axio Imager A2 compound microscope. Images were captured by an Axiocam 305 camera using Zen Pro v3.1 software (Carl Zeiss, Oberkochen, Germany). To more fully record the intricate spore ornamentations, multiple images were stacked using Helicon Focus v8.0.4 Pro (Helicon Soft Ltd 2000, Kharkiv, Ukraine).

Molecular techniques and phylogenetic analyses

From fresh and fungarium collections, small tissue samples were selected for DNA extraction and subsequent DNA sequencing from the *Peziza atrovinosa* specimens (Table 1). These include seven specimens of *P. atrovinosa* from the Farlow Herbarium, Harvard University Herbaria (FH), along with seven specimens from the University of Florida Fungarium (FLAS-F). DNA extraction of the ascomatal samples from fungarium material was performed using either the Qiagen DNeasy Plant Mini Kit (Qiagen, Germantown, Maryland) or a modified CTAB-based chloroform extraction method (Gardes & Bruns, 1993). Rapid extractions were performed on freshly collected material that was stored in alkaline extraction buffer following the

methods of Vandepol *et al.* (2020). Tissues were ground in Microeppendorf 1.5 ml tubes using Kimble's Pellet Pestle (catalogue no.749521-1500) and sterile sand. Standard PCR amplification and Sanger sequencing was performed on most samples.

The genomes of three of the FLAS fungarium specimens were also sequenced on the Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA) at the Genomics Core at Michigan State University (East Lansing, MI, USA), as described below. For all other specimens, DNA dilutions of either 1:10 or 1:100 of DNA template:water were used for polymerase chain reaction (PCR) amplification. For Sanger sequencing of DNA extracts of both fungarium and fresh material, the following gene regions were amplified: (i) the nuclear ribosomal internal transcribed spacer region (ITS1-5.8s-ITS2, abbreviated as ITS) using ITS1F (Gardes & Bruns, 1993) or ITS5 as the 5′ primer and ITS4 (White *et al.*, 1990) as the 3′ primer; (ii) the nuclear ribosomal 28S Large Subunit (LSU) using primers NL1 and NL4 (O'Donnell, 1993) or LROR and LR5 (Hopple & Vilgalys, 1994); (iii) the RNA polymerase II gene (RPB2) using primers RPB2-200036F (Pfister *et al.*, 2008) and RPB2-7CR (Liu *et al.*, 1999) or newly designed RPB2 primers specific for *Peziza atrovinosa* (5′, Patro_RPB2_1528F: CAGCTCCACAACACCCATTG and 3′, Patro_RPB2_2467R: TGAGGAACTCCAT GGAACGTG). The primer design used an RPB2 target sequence was derived from a *P. atrovinosa* genome using the NCBI (National Center for Biotechnology Information) primer design program Primer BLAST (Ye *et al.*, 2012).

PCR amplifications were carried out in a Bio-Rad C1000 Thermal Cycler (Applied Biosystems, Hercules, CA, USA). For PCR, 5 µl of 1/10 dilutions of the fungarium DNA extracts were used as templates in a reaction volume of 20 µl, and 1 µl of the rapid extraction of fresh material was added to a reaction volume of 24 µl. The Bio-Rad Iproof High-Fidelity PCR Master Mix (Applied Biosystems, Hercules, CA, USA, catalogue no. 1725310) was used for PCR amplification of the ITS and LSU regions with thermal cycling parameters as described in Pfister *et al.* (2020). High-fidelity platinum Taq by Invitrogen (Waltham, MA, USA catalogue no.11304011) was used for PCR amplification of the RPB2 region. PCR cycling parameters were described previously in Pfister *et al.* (2020).

The PCR products of all genes were subsequently prepared for PCR purification and Sanger sequencing using GeneWiz Inc. (Cambridge, MA, USA) sequencing facilities or Eurofins Genomics (Louisville, KY, USA). The forward and reverse sequences from each PCR product were edited with Geneious v10.2.3 or Geneious Prime 2024.0.3 (https://www.geneious.com). The newly generated DNA sequences were submitted to the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/; ITS accession numbers in Figure 2, RPB2 and LSU accession numbers in Table 1).

Additional ITS, LSU, and RPB2 sequences were extracted from three de novo genome assemblies of *P. atrovinosa* that were included in an ongoing genome sequencing project of diverse *Pezizomycetes* (Lemmond *et al.*, unpublished). Briefly, genomic DNA extracted from three *P. atrovinosa* specimens (FLAS-F-68805, FLAS-F-69722 and FLAS-F64202) was sequenced with 150bp paired-end sequencing on the Illumina Novaseq 6000 platform at the Genomics Core at Michigan State University. *De novo* assemblies of resulting reads were conducted with a custom bioinformatic pipeline modeled after the Automatic Assembly for the Fungi (AAFTF) pipeline (Stajich & Palmer, 2012). Amino acid sequences of the RPB2 locus were identified in each assembly using the Universal Fungal Core Genome (UFCG) pipeline (Kim *et al.*, 2023) and the corresponding nucleotide sequences were extracted from genome assemblies using Exonerate v2.4.0 (Slater & Birney, 2005). ITS and LSU sequences were extracted from genome assemblies using the BARRNAP tool (Seeman, 2018).

The species delimitation of *P. atrovinosa* and its phylogenetic placement within the *Pezizaceae* was estimated by two separate analyses: a Maximum Likelihood (ML) analysis of aligned ITS DNA sequences and a ML analysis of a concatenated alignment of LSU and RPB2 DNA sequences. For the ITS dataset, we included representative sequences from ascomata, mitotic spore mats, and ectomycorrhizal root tips from *Pezizaceae*, including sequences from collections for a core set of genera (*Cazia*, *Chromelosporiopsis*, *Chromelosporium*, *Legaliana*, *Mycoclelandia*, *Ruhlandiella*, *Tirmania*, and *Velenovskya*) and closely related sequences. To ensure that we included as many closely related taxa as possible we used BLAST to check several sequences from each of these genera, as well as our *P. atrovinosa* sequences, and also used the detailed ITS analyses of Healy *et al.* (2022). We made a special effort to include as many ITS DNA sequences from ectomycorrhizal root tip samples (ECM) as possible. Taxa are listed by their GenBank accession numbers in Figure 2. The ITS DNA sequences were first aligned with MAFFT, through the CIPRES Science Gateway (Miller *et al.*, 2010) or with MUSCLE v5.1 (Edgar, 2022) in Geneious Prime. Maximum Likelihood (ML) phylogenetic analysis, using the GTRGAMMA substitution model, default parameters, and 1000 bootstrap replicates, was performed using the online version of RAxML-HPC2 on XSEDE v8.2.12 (CIPRES, Miller *et al.*, 2010; Stamatakis, 2014).

To assemble the LSU and RPB2 datasets, we downloaded all *Pezizaceae* sequences for which both the LSU and RPB2 regions were available, starting with the backbone dataset from Hansen *et al.* (2005). To recover any additional relevant *Pezizaceae* sequences and sequences closely related to *P. atrovinosa*, we used BLAST with our LSU and RPB2 sequences against the NCBI GenBank database. The ITS and RPB2-LSU alignments are available through Open Science Framework (https://osf.io/arp89/?view_only=a1b027f6704345a8acffced6c4815f65).

Isotopic analysis

Isotopic and compositional properties of two *P. atrovinosa* samples (FLAS–F–64202 and FLAS–F–68216) were measured at the Light Stable Isotope Mass Spectrometry Lab at the University of Florida (Gainesville, FL, USA). For each sample, %C, %N, δ15n and δ13C were measured. All carbon isotopic results were expressed in standard delta notation relative to Vienna Peedee Belemnite (VPDB). All nitrogen isotopic results are expressed in standard delta notation relative to air.

An analysis of δ13C and δ15N values of the *P. atrovinosa* samples was conducted to test the hypothesis of ECM trophism for this group. This analysis used existing isotopic data from 813 fungal specimens of known trophic modes from Mayor *et al.* (2009) as a training dataset for a quadratic discriminant analysis (QDA) model (Birkebak *et al.*, 2013). A QDA model was selected as an appropriate model given the unequal variances of the δ13C and δ15N values used in the training dataset. This QDA model was used to predict the trophic status (either ECM or saprobic) of the two *P. atrovinosa* samples. The QDA analysis was conducted in RStudio (2023.06.2+561) using R v4.3.1 (R core team, 2021) and the qda function in the MASS package (Venables & Ripley, 2002). Data were visualized in R using the ggplot2 package (Wickham, 2016).

Results

Phylogenetic results

The LSU alignment included 69 taxa and 923 characters and the likelihood score for the best tree was -6756.606787. The RPB2 alignment included 74 taxa and 521 characters and the likelihood score for the best tree was -9678.481038. There were no supported incongruences between the two phylogenetic trees, so the two alignments were concatenated. The RPB2-LSU alignment had 74 taxa and 1444 characters and the likelihood score for the best tree was -16675.34586. The RPB2 locus was represented for each taxon, but the LSU was missing for the following five accessions: *Peziza ammophila*, one of the two *P. lobulata* accessions, *P. pseudoammophila*, *P. subviolacea*, and one of the two *Sarcosphaera coronaria* accessions. *Ascobolus crenulatus* (*Ascobolaceae*) served as the outgroup for the *Pezizaceae*. The best tree is shown in Fig. 1. *Peziza atrovinosa*

Table 1 – *Peziza atrovinosa* herbarium specimens and GenBank DNA sequences included in the molecular phylogenetic study

Current Name	Herbarium	Locality	LSU	RPB2
Amylascus cineraceus	FLAS-F-64534	Chile	OQ270081	OQ230425
Amylascus hallingii	NY1491200	Australia	NG_242434	OQ230422
Ascobolus crenulatus	AFTOL-ID_181	unknown	AY544678	DQ470893
Daleomyces exogelatinosus	C:KH-00-029	Denmark	AY500545	AY500501
Daleomyces ligni	CBS 146637, CPC-39110	France	MW883832	MW890073
Daleomyces petersii	TAAM:187584	UK: England	MN737816	MN816681
Daleomyces phillipsii	TAAM:199363	Estonia	MN737802	MN816674
Elaiopezia boudieri	MPU:JCD 954-75	France	MT273595	MT274700
Elaiopezia obtusapiculata	C:TL-6474	Denmark	AY500550	AY500490
Elaiopezia polaripapulata	C:KH-96-11	Denmark	AY500551	AY500515
Geoscypha tenacella (*P. subviolacea*)	TAAM:165082	Estonia	–	MN816685
Geoscypha ampelina	C:KH-00-011	Denmark	AF335127	AY500492
Geoscypha violacea (*P. lobulata*)	FH:KH-03-157	USA	AY500548	AY500495
Geoscypha violacea (*P. lobulata*)	GM 010520142	Italy	–	MN816678
Hansenopezia decora	CNF 2/10621	Croatia	NG_241909	MK673767
Hansenopezia retrocurvata	C:KS-94-182	Denmark	AF335159	AY500516
Hydnoplicata whitei	C, OSC:Trappe 17049	Australia	AF335168	AY500491
Hydnotryopsis sp.	C, OSC:Trappe 17231	USA	AF335116	AY500472
Iodophanus carneus	C:JHP_00-027	Denmark	AY500534	AY500506
Iodophanus hyperboreus	C:Gr-83-06	Greenland	AY500535	AY500458
Iodowynnea auriformis	FH:18510_PAN	India	AF335118	AY500473
Legaliana badia	LY:NV 2019.09.14	France	MT273593	MT274699
Legaliana limnaea	C:HFG_94-2	Denmark	AF335147	AY500518
Malvipezia emileia	GM 03091006	Italy	KU898062	KJ728720
Malvipezia howsei	C:KH-97-98	Denmark	AF335146	AY500493
Pachyphlodes annagardnerae	MIN:925696	USA: IA	KJ775836	OQ230428
Pachyphlodes thysellii	FLAS-F-66243	USA: MN	JN121369	OQ230436
Paragalactinia michelii	C:TL-5692	Denmark	AY500549	AY500494
Paragalactinia succosa	C:KH-97-139	Denmark	AF335167	AY500517
Paragalactinia succosa	C:KH-98-07	Denmark	AF335166	AY500487
Peziza ammophila	LIP:791126	France	–	KX271742
Peziza arvernensis	C:KH-98-12	Denmark	AF335131	AY500497
Peziza azureoides	MPU:JCD 856-75	France	MT273592	MT274698
Peziza badiofusca	C:KH-98-113	Sweden	AF335132	AY500475
Peziza depressa	C:KH-98-28	Denmark	AF335135	AY500474
Peziza echinispora	TURA:JukkaVauras_9110F	Finland	AF335138	AY500496
Peziza ellipsospora	C, OSC:Trappe13017	USA	AF335139	AY500482
Peziza nordica	FH:G01_15	Norway	KU898046	KU898054
Peziza nordica	FH:G07_14	Norway	KU898047	KU898055
Peziza oliviae	OSC:JLF2140	USA: OR	KU898051	KU898059
Peziza oliviae	OSC:JLF2538	USA: OR	KU898050	KU898058
Peziza pseudoammophila	LIP:791104	France	–	KX271743
Peziza saniosa	C:KH-97-137	Denmark	AF335160	AY500476
Peziza varia	C:KH-97-107	Denmark	AF335150	AY500498
Peziza varia	C:KH-97-54	Denmark	AF335134	AY500519
Peziza varia	C:KH-99-04	USA	AF335151	AY500499
Peziza vesiculosa	OSC:100126, AFTOL-ID_507	unknown	DQ470948	DQ470898
Phaeopezia calongei	LIP:0002247	Spain	OL984070	OL984066
Phaeopezia calongei	MA-F-33516	Spain	OL984069	OL984065
Phylloscypha phyllogena	C:KH-99-03	USA	AF335155	AY500480
Phylloscypha kallioi	TUR s.n. (type)	Finland	AF335156	AY500481

Table 1 – (continued)

Current Name	Herbarium	Locality	LSU	RPB2
Plicaria carbonaria	FH:DHP 9215	USA	AY500553	AY500479
Plicaria leiocarpa	AFTOL-ID_1345, CBS-144.92	unknown	DQ842029	DQ842038
Plicaria trachycarpa	C:KH-97-93	Denmark	AY500554	AY500478
Plicariella flavovirens	FLAS-F-61549	USA: MN	JN121375	OQ230429
Purpureodiscus bananincola	FH:V-Demoulin-5529	New Guinea	AF335133	AY500483
Purpureodiscus subisabellinus	C:RK 96-54	Norway	AF335163	AY500484
Purpureodiscus subisabellinus	Winterhoff 8844	Germany	AF335164	AY500485
Rugosporella atrovinosa	FH 00465069	USA: MA	PP663026	PP681916
Rugosporella atrovinosa	FH 00822838	USA: ME	PP663025	PP681915, PP823944
Rugosporella atrovinosa	FLAS-F-64202	USA: OH	MT350464	PP823944
Rugosporella atrovinosa	FLAS-F-68805	Canada: QC	OR134533	PP823942
Rugosporella atrovinosa	FLAS-F-69722	USA: KY	PP808677	PP823943
Ruhlandiella berolinensis	C:Mycoflora_of_Macaronesia 1230	Spain: Canary Islands	AF335175	AY500477
Ruhlandiella patagonica	FLAS-F-62145	Argentina	MG947618	MH156156
Ruhlandiella patagonica	FLAS-F-62148	Chile	MG947619	MH156157
Sarcopeziza sicula	MCVE 25877	Italy	MH704523	MH709116
Sarcosphaera coronaria	C:KS-94-19	Denmark	–	AY500523
Sarcosphaera coronaria	C:KS-94-24A	Denmark	AY500555	AY863001
Terfezia bertae	PA-2022a_AH_51463	Spain	ON009056	ON012516
Terfezia claveryi	FH, OSC:Trappe 3195	Kuwait	AY500558	AY500503
Tirmania nivea	C, OSC:Trappe 23190	Israel	AF335177	AY500525
Tirmania pinoyi	C, OSC:Trappe 13587	Saudi Arabia	AF335178	AY500502
Velenovskya vacini	TUR-A 209626	Italy	ON775566	ON758342

was placed with strong support within a clade of *Pezizaceae* that included *Velenovskya*, *Ruhlandiella*, *Hydnoplicata*, *Tirmania*, *Legaliana*, *Terfezia*, *Peziza ellipsospora* and *Galactinia*. *Peziza atrovinosa* was not supported as a member of any other genus in the RPB2-LSU analysis.

Phylogenetic analyses of the ITS rDNA sequence dataset using ML (Fig. 2) indicate that the *P. atrovinosa* specimens form a highly supported monophyletic clade that is unique among genera of the *Pezizaceae*. All of the ITS rDNA sequences from the *P. atrovinosa* specimens in this study were 98% to 100% similar to each other. Inclusion of ITS rDNA sequences from ECM roots in the phylogeny demonstrate that the mycorrhizal habit is common among many genera in the *Pezizaceae*, including species of *Cazia*, *Chromelosporiopsis*, *Legaliana*, *Mycoclelandia*, *Ruhlandiella*, *Terfezia*, *Tirmania*, and many taxa that are considered currently as "*Peziza*" but require further taxonomic reconsideration.

Isotopic analysis results

Isotopic analysis of two *P. atrovinosa* specimens (FLAS-F-64202 and FLAS-F-68216) predicted an ECM trophic status for the *P. atrovinosa* lineage (Fig. 3). Both samples of *P. atrovinosa* were predicted by the QDA model as belonging to the ECM group (Fig. 3). FLAS-F-64202 (δ15N = 3.56, δ13C = -26.11) was predicted as ECM with 0.95 posterior probability, and FLAS-F-68216 (δ15N = 6.42, δ13C = -25.94) was predicted as ECM with 0.99 posterior probability.

Taxonomy

Rugosporella Pfister, Healy & LoBuglio, *gen. nov*. – MB 854056

Description: Apothecia shallow cupulate, often in groups, hymenium ranging in color from tawny buff to dark brown to nearly black on drying, outer surface concolorous or lighter than the hymenium, scurfy. Flesh thick, composed of *textura intricata* with intermixed globose cells. Outer excipulum with low pustules composed of parallel cells oriented perpendicularly to the outer surface. Ascospores broadly ellipsoid, under 15 µm long, with two guttules, surface ornamented with a regular or irregular reticulum the veins of which become brown at maturity. Asci blue in iodine at the apex without a ring and blue in the upper half. Paraphyses slightly swollen at the tip, containing dark amorphous material.

Etymology: From Latin *rugo* – wrinkled or folded; *spora* – spore; *-ella*, implying the diminutive. Also referring to the name used by Gerard, *Peziza rugospora*, when sending material to M. C. Cooke.

Type: *Peziza atrovinosa* Cooke.

Rugosporella atrovinosa (Cooke) Pfister, Healy & LoBuglio, *comb. nov.* – MB 854057 – Fig. 4, 5.

Basionym: *Peziza atrovinosa* W.R. Gerard ex Cooke, *Bull. Buffalo Acad. Sci.*, 2: 288 (1875).

Typification: Holotype: (K); Isotype: USA. Dutchess Co., Poughkeepsie, *leg.* W. R. Gerard, lectotype K (designated by Rifai, 1968), isolectotype CUP-D-03703 (27-31).

Homotypic synonymies: *Plicaria atrovinosa* (Cooke) D.S. Hone, *Minn. Bot. Stud.*, 4 (2): 79. (1909); *Aleurina atrovinosa* (Cooke) Seaver, *North American cup-fungi (Operculates)*: 101 (1928); *Galactinia atrovinosa* (Cooke) Le Gal, *Bull. Soc. mycol. Fr.*, 78: 207. (1962).

Taxonomic synonymies: *Peziza retiderma* Cooke, *Mycographia*, 1 (4): 176 (1877); *Phaeopezia retiderma* (Cooke) Sacc., *Syll. fung.*, 8: 472. (1889); *Aleurina retiderma* (Cooke) Sacc. & P. Syd., *Syll. fung.*, 16: 739 (1902); *Galactinia retiderma* (Cooke) Le Gal, *Discomycètes de*

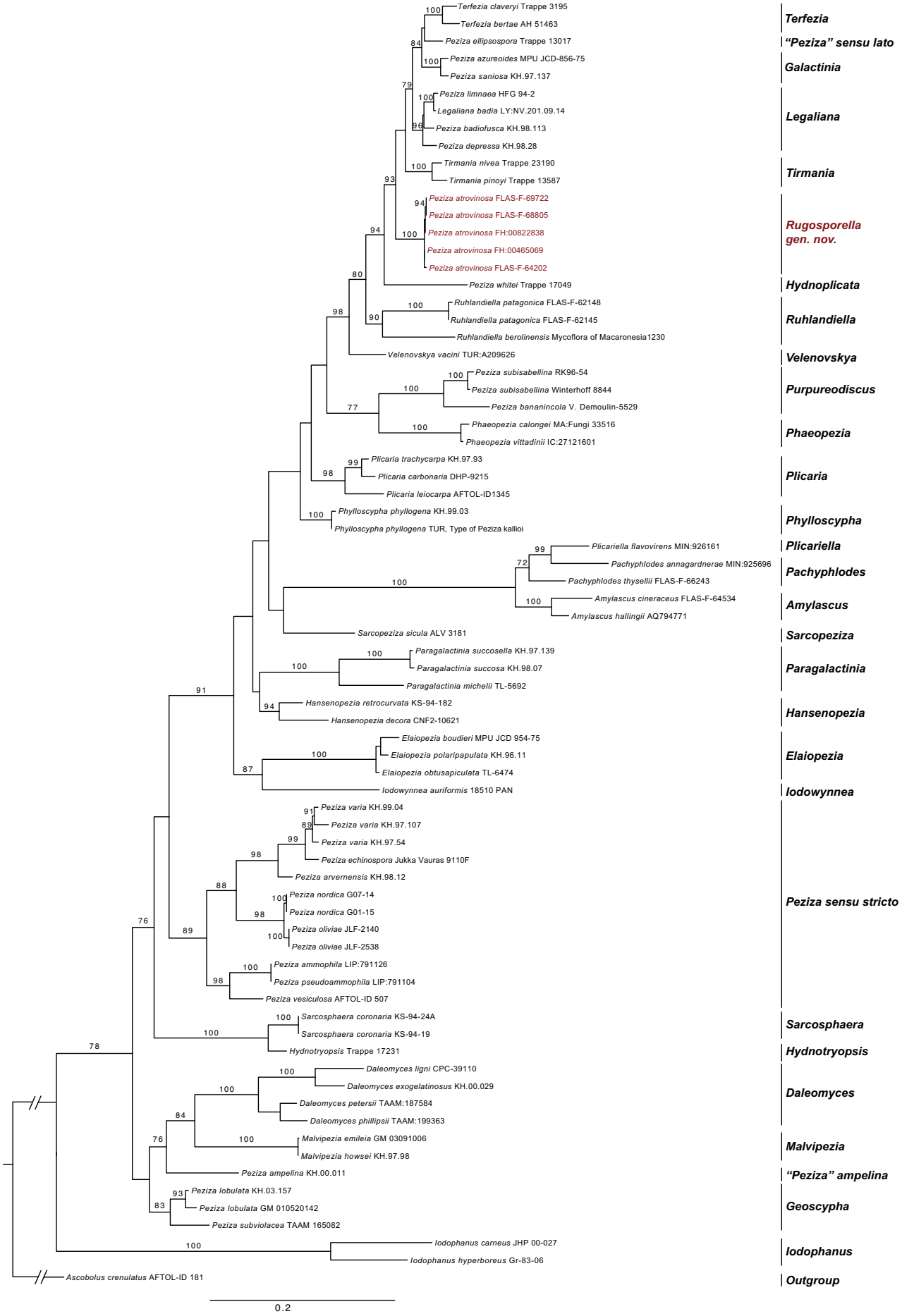

Figure 1 – Maximum Likelihood phylogeny based on concatenated LSU rDNA and RPB2 sequences from *Rugosporella atrovinosa* (text in red) and other genera in the *Pezizaceae*. Fungarium or collector numbers follow species names. Bootstrap support ≥70 is shown on branches. *Ascobolus crenulatus* is the outgroup species.

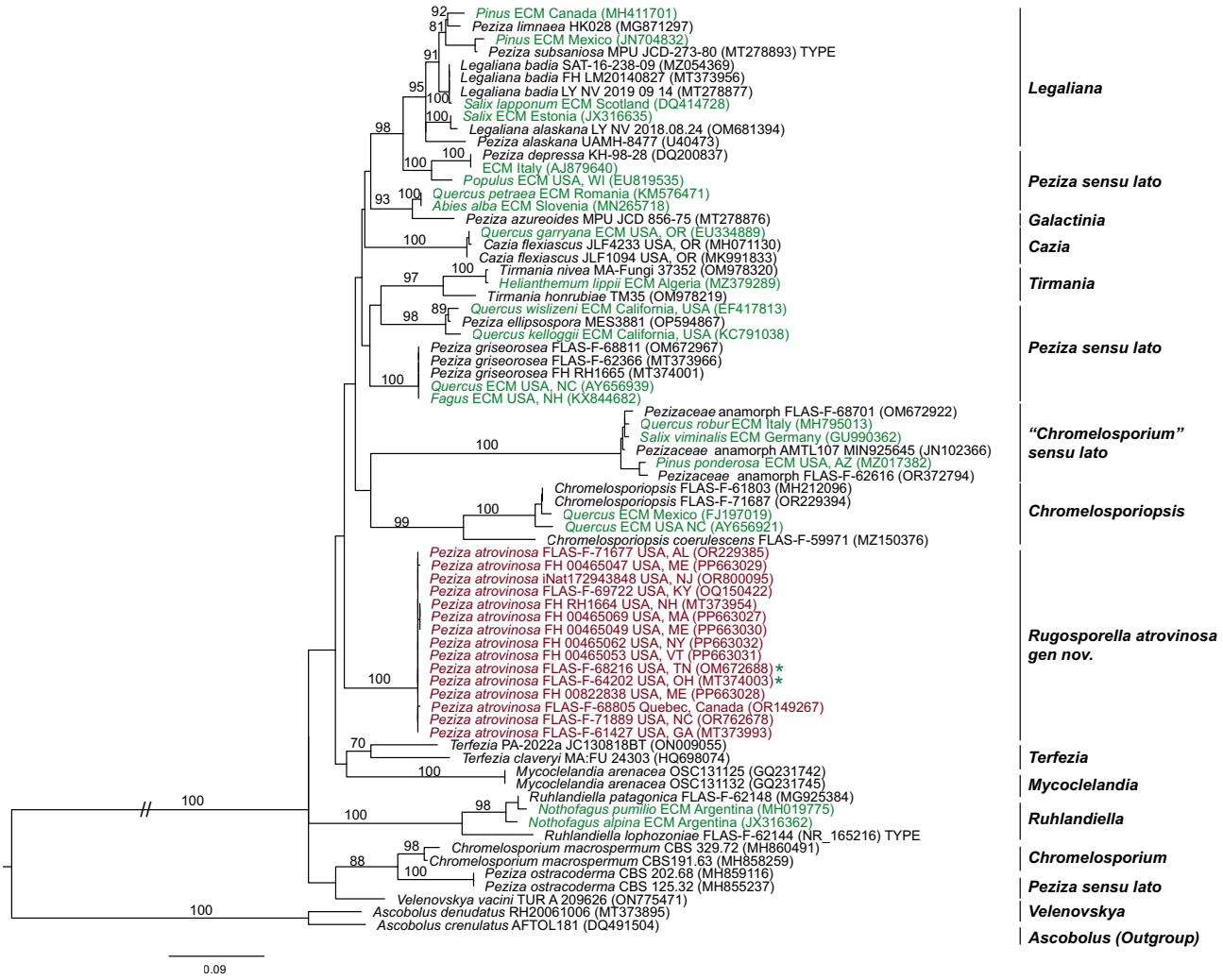

Figure 2 – Maximum Likelihood phylogeny based on ITS rDNA sequences from *Rugosporella atrovinosa* fungarium specimens (text in red) and genera in the *Pezizaceae*. ITS DNA sequences from ectomycorrhizal tissue samples are indicated as "ECM" and are highlighted in green. The two *R. atrovinosa* specimens (FLAS-F-64202 and FLAS-F-68216) with predicted ECM trophic status based on isotopic analysis are indicated with a green asterisk. GenBank numbers associated with each taxon are in parentheses. Bootstrap support ≥70 is shown on branches. *Ascobolus crenulatus* and *A. denudatus* are the outgroup species.

Madagascar: 57 (1953); *Peziza chlamydospora* Ellis & Everh., *Bull. Torrey Bot. Club*, 10: 98 (1883).

Macroscopic features: Apothecia gregarious to cespitose, medium sized 2.5–5 cm, sessile or with a very short stalk. **Disc** concave, smooth, pale brown or smoky, when dry, black to blackish brown, sometimes vinaceous. **Receptacle** cupulate to saucer-shaped, margin entire, sometimes contorted by mutual pressure, outer surface generally black when dry.

Microscopic features: Excipulum composed of large subglobose to pyriform cells, up to 100 µm diam., regularly interspersed with septate hyphae 5–7 µm in diam., up to 25 µm long. **Ectal excipulum** up to 160 µm thick; **medullary excipulum** about 250 µm thick. Toward the outer surface the cells become angular or compressed and are oriented in a somewhat parallel fashion, cells of the outer-most layer elongate to an outer layer of short cells which sometimes produce a pustulate appearance. **Subhymenium** of angular cells. **Hymenium** approximately 200–230 µm thick. **Asci** cylindrical, arising from croziers, apex blued in iodine solutions, of the "i" type of Hansen *et al.* (2001)/ WT type of Van Vooren (2020), 200–260 × 10–12 µm, 8-spored. **Ascospores** uniseriate, biguttulate, ellipsoidal, at first hyaline and smooth-walled, but soon covered with yellow to brown ridges and warts which anastomose to form an irregular reticulum, in some cases an apiculus develops at each end of the spore. The markings reach a height of 2 µm, (13) 13.7–14.8 (15.6) × (7) 7.8–8.8 (9.2) µm including markings. **Paraphyses** rather stout, septate, unbranched, yellowish, 3–4 µm diam below, apex enlarged to 7 µm.

Habitat and phenology: Occurring on soil in mixed forests, often on trail verges, from July through September, in the northern range, and earlier toward the south.

Trophic status: Isotopic analysis strongly suggests this species is ectomycorrhizal.

Distribution: Present in northeastern North America, from Canada south to Alabama, USA and at least as far west as Kentucky, USA.

Specimens studied, including those sequenced: Canada. Quebec, Chelsea, 45.549745 N -75.840063 E +-97 m. WGS84, growing on the ground, mixed forest (Beech, Oak, Maple, Eastern Hemlock), *leg.* Igor Khomenko (2021-998), 15.VII.2021 (FLAS-F-68805). USA. Alabama. Cullman Co., Cullman, County Rd 177, Crane Hill, 33.969733 N -87.124675 E +-8 m, on soil, woods of *Quercus*, *Pinus*, *Fagus*, privet, *Vitis*, *leg.* Cassie Pugh, *det.* Rosanne Healy, 23.IV.2023 (FLAS-F-71677). Connecticut. New Haven Co., New Haven, *leg.* R. Thaxter (sn), *det*. D. H. Pfister, 1888-1889 (FH00465067); New Haven Co., New Haven, on ground, *leg*. R. Thaxter (accession no 929),

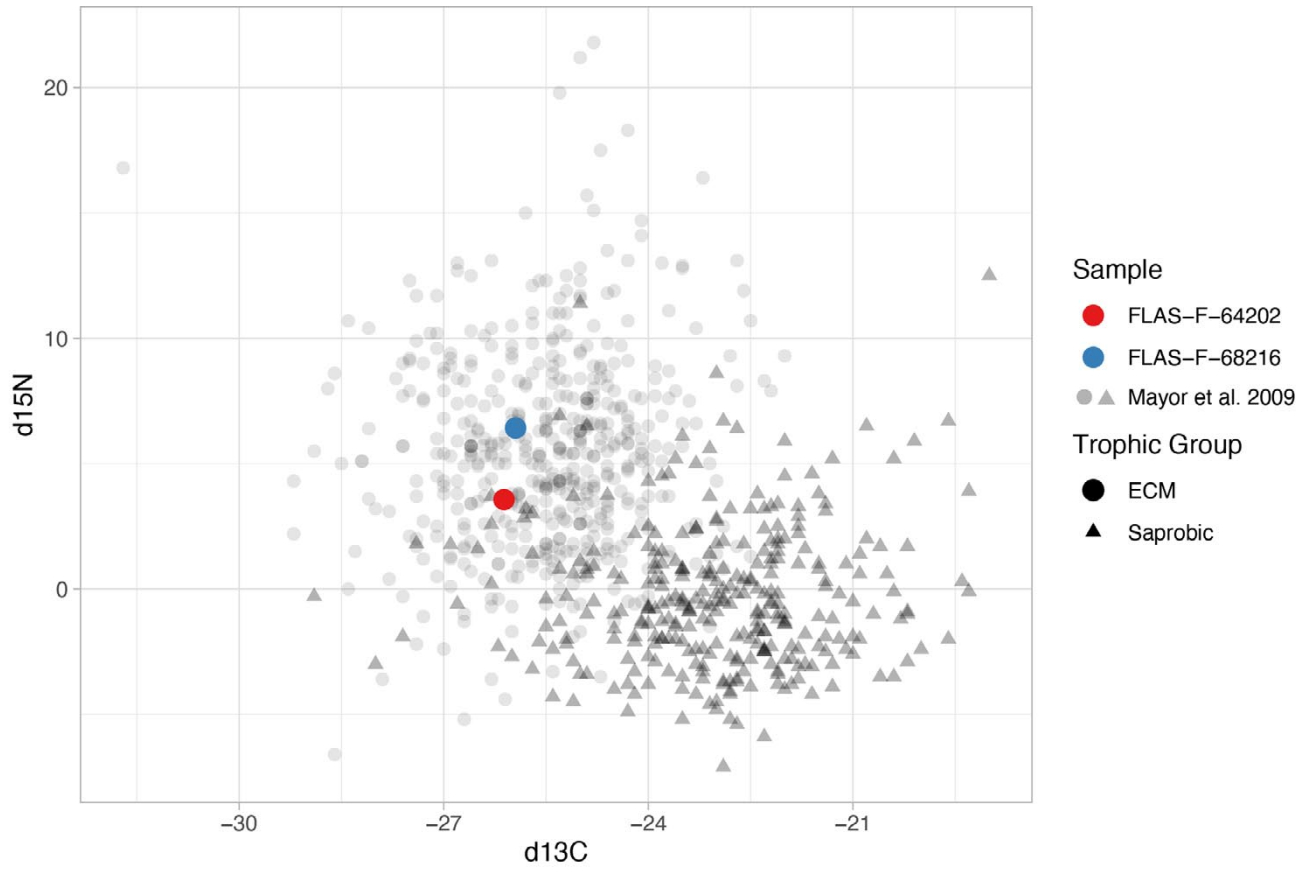

Figure 3 – Plot of δ13C and δ15N values. Grey background points represent data for known ECM (circles) and saprobic (triangles) taxa from Mayor *et al.* (2009) used to train the quadratic discriminant analysis (QDA) model. Red and blue circles represent samples of *Rugosporella atrovinosa* predicted as ECM by the QDA model.

det. D. H. Pfister, VIII. 1890 (FH00465068); Tolland Co., Storrs, near Felton River, on soil, *leg.* D. H. Pfister (sn), 25.VII.1979 (FH00465060). Georgia. Pickens Co., Burnt Mountain Preserve, Burnt Mountain Champion Creek Trail, mixed woods, in moss along trail, *leg.* Rosanne Healy, 25.VIII.2020 (FLAS-F-67101); Greene Co., Eatonton, Rock Hawk/Oconee Archery Range in Oconee Wildlife Management Area, 33.339 N -83.162 E, *leg.* Curtis Peyer, 20.V.2020 (FLAS-F-68169). Kentucky. Laurel Co., Daniel Boone National Forest, Sheltowee Trace Trail, Van Hook Falls, 37.000188 N -84.286292 W, 1050 ft (320 m) a.s.l., on soil, *leg.* Judson Van Wyk, 21.IX.2022 (FLAS-F-69722), immature; Whitley Co., Daniel Boone National Forest, Dog Slaughter Falls trail, 36.903522 N -84.282789 E, 1270 ft (387 m) a.s.l., on soil, *leg.* Gregory Bonito, 20.IX.2022 (FLAS-F-69666). Maine. Aroostook Co., on soil in hardwoods, *leg.* M. E. Bigelow (Barr 1719), *det.* D. H. Pfister, 15.VII.1956 (FH00823575); *ibid.*, 4.VIII.1956 (FH00823575); Aroostook Co., near Madawaska Lake, on soil at edge of trail, *leg.* M. E. Bigelow (Barr 1730), *det.* D. H. Pfister, 18.VIII.1956 (FH00823547); Aroostook Co., near Madawaska Lake, on soil of trail, *leg.* H. E. Bigelow (4037/Barr 1718), *det.* D. H. Pfister, 15.VIII.1956 (FH00823629); Aroostook Co., near Madawaska Lake, on soil of trail, *leg.* H. E. Bigelow (Barr 1810), *det.* D. H. Pfister, 3.IX.1956 (FH00823505); Penobscot Co., Orono, County Rd., *leg.* D. H. Pfister, 13.VIII.1983 (FH00465047); Penobscot Co., East Millinocket, off Rt. 157, on soil, *leg.* H. E. & M. E. Bigelow (Barr 3693), *det.* D. H. Pfister, 26.VIII.1962, 11.VIII.1983 (FH00823633); Penobscot Co., soil, *leg.* H. E. & M. E. Bigelow (Barr 3746), *det.* D. H. Pfister, 2I.X.1962 (FH00823657); Penobscot Co., Orono, on soil on trail, *leg.* D. H. Pfister (FH00465049); York Co., Kittery Point, *leg.* R. Thaxter, *det.* D. H. Pfister, 1911 (FH00465048); York Co., Kittery Point, *leg.* R. Thaxter, s.d. (FH00465054); Washington Co., Beddington, Lead Mountain, *leg.* M. E. Smith, *det.* D. H. Pfister, 5.VIII.2009 (FH00822838). Massachusetts. Berkshire Co., Windsor, Wahconash Falls, *leg.* H. E. and M. E. Bigelow (Barr 2590), *det.* D. H. Pfister, 22.VII.1959 (FH 00465078); *ibid.*, (FH00465079); Essex Co., Rockport, woods near Evans Field, close to train station, beech forest, on soil, *leg.* Michaela Schmull, *det.* D. H. Pfister, 27.VI.2010 (FH00302477); Essex Co., Cape Ann, Dogtown, on soil in white pine and beech forest, *leg.* Lawrence Millman, *det.* D. H. Pfister, 2 IX 2004 (FH 00465052); Franklin Co., Conway State Forest, on soil, *leg.* H. E. Bigelow and M. E. Bigelow, *det.* D. H. Pfister, 2.IX.1965 (FH00465051), *ibid.* (Barr 3010), 23.VII.1961 (FH00465074); *ibid.* (Barr 2767) (FH00465075); Franklin Co., Conway State Forest, *leg.* H. E. & M. E. Bigelow (Barr 3012), *det.* D. H. Pfister, 23.VII.1961 (FH 00465083), ibid. (FH 00465070); Franklin Co., Conway State Forest, *leg.* H. E. Bigelow (Barr 2768), *det.* D. H. Pfister, 9.VIII.1960 (FH00465071); Franklin Co., Conway, *leg.* H. E. & M. E. Bigelow (Barr 3171), *det.* D. H. Pfister, 29.VII.1961 (FH00465082); Franklin Co., Warwich, Mt. Grace State Forest, *leg.* H. E. and M. E. Bigelow (Barr 2604), *det.* D. H. Pfister, 28.VII.1959 (FH00465080); Franklin Co., Mt. Toby, *leg.* H. E. and M. E. Bigelow (Barr 2531), *det.* D. H. Pfister, 4.X.1958 (FH00465081); Franklin Co., Colrain-Heath, *leg.* H. E. & M. E. Bigelow, F. Witham (Barr 2432), *det.* D. H. Pfister, 12.VII.1958 (FH00465073); Franklin Co., Leverett, *leg.* H. E. & M. E. Bigelow (Barr 2492), *det.* D. H. Pfister, 6.VII.1958 (FH 00465077); Hampshire Co., Amherst, Lover's Lane, on soil side of trail, *leg.* H. E. Bigelow (Barr 2430), *det.* D. H. Pfister, 9.VIII.1958 (FH00465076); Hampshire Co., Vicinity Amherst, on soil in woods, *leg.* H. E. Bigelow, M. E. Bigelow, F. Witham (Barr 2404), *det.* D. H. Pfister, 4.VIII.1958 (FH00465072); Middlesex Co., Carlisle, woody debris, *leg.* L. Millman, 7.VIII.2006 (FH00465069); Middlesex Co., Lincoln, around Walden Pond, mixed woods, trail verge, *leg.* Lawrence Millman, *det.* D. H. Pfister, 18.VIII.2008 (FH00822876). New Hampshire. Carroll Co., Pequsket, on Piper Trail, D. H. Linder and R. Singer, *det.* W. L. White, 25 – 26.VII.1941 (FH00465059); Carroll Co., Albany, World Fellowship Center, sandy soil, *leg.* Lawrence Millman, 21.IX.2014 (FH00822872); Carroll Co., Intervale, *leg.* R. Thaxter, VII.1907 (FH00465055); Intervale, VIII.1901, *det.* D. H. Pfister, VIII.1901 (FH00465057); Carroll Co., Sawyer Rock Picnic Area, White Mountain

Figure 4 – Field collection images showing the range of color variation of *Rugosporella atrovinosa* apothecia. A. FLAS-F-67101 B-C. FLAS-F-71677. B shows top view. C shows underside with pustulate receptacle. Photos by Cassie Pugh. D. FLAS-F-68805 photo by Igor Khomenko. E. FLAS-F-68169 photo by Curtis Peyer. Bars: A, D, E = 2 cm; B, C = 1 cm.

National Forest, *leg.* H. E. & M. E. Bigelow (Barr 4042), *det.* D. H. Pfister, 10.VIII.1963 (FH00823658); Coös Co., Shelbourne, *leg.* W. G. Farlow, (FH00465066). New York. Dutchess Co., Poughkeepsie, *leg.* W.R. Gerard, isotype of *Peziza atrovinosa* (CUP-3703); Hamilton Co., Raquette Lake, Brown Tract Road, scattered in sandy soil in road bank, *leg.* T. Baroni (4614), *det.* D. H. Pfister, 19.VIII.1984 (FH00465061); *ibid*., Baroni (4614)(FH00465062), *ibid*., Baroni (4612) (FH00465064). North Carolina. Macon Co., Highlands Biological Station, 35.03 N, 83.12 W, *leg.* J. Kimbrough, as *P. retiderma*, 13.VIII.1982 (FLAS-F-52955). Ohio. Hocking Co., Old Man's Cave State Park, one soil in mixed woods, *leg.* W. B. & E. G. Cooke (46536), *det.* D. H. Pfister, 15.X.1972 (FH 00465063); Hocking Co., Tar Hollow State Park and Forest, 39.3899 N 82.7535 W, on soil, mixed pine-hardwood forest, *leg.* Django Grootmyers (FLAS-F-64202). Pennsylvania. Chester Co., West Chester, 04.VIII.1885 [possible type of *P. chlamydospora*]; Huntingdon Co., Alan Seeger State Monument, *leg.* J. Kimbrough, *det.* R. Korf, 7.VII.1982 (FLAS-F-52966); Philadelphia Co., Philadelphia, *leg.* W. C. Stevenson, IX.1883. [possible type of *P. chlamydospora*]; Pike Co., approx. 6 mi. E of Green tree near Promise Land State Park, on soil with mosses, *leg.* G. Benny, 5.VIII.1986, *det.* J. Kimbrough (as *P. retiderma*) (FLAS-F-55016). Tennessee. Blount Co., Great Smoky Mts. National Park, Parsons Branch Rd. turnaround, Cades Cove, *leg.* L. Ryvarden, *det.* D. H. Pfister (Tenn 25), VIII.1977 (FH00465063); Blount Co., Great Smoky Mts. National Park, Schoolhouse Gap Trail, 35.636233 N -83.736143 E, 1810 ft (552 m) a.s.l., on soil in mixed forest of *Carpinus*, *Betula*, *Tilia*, *Pinus*, *Quercus*, and *Tsuga*, *leg.* R. Healy 16.VIII.2021 (FLAS-F-68216); Carter Co., Burbank, *leg.* R. Thaxter, *det.* D. H. Pfister, VIII-IX.1887 (FH00464046); Cocke Co., Cosby Campground, 35.75543 N, -83.20894 E, on soil under *Acer*, *Liriodendron*, *Cornus*, *leg.* Gregory Bonito, Judson Van Wyk, 12.VIII.2021 (FLAS-F-68274); Sevier Co., near Whaley-Big Greenbrier Cemetery, 35.7 N -83.38 E, 1870 ft (570 m) a.s.l., on soil, *leg.* Sarah Prentice 14.VIII.2021 (FLAS-F-68478). Ver-

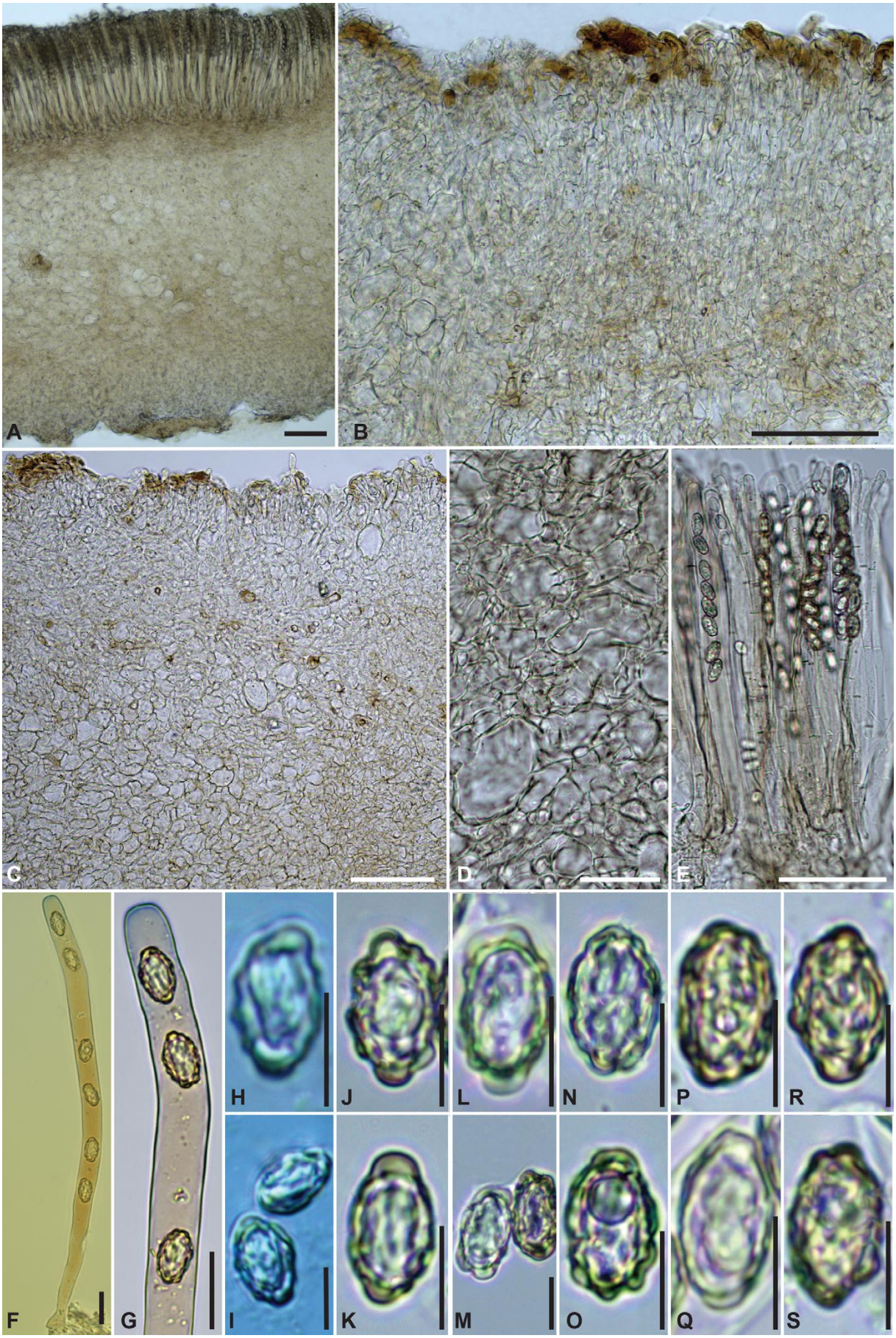

Figure 5 – Microscopy images of *Rugosporella atrovinosa*. A. Through section of apothecium showing excipulum and hymenium (FLAS-F-68478). B. Ectal excipulum (FLAS-F-64202). C. Ectal and medullary excipulum (FLAS-F-64202). D. Higher magnification of medullary excipular cells (FLAS-F-68169). E. Asci and paraphyses (FLAS-F-68216). F, G. Ascus showing amyloid reaction in Melzer's solution (FLAS-F-64202). H, I. Differential interference contrast of ascospores (FLAS-F-68805). J, K. Ascospores (FLAS-F-69666). L, M (FLAS-F-68169). N. (FLAS-F-64202) O. (FLAS-F-69666). P, Q, R, S. Ascospores of isotype for *Peziza atrovinosa* (CUP-D-3703). Bars: A, B, C = 100 µm. D, E = 50 µm. F, G = 20 µm. L-S = 10 µm.

mont. Chittenden Co., Burlington, Indian Hill, *leg.* D. H. Pfister, 30.VIII.2000 (FH00465053); Lamoille Co., Stowe, Covered Bridge Rd, *leg.* H. E. & M. E. Bigelow (Barr 4386), *det.* D. H. Pfister, 23.VII.1964 (FH00823610); Lamoille Co., Ranch Brook, near Mt. Mansfield, *leg.* H. E. & M.E. Bigelow (Barr 4403), *det.* D. H. Pfister, 27.VII.1964 (FH 00823540); Rutland Co., Paulet, Haystack Mt., *leg.* C. W. Dodge (1864), 22.IX.1922 (FH00465080). West Virginia. Braxton Co., on US19, 3-4 mi S of Sutton, *leg.* J. Kimbrough, 12.VIII.1982 (FLAS-F-52961).

Discussion

A note on the authorship of *P. atrovinosa*. In the original publication Cooke (1875) lists the author of *P. atrovinosa* as "Cooke." It is also said to be from New Jersey, presumably a mix up with Ellis whose collections are also listed in Cooke's paper. *Peziza griseorosea* W.R. Gerard that precedes *P. atrovinosa* in Cooke's list is attributed to Gerard and gives the location as New York. Cooke (1877) corrected the locality later in this series to indicate New York as the locality and Gerard as the collector. Saccardo (1889) lists the authorities as Gerard & Cooke, and he gives the location as "ad terram in Britannia." Cooke (1876) illustrated *P. atrovinosa* (Fig. 6) in his *Mycographia* and there he indicated "Gerard" as the author. We know from fungarium annotations that Gerard sent material to Cooke under the name *P. rugospora*, a name that had previously been used by Sowerby for a different species.

Rugosporella atrovinosa has been confused with *Legaliana badia*. It has been shown through molecular phylogenetic analysis that *R. atrovinosa* is placed outside *Legaliana* in an independent, supported clade. *Rugosporella atrovinosa* is situated within a larger clade that includes *Legaliana* and several other species in genera that are both epigeous and hypogeous (Fig. 1). No environmental or ectomycorrhizal root tip sequences have been recorded for *R. atrovinosa*. However, this larger clade includes species that are documented to be mycorrhizal, and we have provided isotopic evidence which suggests that *R. atrovinosa* is ectomycorrhizal as well.

Judging by sequence BLASTn searches there are several collections of *R. atrovinosa* that are misidentified on GenBank as *Phylloscypha phyllogena* (Cooke) Van Vooren. The spore ornamentation of *P. phyllogena* differs from both *Rugosporella atrovinosa* and *Legaliana badia* in that it is made up of isolated warts. Additionally, *P. phyllogena* is a spring fruiting fungus that has violet tints at the base of the receptacle.

One of the species that has been considered along with *Rugosporella atrovinosa* because of the highly marked, pigmented ascospores is *Plicariella vacini* Velen., now *Velenovskya vacini* (Velen.) Albanese, Boragine, M. Carbone & P. Alvarado, based on a molecular phylogenetic study (Albanese *et al.*, 2022). Moravec & Spooner (1988) discuss this species, provide a description and compare it to *R. atrovinosa*. The two differ in morphology and in habitat. The yellow-brownish ascospores of *V. vacini* are ornamented with coarse "thick wing-like obtuse to pyramidal warts" (Albanese *et al.*, 2022) rather than a reticulum. Also, *V. vacini* occurs on burned areas, whereas *R. atrovinosa* is found directly on forest soil. Finally, our study shows that *V. vacini* is distantly related to our new genus.

Three names deserve further discussion. Two are represented by specimens collected within the known range of *Rugosporella atrovinosa*. Citing authentic material in the Farlow Herbarium, Pfister (1978a) considered *Peziza chlamydospora* Ellis & Everh. to be a synonym of *R. atrovinosa*. A restudy of that material confirms this synonymy. The type specimen was collected in eastern Pennsylvania, USA. The other species synonymized here is *P. retiderma*. The type collection of *Peziza retiderma* was collected in South Portland, Maine, USA by Charles Bowen Fuller, the longtime curator of the defunct Portland Society of Natural History. Cooke's (1877) illustration is provided here as Fig. 7. The type specimen was studied by Moravec & Spooner (1988). The name often has been used interchangeably with *P. atrovinosa* in North America. Our proposed synonym reflects our view that these two taxa fall within the range of variation of *R. atrovinosa* and also occur in the same geographical area. No collections, other than the type specimen, have been authoritatively identified as *P. retiderma* in North America. A third name deserves mention. *Aleurina stipitata* Cash is an illegitimate name; it is a later homonym of *A. stipitata* Rodway, a species described from Tasmania. Cash's specimens were collected on soil in Panama. Pfister (1979) studied the type specimen and pointed out its similarity to *P. atrovinosa* and *P. retiderma*. Resolution of its identity awaits study of additional material from Central America.

There are reports of *Peziza atrovinosa* from Mexico and Central America (Denison, 1963; Medel *et al.*, 2013; Sanchez *et al.*, 2005). At least some of these are on burned areas and are being re-examined; the habitat suggests that they may belong to the genus *Velenovskya*. Le Gal (1953) named collections from Madagascar as *P. retiderma*. A re-examination of these Malagasy collections shows that the ascospore ornamentation differs from *R. atrovinosa* and thus, from *P. retiderma* as we have interpreted it. Specimens studied are: Madagascar. Mandraka, forêt orientales, 2 et 30 mars 1940, as cited by Le Gal, CUP-K-3992, CUP-K-3993, as *Galactinia retiderma* (Cooke) Le Gal. There are currently no sequences available for the Madagascar collections. *Peziza retiderma* was considered the type of the illegitimate genus *Aleurina* (Sacc.) Sacc. & P. Syd. (1902). Eckblad (1968) considered this to be the case, but the earlier *Aleurina* Massee is now recognized as the accepted genus and circumscribes a group of ectomycorrhizal taxa known primarily from the Southern Hemisphere (Zhuang & Korf, 1986). The type is *Aleurina tasmanica* Massee.

Acknowledgments

We join in celebrating Trond Schumacher recognizing his contributions to the understanding of the systematics and ecology of the *Pezizomycetes*.

We thank Kevin Childs and the MSU RTSF Genomics Core for assistance with sequencing genomic DNA samples. We thank Jason Curtis and the staff at the University of Florida Stable Isotope Mass Spec Lab for processing isotope samples.

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Authors' contributions

DHP conceived the project, examined specimens and literature, and wrote the preliminary manuscript; BRL extracted the RPB2 sequences from low coverage genomes of FLAS-F *R. atrovinosa*, provided the isotopic data and analyses, reviewed the manuscript and obtained funding; RH performed sequencing, measured FLAS-F specimens, performed micrography, and analyses, reviewed the manuscript, prepared the figures and provided funding; KFL performed sequencing and analyses, prepared figures, and reviewed the manuscript; GB provided isotopic data and reviewed manuscript and obtained funding; MES reviewed the manuscript, gave advice and provided funding.

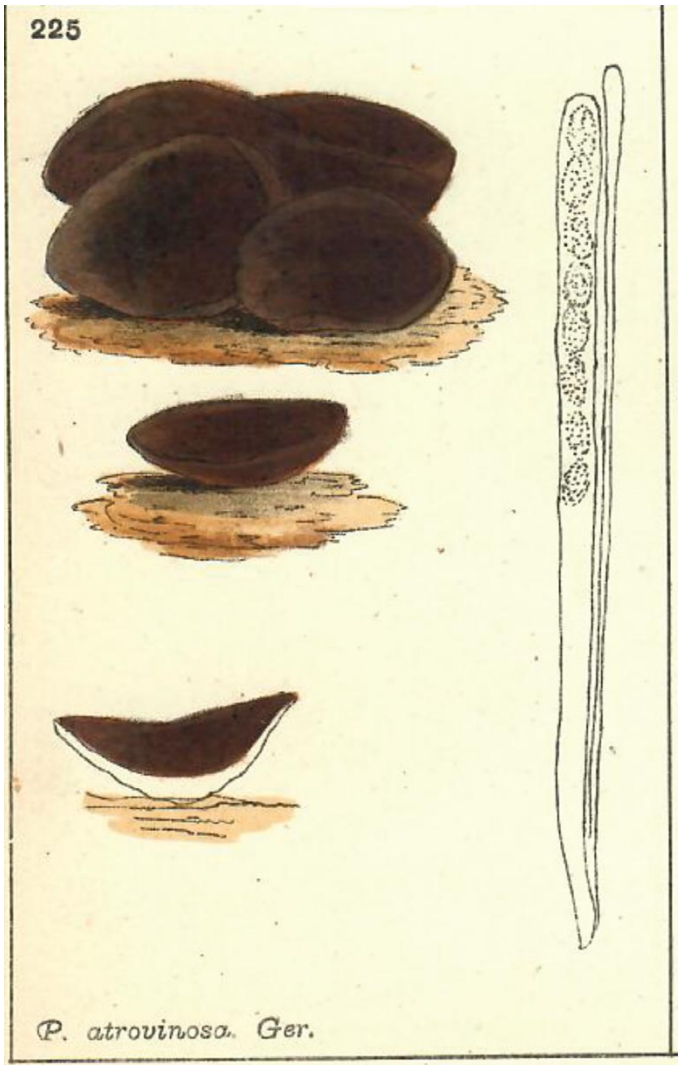

Figure 6 – Illustration of *Peziza atrovinosa* by M. C. Cooke (*Mycographia*, pl. 57, fig. 225. 1876)

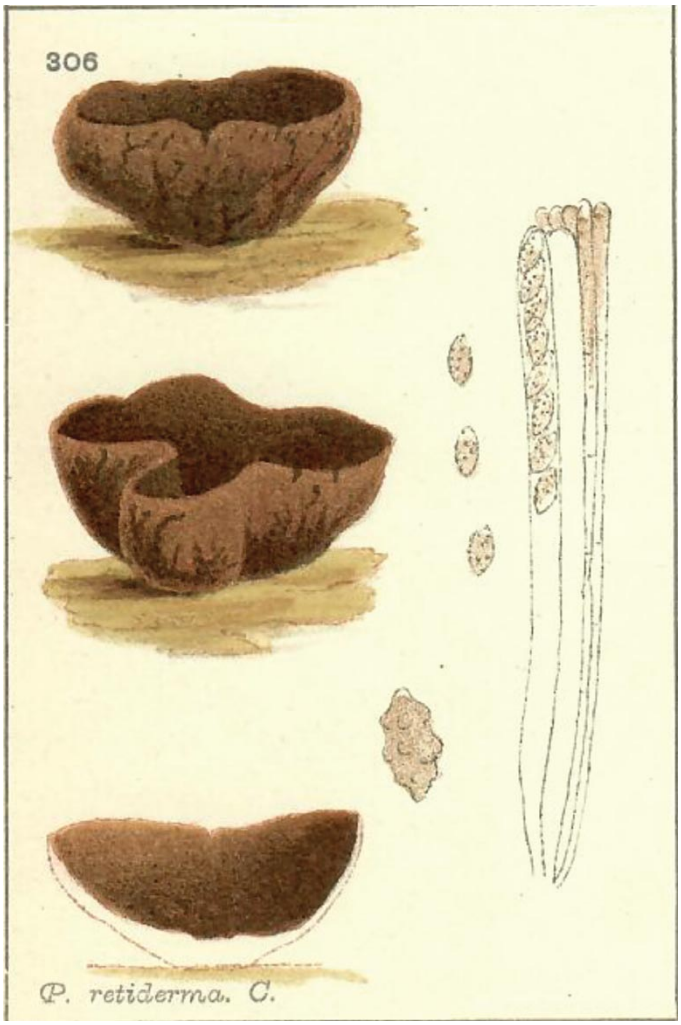

Figure 7 – Illustration of *Peziza retiderma* by M. C. Cooke (*Mycographia*, pl. 79, fig. 306. 1877)

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