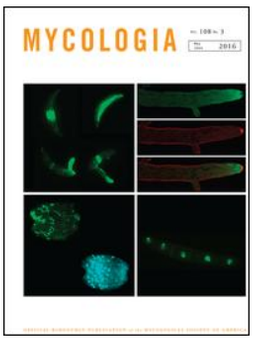

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Systematic study of truffles in the genus *Ruhlandiella*, with the description of two new species from Patagonia

Nattapol Kraisitudomsook [a], Rosanne A. Healy [a], Alija B. Mujic [a], Donald H. Pfister [b], Eduardo R. Nouhra[c], and Matthew E. Smith [a]

[a]Department of Plant Pathology, University of Florida, Gainesville, Florida 32611; [b]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138; [c]Instituto Multidisciplinario de Biologia Vegetal (CONICET), Universidad Nacional de Cordoba, Cordoba 5000, CC 495, Argentina

ABSTRACT

Ruhlandiella is a genus of exothecial, ectomycorrhizal fungi in the order Pezizales. Ascomata of exothecial fungi typically lack a peridium and are covered with a hymenial layer instead. *Ruhlandiella* species have nonoperculate asci and highly ornamented ascospores. The genus was first described by Hennings in 1903 to include the single species, *R. berolinensis*. Since then, mycologists have uncovered *Ruhlandiella* species in many locations around the globe, including Australia, Spain, Italy, and the USA. Currently, there are four recognized species: *R. berolinensis, R. peregrina, R. reticulata*, and *R. truncata*. All were found near *Eucalyptus* or *Melaleuca* trees of Australasian origin. Recently, we discovered two new species of *Ruhlandiella* in Nothofagaceae forests in South America. They regularly form mitotic spore mats directly on soil in the forests of Patagonia. Here, we formally describe these new species and construct the phylogeny of *Ruhlandiella* and related genera using a multilocus phylogenetic analysis. We also revise the taxonomy of *Ruhlandiella* and provide an identification key to accepted species of *Ruhlandiella*.

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Exothecium; hypogeous fungi; Nothofagaceae forests; Pezizaceae; Pezizales; *Sphaerosoma*; *Muciturbo*; 3 new taxa

INTRODUCTION

Ruhlandiella (Pezizaceae) species are exothecial, truffle-like fungi. Exothecial fungi are essentially "inside-out" truffles, i.e., their ascomata lack a peridium and are instead covered with a hymenial layer (Weber et al. 1997). This ascoma structure is different from the closest known relatives of *Ruhlandiella*, which are either epigeous cup fungi or hypogeous truffles enclosed by a peridium (Healy et al. 2013). Like many truffles, species of *Ruhlandiella* have highly ornamented ascospores and asci that lack opercula (Dissing and Korf 1980). The genus was first introduced by Hennings (1903) who described the type species *Ruhlandiella berolinensis* from the Berlin Botanical Garden in Germany (Dissing and Korf 1980). This species was later identified as an ectomycorrhizal symbiont of *Eucalyptus* trees; it fruited abundantly after wildfires and formed ectomycorrhizae on seedlings in a greenhouse experiment (Warcup 1990).

Dissing and Korf (1980) indicated that the holotype of *R. berolinensis* is lost and thus designated a collection from a eucalypt plantation in the Canary Islands as neotype (CUP-MM-1230). Since that time, *Ruhlandiella* specimens have been collected in Australia, Italy, Spain, and the USA (Dissing and Korf 1980; Galán and Moreno 1998; Rubio et al. 2010; Lantieri et al. 2012). All collections were found near *Eucalyptus*, *Melaleuca*, or other ectomycorrhizal Myrtaceae trees of Australasian origin.

Based on morphology, Galán and Moreno (1998) and Hansen et al. (2005) suggested that *Muciturbo* may be a synonym of *Ruhlandiella*. However, this claim has not been addressed using molecular phylogenetic data. The genus *Muciturbo* was described by Warcup and Talbot (1989) based on *M. reticulatus*. Recently, Rubio et al. (2010) transferred two species of *Muciturbo* (*M. reticulatus* and *M. truncatus*) to *Ruhlandiella* based on morphological similarities between the two genera (e.g., the exothecial ascomata, dextrinoid reaction of the young asci, similar spore ornamentation, and paraphyses with gelatinous sheaths that significantly exceed the asci in length). Furthermore, an Australian specimen identified as "*Muciturbo* sp." was closely related to *R. berolinensis* in a 28S rDNA phylogeny (Healy et al. 2013). Several additional species have also been treated in *Ruhlandiella*. Hirsch (1983) transferred *Boudiera parvispora*, an apothecial fungus from India, to *Ruhlandiella*. Two species, *Ruhlandiella hesperia* and

CONTACT Nattapol Kraisitudomsook kraisin@ufl.edu
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Sphaerosoma fuscescens, were treated as potential synonyms of *R. berolinensis* (Dissing and Korf 1980; Rouppert 1909). However, none were studied using molecular phylogenetic data.

In 1905–1906, during an expedition in Nothofagaceae forests in South America, Roland Thaxter found *Ruhlandiella*-like ascomata that he referred to as "pearly livid white fungus hypogeous" (Halling 1981). Healy et al. (2013) also reported finding mitotic spore mats of *Ruhlandiella* in Nothofagaceae forests across Patagonia. These mitotic spore mats were morphologically similar to the *Muciturbo* anamorphs described and illustrated by Warcup and Talbot (1989). Recently, we discovered several new collections of gelatinous exothecial fungi in Nothofagaceae forests in South America. These fungi match the general morphology of *Ruhlandiella* but are distinct from all known *Ruhlandiella* species.

In this paper, we critically review the taxonomy and systematics of *Ruhlandiella* based on all available morphological and phylogenetic data. We provide new molecular data from as many *Ruhlandiella* species as possible and provide descriptions of two new species of *Ruhlandiella* from Nothofagaceae forests of southern South America. Lastly, we analyze the phylogeny of *Ruhlandiella* based on five informative loci: the nuc rDNA internal transcribed region (ITS1-5.8S-ITS2 = ITS) and 28S gene (28S), the largest subunit of RNA polymerase II (*RPB1*), the second largest subunit of RNA polymerase II (*RPB2*), and the β-tubulin protein–coding gene *(TUB1*).

MATERIALS AND METHODS

Ascomata and mitotic spore mats of *Ruhlandiella* were collected in Patagonia, Chile and Argentina, from 2008 to 2017. *Nothofagaceae*-dominated forests are common across large swaths of Patagonia, and the region has a cool-temperate climate, with mean annual temperatures ranging from 3 to 12 C (Paruelo et al. 1998). Annual precipitation varies across the region, with an average of 2300 mm of rainfall per year (Vivanco and Austin 2008).

Hypogeous fungi were located by searching through leaf litter and soil using a truffle rake. Samples were placed in plastic boxes and transported to the laboratory within 8 h. Macroscopic photos of fresh specimens were taken in the field and in the laboratory. Samples were then dried on a forced-air dryer for approximately 24 h. All samples were stored in plastic bags with silica gel, and pieces of some samples were also stored in cetyltrimethylammonium bromide (CTAB) solution to preserve the DNA (Gardes and Bruns 1993). Specimens are accessioned at the Fungal Herbarium of the Florida Museum of Natural History (FLAS) in the USA, the Herbario del Museo Botánico de Córdoba (CORD) in Argentina, and the Museo Nacional de Historia Natural de Chile (SGO) in Chile. Additional specimens were received as loans from the following herbaria: the Plant Pathology Herbarium at Cornell University (CUP), the Farlow Herbarium at Harvard University (FH), the University of California Herbarium (UC), the Oregon State University Herbarium (OSC), the Royal Botanic Gardens (K), the Swedish Museum of Natural History (S), and the private herbarium of Ángel Suárez from Asturias, Spain (Rubio et al. 2010).

Dried material was rehydrated, hand-sectioned with a razor blade, and mounted in water, 3% KOH, cotton blue, or Melzer's reagent. Images were captured using a QImaging MicroPublisher 3.3 RTV digital camera (British Columbia, Canada) mounted on a Nikon Optiphot light microscope (Tokyo, Japan). Images were edited in Adobe Photoshop CS5.1 (San Jose, California). Relevant morphological characters, including hyphae, ascospores, spore ornamentation, asci, and paraphyses, were studied, and their dimensions assessed based on 20 individual measurements at various magnifications. Microscopic features were compared with the known species of *Muciturbo, Ruhlandiella*, and *Sphaerosoma* based on original descriptions and type specimens when available. Image plates were created with Adobe Illustrator CS5.1.

Clean fungal tissues were taken from the inside of fresh or dried specimens. DNA was then extracted using a modified CTAB method (Gardes and Bruns 1993). Polymerase chain reactions (PCRs) of the ITS were performed using forward primer ITS1F and reverse primer ITS4 (White et al. 1990) and Phusion Hot Start Flex DNA Polymerase standard protocol (New England Biolabs, Ipswich, Massachusetts). Primer pairs ITS1F-ITS2 and ITS3-ITS4 were used if the ITS1F-ITS4 primer pair did not yield amplicons. PCR of the 28S was performed using the same protocol with forward primer LROR (Hopple and Vilgalys 1994) and reverse primer LR5F (Tedersoo et al. 2008). PCR of *RPB1* was performed using the forward primer Af and the reverse primer Cr (Matheny et al. 2002) following a touchdown protocol (Hansen and Olariaga 2015). *RPB2* was amplified using the forward primer P6Fa and reverse primer P7R, whereas *TUB1* was amplified using the forward primer PB1a and reverse primer PB42Fa (Hansen et al. 2005). Both genes were amplified with the touchdown protocol from Bonito et al. (2013).

PCR products were visualized on 1.5% agarose gels stained with SYBR Green I (Molecular Probes, Eugene, Oregon). Amplicons were cleaned with EXO (exonuclease I) and AP (antarctic phosphatase) enzymes (New

England Biolabs) (Werle et al. 1994) and sequenced by the University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, Florida) or GENEWIZ (South Plainfield, New Jersey). Sequences were then edited with Sequencher 5.0.1 (Gene Codes, Ann Arbor, Michigan) and aligned in Mesquite 3.2 (Maddison and Maddison 2018) with the aid of MUSCLE 3.8.31 (Edgar 2004). Missing characters and introns in the multilocus data set were removed by Gblocks 0.91b (Castresana 2000) using the default parameters and "with-half" gap option, i.e., characters with data missing in more than half of all taxa are removed. We concatenated the 28S, *RPB1, RPB2*, and *TUB1* genes with the Super-Aligner code into a single matrix (Mujic et al. 2019). Because Gblocks was too conservative, the ITS alignment was edited manually to exclude gaps and ambiguous regions. All final alignments were exported and submitted to TreeBASE (study no: S22302).

The concatenated alignment was analyzed with maximum likelihood (ML) and Bayesian Inference (BI) methods. Both were performed in the Cyberinfrastructure for Phylogenetic Research Science Gateway (CIPRES) 3.1 (Miller et al. 2010). ML was run on RAxML 8.2.10 (Stamatakis 2014) with 1000 bootstrap iterations and the GTRGAMMA model. The ITS alignment was run separately with the same ML parameters. The concatenated alignment was then partitioned into 28S, *RPB1, RPB2*, and *TUB1* matrices for BI analysis. Evolutionary models for each partition were estimated independently by jModelTest 2 2.1.6 (Darriba et al. 2015). The GTR+I+G model was selected for all partitions. BI was run on MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) with a chain length of 1 million generations, sampling frequency 1000 with the first 25% of samples being discarded as the burn-in. The rest of the parameters were set to defaults. Phylogenetic trees for both ML and BI were visualized and rooted in FigTree 1.4.3 (Rambaut 2016) using multiple genera in the Pyronemataceae as the outgroup (Hansen et al. 2005). The ITS phylogeny was midpoint-rooted. Nodes were considered strongly supported when the bootstrap values were ≥70% or posterior probability values were ≥0.95. Final trees were edited in Adobe Illustrator CS5.1 (San José, California).

RESULTS

Phylogenetic analyses.—The concatenated multilocus alignment comprised 56 specimens (TABLE 1) with a total of 2875 nucleic acid sites (28S: 721 sites, *RPB1*: 729 sites, *RPB2*: 691 sites, and *TUB1*: 734 sites). The ITS alignment comprised 34 specimens with 600 nucleic acid sites. Phylogenetic trees based on the multilocus alignment using ML and BI methods were congruent except for a minor and unsupported incongruence within the *Peziza succosa* clade. The multilocus phylogeny indicated that all *Ruhlandiella* species form a supported clade, with other taxa in the /terfezia-peziza depressa lineage (FIG. 1). *Ruhlandiella* was clearly divided into two clades. The first was the Australasian clade, which includes *R. berolinensis, R. truncata, R. reticulata, R. peregrina*, and an unnamed species. In the case of the unnamed species, one ascoma from New South Wales, Australia, which was initially identified as *R. berolinensis* (OSC-60136), was strongly supported as a unique taxon (FIG. 1). The other clade included the two new South American species, *R. patagonica* and *R. lophozoniae*. Ten mitotic spore mat samples clustered together in the *R. patagonica* clade, and one mitotic spore mat sample (MES-1255) was resolved in the *R. lophozoniae* clade. A phylogenetic tree based on ITS (FIG. 2) placed sequences of ectomycorrhizal root tips within a well-supported clade that includes both spore mats and ascomata, indicating that *R. patagonica* is an ectomycorrhizal fungus associated with the Nothofagaceae.

TAXONOMY

Ruhlandiella patagonica Kraisit., Pfister, Healy & M.E. Sm., sp. nov. FIG. 3
MycoBank MB824729

Typification: CHILE. MAGALLANES: Magallanes Forest Reserve, near the park guard (53°8′34.6″S, 71°0′17.5″W), 343 m above sea level (asl), by a small creek in a *Nothofagus pumilio* forest with *N. betuloides* at forest edges, under soil leaf litter, with abundant mitotic spore mats nearby, 6 Apr 2017, *A.B. Mujic MES-2502* (**holotype** SGO-168848). **Isotype** FLAS-F-62147.

Etymology: patagonica, referring to the location (Patagonia) where this novel species was discovered.

Diagnosis: Ascomata hypogeous, exothecial, asci 340–430 × 32–40 µm, ascospores globose, 22–36 µm, reticulate, yellow-brown, ornamentation up to 4 µm, paraphyses covered with a gelatinous sheath, spore mats abundant, found in Nothofagaceae forests.

Ascoma an exothecium roughly 3–5(–7) mm diam, globose, somewhat convoluted, pearly white and moist when fresh (becoming yellowish to pale brown in age or with drying), with short, thin hyphal cords to which soil debris adhere in some specimens. Asci lacking opercula, cylindrical or cylindrical-clavate, 270–400 × 32–40 µm (mean ± SD = 306.2 × 34.5 ± 34.47 × 4.30 µm), attenuate at base, usually (ca. 90%) 8-spored, persistent, content dextrinoid when young, inamyloid when young and mature. Ascospores globose, 20–38 µm diam (mean ±

Table 1. Taxa used in this study with their corresponding herbaria and GenBank accession numbers.

Taxon name	Collection (ID) number	Herbarium accession number	GenBank accession number					Reference
			ITS	LSU	β-tubulin	RPB2	RPB1	
Amylascus tasmanicus	Trappe 18084	C, dupl. OSC	—	AF335113	AY513297	AY500465	—	Hansen et al. 2005
Ascobolus carbonarius	KH-00-008	C	—	AY500526	AY513298	AY500459	—	Hansen et al. 2005
Boudiera tracheia	Rana 79.049	C	—	AY500530	AY513301	AY500507	—	Hansen et al. 2005
Byssonectria terrestris	KS-94-4	C	—	AY500531	AY513302	AY500504	—	Hansen et al. 2005
Hydnotryopsis sp.	Trappe 17231	C, dupl. OSC	—	AF335116	AY513305	AY500472	—	Hansen et al. 2005
Iodophanus carneus	JHP 00.027	C	—	AY500534	AY513306	AY500506	—	Hansen et al. 2005
Iodowynnea auriformis	18510 PAN	FH	—	AF335118	AY513309	AY500473	—	Hansen et al. 2005
Marcelleina persoonii	TL-5696	C	—	AY500537	AY513311	AY500464	—	Hansen et al. 2005
Otidea umbrina	KH-01-09	C	—	AY500540	AY513314	—	—	Hansen et al. 2005
Pachyella babingtonii	KS-94-45	C	—	AF335122	AY513316	AY500522	—	Hansen et al. 2005
Pachyella violaceonigra	s.n.	FH	—	AF335125	AY513321	AY500470	—	Hansen et al. 2005
Peziza ampellina	KH 00.011	C	—	AF335127	AY513323	AY500492	—	Hansen et al. 2005
Peziza badiofusa	KH-98-113	C	—	AF335132	AY513326	AY500475	—	Hansen et al. 2005
Peziza depressa	KH-98-28	C	—	AF335135	AY513328	AY500474	—	Hansen et al. 2005
Peziza ellipsospora	Trappe 13017	C, dupl. OSC	—	AF335139	AY513330	AY500482	—	Hansen et al. 2005
Peziza gerardii	DHP-02-495	FH	—	AY500547	AY513333	AY500471	—	Hansen et al. 2005
Peziza lobulata	KH 03.157	FH	—	AY500548	AY513336	AY500495	—	Hansen et al. 2005
Peziza natrophila	JHP 93.021	C	—	AF33513	AY513339	AY500486	—	Hansen et al. 2005
Peziza obtuspiculata	TL-6474	C	—	AY500550	AY513340	AY500490	—	Hansen et al. 2005
Peziza phyllogena	KH-99-03	C	—	AF335155	AY513341	AY500480	—	Hansen et al. 2005
Peziza subisabellina	RK 96.54	private herb. Roy Kristiansen	—	AF335163	AY513347	AY500484	—	Hansen et al. 2005
Peziza succosa	KH-98-07	C	—	AF335166	AY513350	AY500487	—	Hansen et al. 2005
Peziza varia	KH-97-54	C	—	AF335134	AY513352	AY500519	—	Hansen et al. 2005
Peziza vesiculosa	JV 95-652	C	—	AY500552	AY513355	AY500489	—	Hansen et al. 2005
Peziza whitei	Trappe 17049	C, dupl. OSC	—	AF335168	AY513356	AY500491	—	Hansen et al. 2005
Plicaria trachycarpa	KH-97-93	C	—	AY500554	AY513360	AY500478	—	Hansen et al. 2005
Smardaea amethystina	KH-97-132	C	—	AF335176	AY513364	—	—	Hansen et al. 2005
Tirmania pinoyi	Trappe 13587	C	—	AF335178	AY513368	AY500502	—	Hansen et al. 2005
***Ruhlandiella berolinensis* (neotype)**	MM-1230	CUP-MM-1230	MG925393	MG947628	AY513361	AY500477	MH156171	This paper
Ruhlandiella berolinensis	SPN-01	FLAS-F-62154	MG925392	MG947627	MH156187	MH155172	MH156172	This paper
Ruhlandiella berolinensis	UCB-928	UC-1576349	MG925394	MG947629	MH156188	MH155173	—	This paper
Ruhlandiella lophozoniae	MES-1255	FLAS-F-62133	KY462448	MG947625	MH156185	MH155170	—	This paper
***Ruhlandiella lophozoniae* (type)**	MES-1341	CORD-C-6465 (holotype), FLAS-F-62144 (isotype)	MG925391	MG947626	MH156186	MH155171	—	This paper
Ruhlandiella patagonica	DHP-AR-17	FH-290549	MG925390	—	—	—	—	This paper
Ruhlandiella patagonica	DHP-CH-28	FH-00284821	MH014963	—	—	—	—	This paper
Ruhlandiella patagonica	DHP-CH-42	FH-00284833	MG925376	MG947609	—	—	—	This paper
Ruhlandiella patagonica	MES-1187	FLAS-F-62138	KY462435	—	—	MH155159	—	This paper
Ruhlandiella patagonica	MES-1273	CORD-C-00005143	KY462451	MG947613	—	MH155160	—	This paper
Ruhlandiella patagonica	MES-1277	FLAS-F-62152	MG925380	MG947614	—	MH155161	—	This paper
Ruhlandiella patagonica	MES-1284	CORD-C-00005113	MG925381	MG947615	—	MH155162	—	This paper
Ruhlandiella patagonica	MES-1571	FLAS-F-62141	MG925382	MG947616	—	MH155163	—	This paper
Ruhlandiella patagonica	MES-1702	FLAS-F-62151	—	MG947617	—	MH155164	—	This paper
Ruhlandiella patagonica	MES-2159	FLAS-F-62145	MG925383	MG947618	MH156179	MH155165	—	This paper
Ruhlandiella patagonica	MES-2284	FLAS-F-62148	MG925384	MG947619	MH156180	MH155166	MH156168	This paper
***Ruhlandiella patagonica* (type)**	MES-2502	SGO-168848 (holoype), FLAS-F-62147 (isotype)	MG925385	MG947620	MH156181	MH155167	MH156169	This paper
Ruhlandiella patagonica	MES-2543	FLAS-F-62146	MG925386	MG947621	—	—	—	This paper
Ruhlandiella patagonica	MES-2553	FLAS-F-62153	MG925387	MG947622	MH156182	MH155168	—	This paper
Ruhlandiella patagonica	MES-2682	FLAS-F-62150	MG925388	MG947623	MH156183	MH155169	MH156170	This paper
Ruhlandiella patagonica	MES-2854	FLAS-F-62149	MG925389	MG947624	MH156184	—	—	This paper
Ruhlandiella patagonica	MES-556	FLAS-F-59465	JX414205	JX414178	MH156174	MH155153	—	This paper
Ruhlandiella patagonica	MES-571	FLAS-F-59464	JX414206	JX414179	MH156175	MH155154	—	This paper
Ruhlandiella patagonica	MES-572	FH-940315	JX414207	—	—	—	—	This paper

(*Continued*)

Table 1. (Continued).

Taxon name	Collection (ID) number	Herbarium accession number	GenBank accession number					Reference
			ITS	LSU	β-tubulin	RPB2	RPB1	
Ruhlandiella patagonica	MES-580	FH-940318	JX414209	—	—	MH155155	—	This paper
Ruhlandiella patagonica	MES-581	FH-940319	JX414210	—	—	—	—	This paper
Ruhlandiella patagonica	MES-900	FLAS-F-62134	MG925377	MG947610	MH156176	MH155156	—	This paper
Ruhlandiella patagonica	MES-901	FLAS-F-62135	MG925378	MG947611	MH156177	MH155157	—	This paper
Ruhlandiella patagonica	MES-954	FLAS-F-62136	MG925379	MG947612	MH156178	MH155158	—	This paper
Ruhlandiella peregrina	KM-167991	FH-00301074	JF343549	—	—	—	—	This paper
Ruhlandiella reticulata	SPN-02	FLAS-F-62155	MG925396	MG947631	MH156190	MH155174	MH156173	This paper
Ruhlandiella sp.	OSC-60136	OSC-60136	MG925395	MG947630	MH156189	—	—	This paper
Ruhlandiella truncata	H-5715	OSC	MG925397	MG947632	MH156191	—	—	This paper

SD = 27.7 ± 4.30 µm) excluding ornamentation, biseriate when young, uniseriate when mature, at maturity light brown, highly ornamented, reticulate 2–4 µm high. Paraphyses numerous, filiform, frequently septate, 6–8 µm wide at tip, at maturity exceeding asci in length by 120–140 µm, covered by a gelatinous sheath.

Mitotic spore mats white becoming pale pink when mature, typically produced on soil but sometimes on woody debris in Nothofagaceae-dominated forests, in both disturbed and undisturbed areas. Spore mass dry, tufted, powdery, dense with hyphae and spores, lacking a peridium. Spore mat hyphae 5–7 µm diam, unchanging at maturity, dichotomously branching with unequal lengths, irregularly coralloid. Most hyphae are entirely sporogenous by maturity. Denticles 1–2 µm long, more or less evenly spaced along the diam and length of the hypha. Mitotic spores holoblastic, borne singly on denticles, produced simultaneously on a given hypha. Spores 4–5.5 µm diam (mean ± SD = 5.03 ± 0.61 µm), globose to subglobose, smooth. All parts hyaline when viewed under a light microscope.

Habitat: Buried in soil and leaf litter, Nothofagaceae-dominated forests across western Patagonia, ascomata growing singly or in groups. Molecular phylogenetic inference strongly infers that this species is an ectomycorrhizal symbiont of trees in the Nothofagaceae (FIG. 2).

Distribution: Patagonian region of Chile and Argentina.

Other specimens examined: Ascomata: CHILE. MAGALLANES: Punta Arenas, near a coal mine (near or inside today's Reserva Nacional Magallanes), about 3.5–4.5 km from the city, in a *Nothofagus* forest, 7 Mar 1906, *R. Thaxter Hypogeous No.8*; Punta Arenas, Rio Las Minas, at the overlook, near *Nothofagus* sp. trees, on soil, 19 Mar 2008, *M.E. Smith* and *D.H. Pfister CH-28* (FH-00284821); ibid., *CH-42* (FH-00284833); Rio Santa Maria, south of Reserva San Juan and Fuerte Bulnes (53°40′27.7″S, 70°59′21.6″W), 17 m asl, in a forest dominated by *Nothofagus betuloides* but with some *N. pumilio*, on soil, 1 Apr 2017, *M. E. Smith* and *A.B. Mujic MES-2284* (FLAS-F-62148, SGO-168849); Magallanes Forest Reserve, near the park guard house (53°8′34.3″S, 71°0′21.9″W), 341 m asl, in *N. pumilio* forest, along banks of a creek, fruiting on a slope of soft quartz-filled soil, 17 Apr 2017, *A.B. Mujic MES-2543* (FLAS-F-62146); ibid., except 7 Apr 2017, *D.H. Pfister MES-2553* (FLAS-F-62153); LOS LAGOS: Sendero La Princesa, Anticura, Puyehue National Park, near *N. dombeyi*, on soil, 5 May 2016, *M.E. Smith MES-1702* (FLAS-F-62151); Sendero Pampa Frutilla connector trail (40° 40′18.2″S, 72°9'14.8″W), 486 m asl, in a mixed *Valdivian* forest, under *N. dombeyi*, on soil, fruiting near mitotic spore mats, 7 Apr 2017, *A.B. Mujic MES-*

Figure 1. Phylogram of *Ruhlandiella* and related genera (Pezizales) obtained from maximum likelihood analysis of four concatenated genes (28S, *RPB1, RPB2, TUB1*). Numbers next to nodes represent Bayesian posterior probabilities followed by ML bootstrap support values. Bootstrap values ≥70% and posterior probabilities ≥0.95 are shown here. Sequences of type specimens are highlighted in bold. Black squares (■) represent mitotic spore mat samples. Gray bars indicate the geographic origin of specimens.

2682 (FLAS-F-62150); Puyehue National Park, in an old-growth *Podocarpus nuvigena* forest with *N. dombeyi*, on soil, 14 Apr 2017, *C. Truong MES-2854* (FLAS-F-62149). ARGENTINA. RÍO NEGRO: Parque Nacional Nahuel Huapi at Los Rápidos, near *N. antarctica*, on soil, 20 Mar 2012, *M.E. Smith* and *D. H. Pfister DHP AR-17* (FH-290549); ibid., May 2016, *R. A. Healy MES-2159* (FLAS-F-62145, CORD-C-6466); NEUQUÉN: Lanin National Park, north of Lago Lacar about half way between San Martin and the Hua Hum,

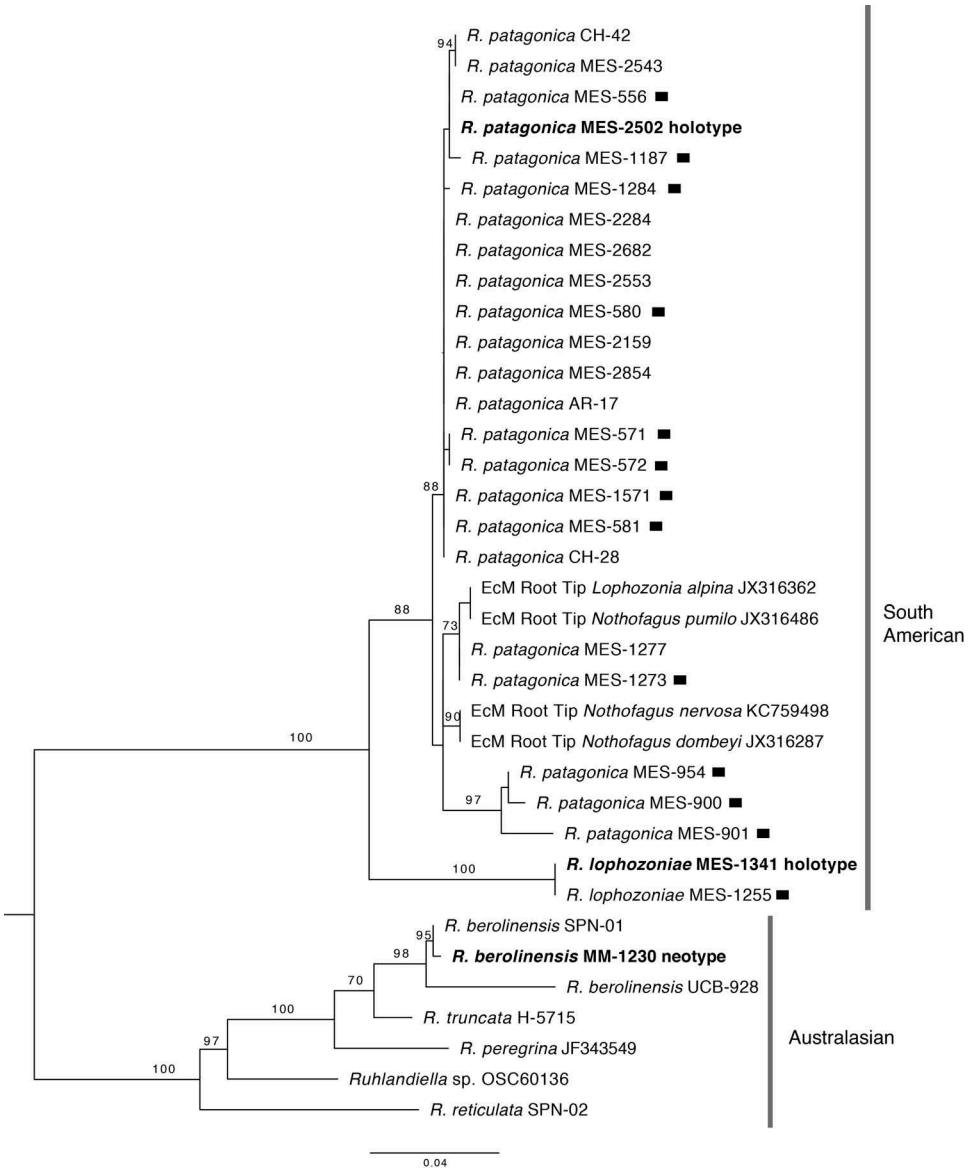

Figure 2. Phylogram of *Ruhlandiella* species obtained from the ITS rDNA alignment based on maximum likelihood (ML) analysis. Numbers next to nodes indicate ML bootstrap support values. Bootstrap values ≥70% are shown here. Sequences of type specimens are highlighted in bold. Black squares (■) represent mitotic spore mat samples. Gray bars indicate the geographic origin of specimens. EcM = ectomycorrhizal.

near *Lophozonia alpina* and *L. obliqua*, on soil, 15 May 2015, *R.A. Healy MES-1277* (FLAS-F-62152).

Mitotic spore mats: CHILE. AYSÉN: Melimoyu Patagonia Sur Reserve, Mirador trail, on soil in mixed forest with *N. dombeyi*, 13 Mar 2012, *M.E. Smith* and *D.H. Pfister MES-556* (FLAS-F-59465); Patagonia Sur Reserve, Valle California, on soil in pure *Nothofagus* forest, 15 Mar 1012, *M.E. Smith* and *D.H. Pfister MES-571* (FLAS-F-59464) and *MES-572*; LOS LAGOS: Puyehue National Park, below Antillanca on edge of road, on soil, near *N. pumilio*, 3 May 2016, *MES-1571* (FLAS-F-62141); EL RANCHO, along the T-80 road, between La Unión and Hueicolla (close to, but not inside, the Monumento Natural Alerce Costero), about 500 m asl, directly on soil, in mixed forest with *Nothofagus dombeyi, Lophozonia alpina*, and Myrtaceae, 1 May 2015, *R.A. Healy MES-900* (FLAS-F-62134, SGO-168850) and *MES-901* (FLAS-F-62135, SGO-168851); Entrance of Parque Nacional Alerce Costero, 893 m asl, on soil, in mixed forest with *N. dombeyi, Lophozonia alpina*, and Myrtaceae, 2 May 2015, *R.A. Healy MES-954* (FLAS-F-62136). ARGENTINA. RÍO NEGRO: Bariloche, near Llao Llao Hotel, on soil, near *N. pumilio*, 17 Mar 2012, *M. E. Smith* and *D.H. Pfister, MES-576* (FLAS-F-59466); Parque Nacional Nahuel Huapi at Los Rápidos, on soil, in pure *Nothofagus* forest, 18 Mar 2012, *M.E. Smith* and

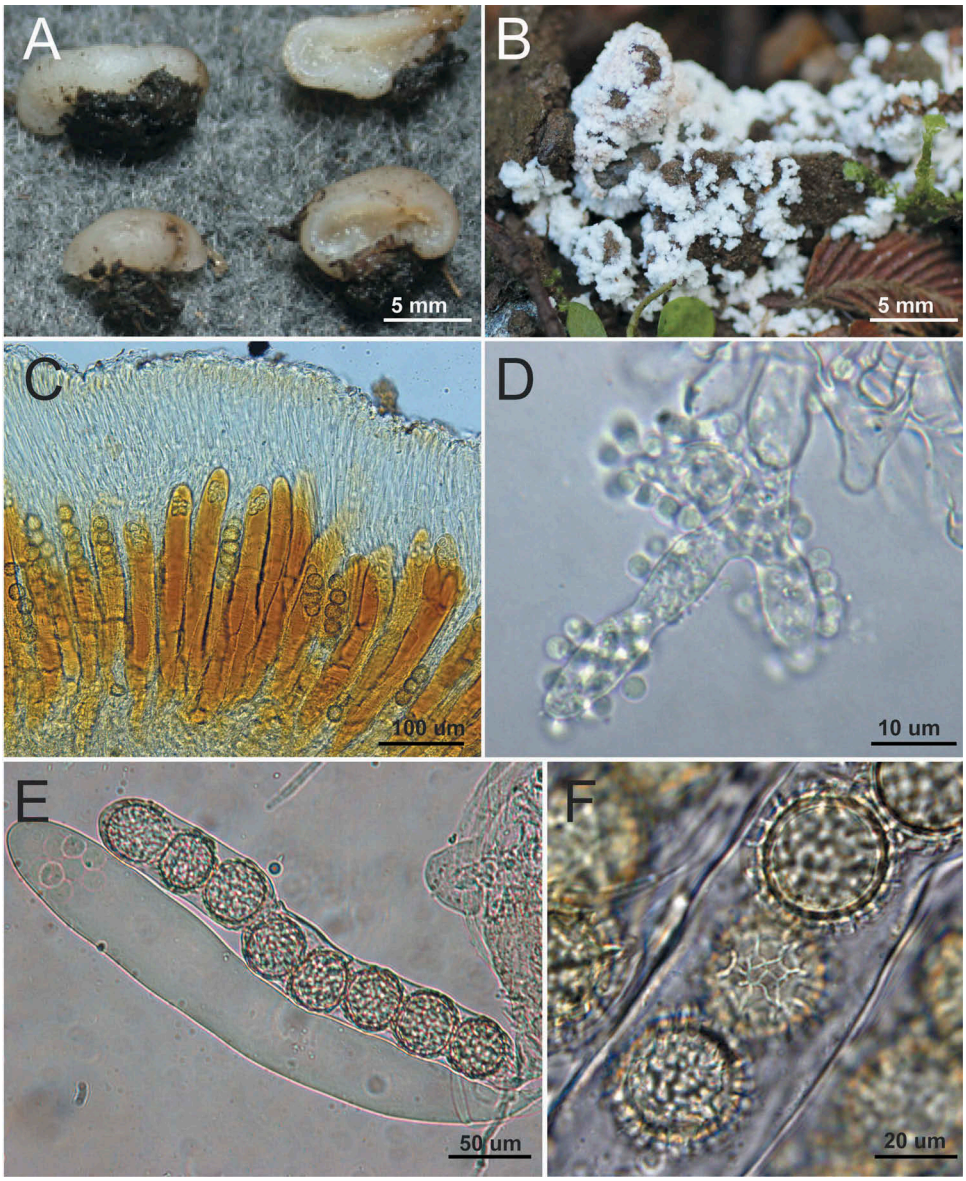

Figure 3. Morphology of *Ruhlandiella patagonica*. A. Fresh halves of two ascomata (MES-2159) showing the outer hymenial layer and the lack of peridium. B. Fresh mitotic spore mats (MES-900) growing directly on soil in the field showing pinkish white color. C. Section in Melzer's reagent from an ascoma (CH-28) showing young dextrinoid asci and paraphyses that far exceed the asci in length. D. Light micrograph of mitotic spore mat (MES-1571) showing dichotomous hyphal branching and mitospores. E. Mature ascus showing uniseriate ascospores. F. Ascospores showing light yellowish pigmentation and reticulate ornamentation.

D.H. Pfister MES-580 and *MES*-581; ibid., 10 May 2015, *D.H. Pfister MES-1187* (FLAS-F-62139); Lago Escondido, side of the road, on soil, near *Nothofagus dombeyi*, 14 May 2015, *D.H. Pfister MES-1273* (CORD-C-00005143); NEUQUÉN: Parque Nacional Lanín, Lago Queñi area, copiously sporulating on exposed soil under bank along the lakeshore, near *N. dombeyi*, 15 May 2015, *R.A. Healy MES-1284* (CORD-C-00005113).

Notes: Ruhlandiella patagonica is the first *Ruhlandiella* species discovered in South America. All *R. patagonica* ascomata were found near Nothofagaceae trees in Patagonia. Although the ascomata are widely distributed across western Patagonia, the mitotic spore mats are much more common.

Ruhlandiella lophozoniae Kraisit., Pfister, Healy & M. E. Sm., sp. nov. FIG. 4

MycoBank MB834730

Typification: ARGENTINA. NEUQUÉN: Lanín National Park, north of Lago Lacar about half way

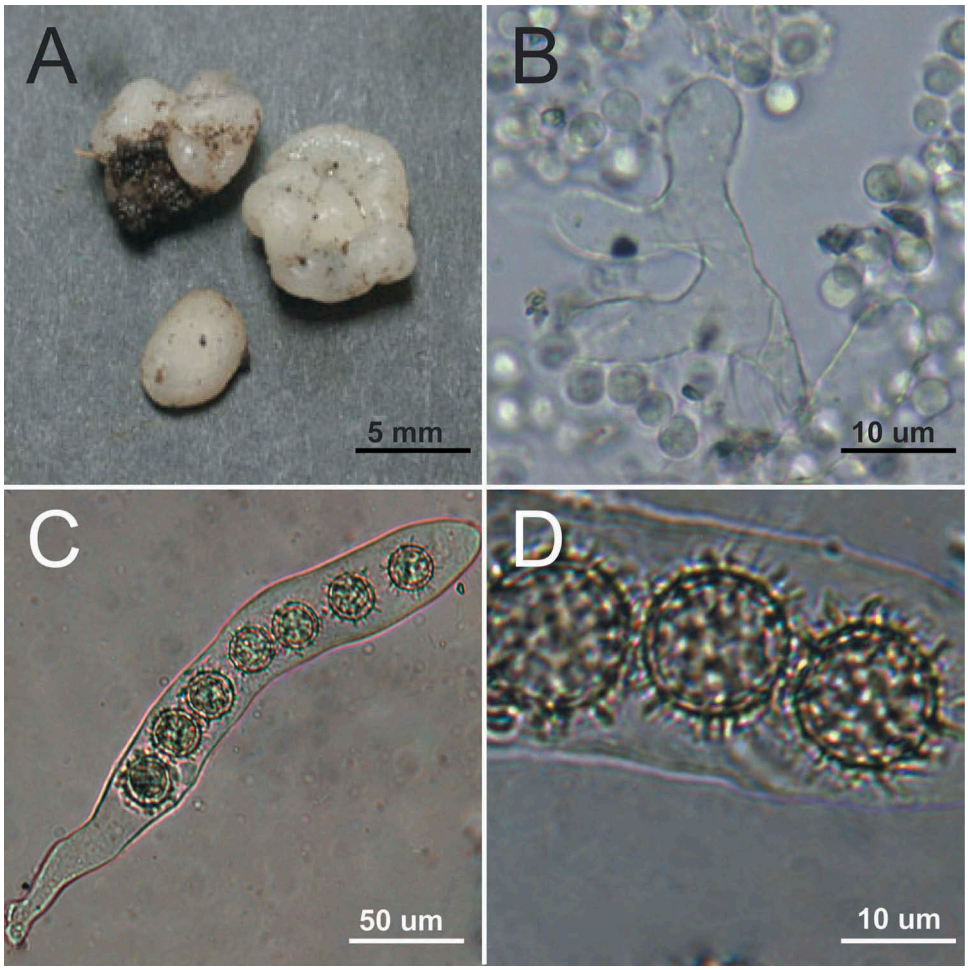

Figure 4. Morphology of *Ruhlandiella lophozoniae*. A. Three fresh ascomata of holotype specimen (MES-1341). B. Light micrograph of mitotic spore mat (MES-1255) showing dichotomous hyphal branching and mitospores. C. Mature ascus (MES-1341) with uniseriate ascospores. D. Mature ascospores showing ornamentation that is less reticulate and spinier in appearance than the ornamentation found in *Ruhlandiella patagonica*.

between San Martin and the Hua Hum pass (53°40′ 27.7″S, 70°59′21.6″W), with *Lophozonia alpina* and *L. obliqua*, on soil, 18 May 2015, *M.E. Smith and R.A. Healy MES-1341* (**holotype** CORD-C-6465). I**sotype**: FLAS-F-62144.

Etymology: Lophozonia (Latin), a genus of Nothofagaceae (Heenan and Smissen 2013), referring to the dominant ectomycorrhizal host tree species where the specimen was discovered.

Diagnosis: Ascomata hypogeous, exothecial, asci 180–230 × 20–36 µm, inamyloid, ascospores globose, 15–22 µm, reticulate, pale brown, ornamentation up to 8 µm high, paraphyses covered with gelatinous sheath, ascomata and spore mats rare, found in Nothofagaceae forests near *Lophozonia* trees.

Ascoma an exothecium roughly 4–6 mm diam, globose, convoluted and brain-like, pearly white, soft, and moist when fresh, with short and thin mycelial cords to which soil debris adhere. Asci lacking opercula, clavate or cylindrical-clavate, 180–230 × 20-36 µm (mean ± SD = 222.2 × 26.9 ± 30.6 × 5.8 µm), attenuate at base, usually (ca. 90%) 8-spored, but occasionally 7-spored, persistent, content dextrinoid when young, inamyloid. Ascospores globose, 15–22 µm (mean ± SD = 18.9 ± 2.4 µm) excluding ornamentation, uniseriate at all stages, hyaline to pale yellow at maturity, highly ornamented, reticulate 6.5–8 µm high. Paraphyses abundant, filiform, frequently septate, 6–8 µm wide at tip, covered with a gelatinous sheath, at maturity exceeding asci in length by 40–80 µm.

Mitotic spore mat: Morphology indistinguishable from *R. patagonica*. Spore mass pale pink, dry, powdery. Hyphae 5–7 µm wide diam, unchanging at maturity, dichotomously branching with unequal lengths. Most hyphae entirely sporogenous at maturity. Denticles 1–2 µm long. Spores 4–5.5 µm diam (mean ± SD = 5.10 ± 0.67 µm), globose to subglobose, smooth. All parts hyaline when viewed under a light microscope.

Habitat: Buried in soil or leaf litter, in a Nothofagaceae-dominated forest where *Lophozonia obliqua* was present.

Distribution: Known only from Lanín National Park, Argentina. Despite extensive sampling of spore mats south of this region, we did not document many collections of this species. This suggests that *R. lophozoniae* may be rare or could be more common in the northern range of Nothofagaceae in Chile and Argentina.

Mitotic spore mat: ARGENTINA. NEUQUÉN: South of Villa La Angostura, side of the road, pinkish, on soil, near *N. dombeyi*, 13 May 2015, *R.A. Healy MES-1255* (FLAS-F-62133).

Notes: Ruhlandiella lophozoniae appears to be rare. Only a few ascomata and one mitotic spore mat sample were found. The teleomorph was discovered in the northern part of Patagonia (Lanín National Park, Argentina) where *Lophozonia* is present.

Ruhlandiella verrucosa (Warcup & Talbot) Kraisit., Pfister, Healy & M.E. Sm., comb. nov.

≡ *Muciturbo verrucosus* P.H.B. Talbot, Mycological Research 92:96. 1989 (Basionym).
MycoBank MB824728

Typification: AUSTRALIA. NEW SOUTH WALES: Nymagee, ex solo sub *Acacia*, Jun 1978, *J.H. Warcup ADW 16982*.

Notes: This species is morphologically similar to *Ruhlandiella truncata*, particularly the truncate nature of ascospore ornamentation. However, *R. truncata* and *R. verrucosa* differ in ascospore size and in the length of the asci (TABLE 2).We do not know how these two species are related phylogenetically. A morphological and molecular study of the type of *Ruhlandiella verrucosa* is needed to clarify the relationship of this species with others in the genus.

Specimen recommended for further study.—*Ruhlandiella* sp. OSC-60136

Ascoma and exothecium: Asci lacking opercula, clavate or cylindrical-clavate, 300–380 × 20–24, attenuate at base, usually 8-spored, evanescent, contents dextrinoid, mature asci weakly amyloid in Melzer's reagent, especially at the tips. Ascospores globose, 18–22 µm diam excluding ornamentation, uniseriate, dark brown at maturity, reticulate 2–3 µm high. Paraphyses numerous, filiform, frequently septate, exceeding asci at maturity by 60–100 µm.

Specimens examined: AUSTRALIA. NEW SOUTH WALES: Nungatta State Forest, unnamed track, 1.9 km northeast of junction of Nungatta and Poole roads, 1.5 km southeast of junction of Poole Road buried in soil and leaf litter, 1 Oct 1996, *A.W. Claridge-1366/Trappe 19838* (OSC-60136).

Notes: We chose not to describe this specimen as a new species because there are other species described from southern Australia that fit the general description of *Ruhlandiella*, including *Sphaerosoma alveolatum, S. mucidum, S. tasmanica*, and *S. trispora*. We were unable to locate the type specimens of these species to compare with OSC-60136. A careful analysis of the previously described species and additional collections from this region are needed before this species can be definitively identified (see Discussion). No associated spore mats were reported for this specimen.

Exothecial species excluded from *Ruhlandiella*.—There has been much confusion regarding the taxonomy of *Ruhlandiella*. Here, we provide an overview of our morphological analyses of exothecial species in Pezizales and discuss why we consider these species to be classified outside *Ruhlandiella*.

Rouppert (1909) suggested that *Sphaerosoma fuscescens* was a synonym of *Ruhlandiella berolinensis* because of its reticulate ascospores. However, Setchell (1910) pointed out that the specimen that Rouppert examined was not the type of *S. fuscescens*. Dissing and Korf (1980) also mentioned that *S. fuscescens* has been misidentified as *R. berolinensis*. After studying the isotype of *Sphaerosoma fuscescens* (F-41729), we agree with Dissing and Korf (1980) that *S. fuscescens* does not belong to *Ruhlandiella* because of three morphological differences (FIG. 5). First, ascospores of *S. fuscescens* are mostly hyaline, but those of *Ruhlandiella* are generally pigmented with shades of brown. Second, asci of *S. fuscescens* retain their dextrinoid reaction after they have reached maturity, whereas this reaction only occurs in immature asci of *Ruhlandiella* (TABLE 2). Lastly, paraphyses of *S. fuscescens* are not obviously gelatinous and only slightly exceed the asci in length, whereas those of *Ruhlandiella* are obviously gelatinous and project considerably beyond the asci. Therefore, we conclude that *S. fuscescens* is unlikely to be a synonym of *Ruhlandiella berolinensis*. Nevertheless, based on morphology, it is possible that *Sphaerosoma* is closely related to *Ruhlandiella* and this group of fungi is probably more diverse than previously thought. More sampling and molecular data are needed to better understand phylogenetic relationships among species of *Ruhlandiella* and *Sphaerosoma*.

Hirsch (1983) transferred *Boudiera parvispora* to *Ruhlandiella* because of its similar ascus and ascospore morphology. We analyzed the morphology of the type specimen of *B. parvispora* (K-236288) and

Table 2. Comparative morphological characteristics of described *Ruhlandiella* species.

Taxon	Distribution	Canopy tree	Ascus size (µm)	Reaction of asci in iodine	Ascospore size (µm)	Mature spore color	Ornamentation	Paraphyses	Ascoma color
Ruhlandiella patagonica[a] sp. nov.	Chile and Argentina	*Nothofagus, Lophozonia* (confirmed hosts)	340–430 × 32–40	Mature none, immature dextrinoid	22–36 w/o ornamentation	Cream yellow	Alveolate-reticulate 2–4 µm high	Gelatinous, exceeding asci by 120–140 µm	White turning dark brown
Ruhlandiella lophozoniae[a] sp. nov.	Argentina	*Lophozonia obliqua*	180–230 × 20–36	Mature none, immature dextrinoid	15–22 w/o ornamentation	Light brown	Reticulate up to 8 µm high	Gelatinous, exceeding asci by 40–80 µm	White turning dark brown
Ruhlandiella berolinensis Hennings (1903)	Australia, Germany, Spain, USA	*Eucalyptus, Melaleuca*	240–280 × 26–33	Mature weakly amyloid, immature dextrinoid	17–20 w/o ornamentation	Dark brown	Reticulate-areolate, highly ornamented 3.2–4 µm high	Gelatinous, exceeding asci by 60–100 µm	Dingy brownish lilac
Ruhlandiella peregrina Lantieri & Pfister (2012)	Italy	Unknown[c]	230–300 × 30–32.5	Mature none, immature dextrinoid	15–19 w/o ornamentation	Brown	Incompletely reticulate, ridges 2–3 µm high	Gelatinous, exceeding asci by 50 µm	Brownish with vinaceous tints
Ruhlandiella reticulata[b] (Talbot) Rubio et al. (2010)	Australia, Spain	*Eucalyptus*	290–405 × 30–45	Mature weakly amyloid, immature dextrinoid	20–32.5 w/o ornamentation	Blackish brown	Highly reticulate flanges 3.5–6 µm high	Gelatinous, exceeding asci by 90–135 µm	White turning dark brown/black
Ruhlandiella truncata (Talbot) Rubio et al. (2010)	Australia, Spain	*Eucalyptus*	230–300 × 38–45	Mature weakly amyloid, immature dextrinoid	22–25 w/o ornamentation	Blackish brown	Not reticulate, truncate, warts 3.0–3.5 µm high	Gelatinous, exceeding asci by 100–140 µm	White turning dark brown/black
Ruhlandiella verrucosa (Talbot) comb. nov.	Australia	*Eucalyptus*	300–350 × 60–80	None reported	30–39 with ornamentation	Blackish brown	Not reticulate, warts up to 3 µm high	Gelatinous, exceeding asci by 80–100 µm	White turning black

[a]Mitotic spore mats reported in nature, white when immature, becoming dusky pink and powdery when mature, hyphae septate, dichotomously branched (this paper).
[b]Mitotic spore mats reported in culture, pale dusky pink, powdery, hyphae irregularly branched (Warcup and Talbot 1989).
[c]The authors suspect that ectomycorrhizal Myrtaceae were present on site (personal communication).

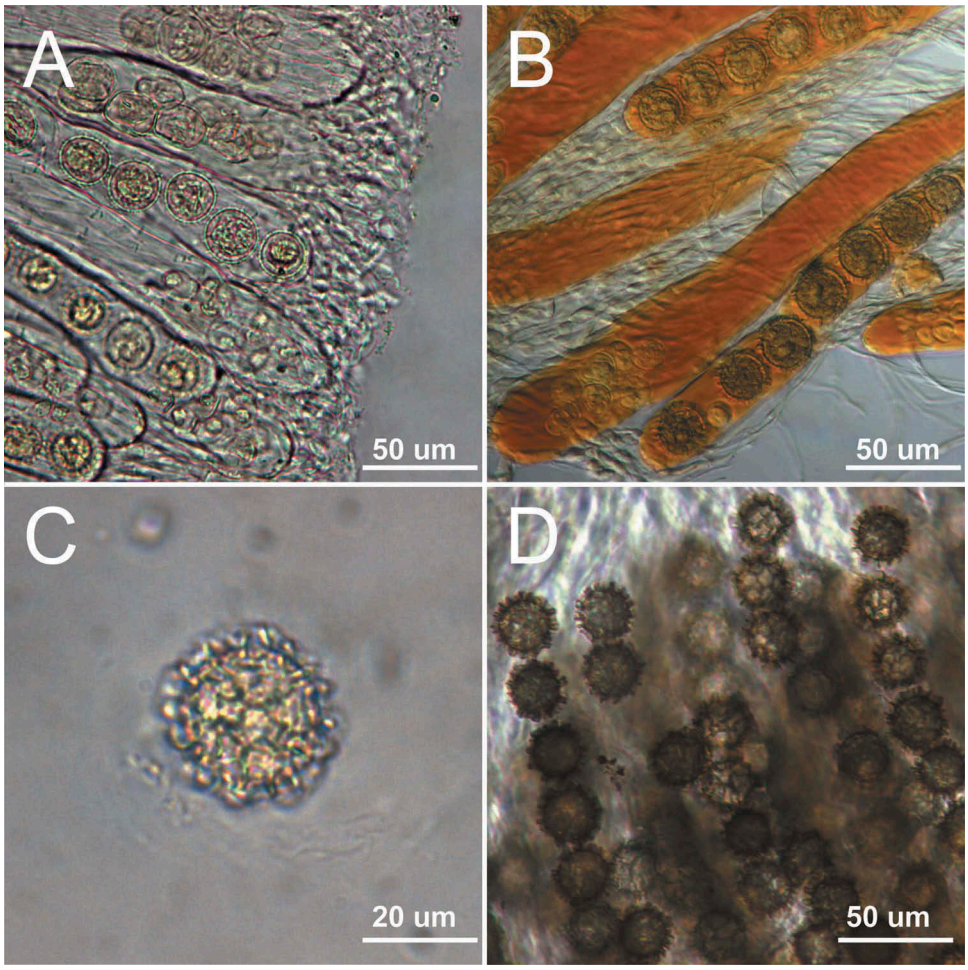

Figure 5. Morphological comparison of *Sphaerosoma fuscescens* and *Ruhlandiella berolinensis*. A. Section of *S. fuscescens* (neotype: F-41729) showing paraphyses that only slightly exceed the asci in length. B. Mature asci of *S. fuscescens* (F-41729) in Melzer's reagent showing dextrinoid response. C. Ascospore of *S. fuscescens* (F-41729) showing the light coloration and short reticulate ornamentation. D. Asci of *R. berolinensis* (neotype: MM-1230) showing ascospores that are highly pigmented and have distinctive reticulate spore ornamentation.

studied the original description by Thind et al. (1974). Several characters of this species do not match the characteristics of *Ruhlandiella*. First, there are no indications that apothecia of *B. parvispora* are gelatinous or exothecial, which are the diagnostic characters of *Ruhlandiella*. Second, the paraphyses of *B. parvispora* appear to be naked, whereas paraphyses of *Ruhlandiella* species are covered with gelatinous sheaths. Third, the ascospores of *B. parvispora* contain dark oil droplets. This character was not detected in any of *Ruhlandiella* species we examined. Finally, no ectomycorrhizal Myrtaceae or Nothofagaceae hosts were documented in the region of India where the specimen was collected. Based on morphological and biogeographic evidence, we conclude that this species does not belong to *Ruhlandiella* and should remain in *Boudiera*. Nonetheless, molecular documentation will help to further resolve the placement of this species.

DISCUSSION

Prior to the Cretaceous period (~100 million years ago [MYA]), Australia and South America were once united via Antarctica and formed a supercontinent known as "Gondwanaland" (Raven and Axelrod 1972) or simply Gondwana. South America and Australia remained connected through Antarctica until about 35 MYA (Sanmartín and Ronquist 2004). The South America–Australasia separation is hypothesized to have facilitated vicariant diversification in several groups of animals and plants (Sanmartín and Ronquist 2004). For instance, several molecular analyses show that *Lophozonia* (Nothofagaceae) diverged into South

American and Australasian clades around the time of the Gondwanan breakup (Swenson et al. 2001; Knapp et al. 2005).

A similar evolutionary pattern is also observed in several ectomycorrhizal fungi. For instance, Truong et al. (2017) concluded that the southern temperate clade of *Amanita* probably diverged into Australasian and southern South American subclades as a result of the Gondwana separation. Similar lineage-diverging patterns are also observed in the *Hysterangium* II clade (Hosaka et al. 2008), the /aleurina lineage (Tedersoo and Smith 2013), and the /gymnohydnotrya clade of Tuberaceae (Bonito et al. 2013). All of these clades contain ectomycorrhizal truffle-like fungi that are found in both Australia and South America.

No previous studies have explored the biogeography of *Ruhlandiella* species. We postulate that a vicariant event occurred in *Ruhlandiella*, i.e., diverging into Australasian and southern South American subclades following the final separation of Gondwana, which occurred roughly 35 MYA (Sanmartín and Ronquist 2004). Nonetheless, a time-calibrated phylogeny is needed to test this hypothesis. An alternative hypothesis is that both *R. patagonica* and *R. lophozoniae* were always present in Australia and Antarctica prior to the Gondwanan breakup, but they either went extinct or have not been discovered yet in Australasia because of their hypogeous nature and small ascoma size. Sampling of mitotic spore mats from Nothofagaceae forests in Australasia could be a rapid way to identify additional diversity within *Ruhlandiella*.

Our study is the first official report of *Ruhlandiella* from Nothofagaceae forests and the only verified report of *Ruhlandiella* species native to the Americas. All specimens were collected in southern South America (Argentina and Chile), suggesting that both *R. patagonica* and *R. lophozoniae* are endemic to the Nothofagaceae forests of Patagonia. Based on existing unpublished notes and collections preserved at the Farlow Herbarium (FH-00284184 in liquid preservative), it appears that Roland Thaxter was the first to document *R. patagonica* during his trip to Punta Arenas, Chile, in 1906 (Halling 1981). Thaxter's collections morphologically match *R. patagonica* and were found in the same locality and time of year as our *R. patagonica* samples.

The other South American species we describe here, *R. lophozoniae*, is also part of the South American clade (FIG. 1). *Ruhlandiella lophozoniae* is morphologically similar to *R. patagonica* except that *R. lophozoniae* has smaller asci and ascospores but with higher spore ornaments (TABLE 2). Both ascomata and mitotic spore mats of *R. lophozoniae* seem rare, but this could be a result of either seasonality or sampling effort, or both of these factors.

A phylogeny based on ITS of ectomycorrhizal root tip sequences from previous studies (Fernández et al. 2013; Nouhra et al. 2013) indicated that *R. patagonica* is an ectomycorrizal fungus associated with *Nothofagus* and *Lophozonia* species (FIG. 2). We suspect that *Ruhlandiella* species are probably involved in the early establishment of Nothofagaceae seedlings. An experiment by Fernández et al. (2013) found that *Nothofagus nervosa* (*Lophozonia alpina*) seedlings were naturally colonized by a fungus then identified as "*Peziza* sp. 2 (KC759498)." Our ITS phylogeny reveals that "*Peziza* sp. 2" is actually *R. patagonica* (FIG. 2). More ecological data on *Ruhlandiella* species are needed to assess their colonization potential on seedlings and saplings of other Nothofagaceae species.

All of the previously described *Ruhlandiella* species belong in the Australasian clade (FIG. 1). As far as we know, all species in this clade form ectomycorrhizal associations with *Eucalyptus* and other ectomycorrhizal members of the Myrtaceae, but it is possible that these species may also associate with Nothofagaceae and other hosts in Australasia. Available data suggest that these fungi were introduced to Europe and North America from Australia along with their host plants (Vellinga et al. 2009).

Our study and others (Warcup and Talbot 1989; Healy et al. 2013) show that *Ruhlandiella* species produce mitotic spore mats. Although the function of the mitospores is unknown, we hypothesize that they may act as conidia and therefore serve as a major dispersal mechanism. However, we cannot rule out the possibility that these spores are spermatia that play a role in sexual reproduction. If these mitotic spores can serve as conidia, then this could explain why species of *Ruhlandiella* have been so successful at dispersing to *Eucalyptus* plantations in Europe and North America. Attempts to germinate mitospores from species of ectomycorrhizal Pezizaceae are so far unsuccessful (Healy et al. 2013), although at least one member of the Pezizales (*Sphaerosporella brunnea*, Pyronemataceae) produces conidia capable of germinating on several kinds of agar media and initiating the formation of ectomycorrhizae (Sánchez et al. 2014). More experiments are needed to understand the biological functions of these mitotic spores.

Dissing and Korf (1980) suggested that *Ruhlandiella berolinensis* is a synonym of *Ruhlandiella hesperia*, which was described by Setchell (1910) from California. We obtained DNA sequences from a more recent and morphologically similar specimen also collected near *Eucalyptus* trees in northern California (UC-1576349).

Multilocus analysis places this specimen in the same clade as the *R. berolinensis* neotype (FIG. 1). This evidence strongly supports the hypothesis that *R. berolinensis* was introduced to North America.

Several studies suggest that *Muciturbo* is synonymous with *Ruhlandiella* (Hansen et al. 2005; Rubio et al. 2010), but previous evidence was inconclusive (Healy et al. 2013). Our multilocus phylogeny shows that *M. reticulatus* and *M. truncatus* are nested within the Australasian clade of *Ruhlandiella* with high support values (FIG. 1). We conclude that Rubio et al. (2010) were correct in their assessment that *Muciturbo* is congeneric with *Ruhlandiella*. Because *M. reticulatus* is the type species of *Muciturbo* (Warcup and Talbot 1989), this genus is no longer accepted. Thus, we also transfer *M. verrucosus* to *Ruhlandiella* (as *R. verrucosa*) and recognize all three described species of *Muciturbo* as members of *Ruhlandiella*.

One *Ruhlandiella* sample from New South Wales, Australia (OSC-60136), was originally identified as *R. berolinensis*, but molecular analysis indicates that it is sister to the rest of the Australasian clade (FIG. 1). The sample was recovered in a *Eucalyptus* forest and is morphologically similar to *R. berolinensis* but differs in ascospore size (TABLE 2). There are other records of *Ruhlandiella*-like specimens recovered from southern Australia. These include *Sphaerosoma alveolatum* (McLennan and Cookson 1923), *S. mucida* (Hansford 1956), *S. tasmanica* (Rodway 1919), and *S. trispora* (McLennan and Cookson 1926). The descriptions of these species match entirely or partly with the current concept of *Ruhlandiella*. However, the asci of *S. trispora* only contain three or four ascospores, and the paraphyses of *S. alveolatum* are dichotomously branched at the tips. These characters are not found in any described *Ruhlandiella* species. We were unable to locate any specimens of the aforementioned species. In the absence of molecular and morphological analyses, it is unclear whether any are *Ruhlandiella* species. Because we cannot confirm that OSC-60136 is undescribed, we leave it as an unidentified *Ruhlandiella* species. More Australian collections of *Ruhlandiella* are needed to determine the identity of this specimen and to clarify the taxonomic placements of the various *Sphaerosoma* species mentioned above.

KEY TO DESCRIBED SPECIES OF *RUHLANDIELLA*

1. Ascomata found in South America, near *Nothofagus, Lophozonia*, or other ectomycorrhizal Nothofagaceae..... 2
1.' Ascomata found in Australia, Europe, North America, or elsewhere near *Eucalyptus* or other ectomycorrhizal Myrtaceae..... 3
2. Asci 340–430 µm long, ascospores pale yellow, 22–36 µm diam, reticulate 2–4 µm high, widespread in Patagonia..... *R. patagonica*
2.' Asci 180–230 µm long, ascospores pale brown, 15–22 µm diam, reticulate 4–8 µm high, apparently rare and known only from northern Patagonia..... *R. lophozoniae*
3. Ascomata flattened, measuring roughly 3 × 2 mm, white when young but turning dark purple or black..... *R. reticulata*
3.' Not as above, ascomata subglobose to globose, convoluted, light tan, brown, or reddish brown..... 4
4. Ascomata relatively large, 1–3 cm diam, highly convoluted and brain-like, common and known from *Eucalyptus* plantations on several continents..... *R. berolinensis*
4.' Ascomata small, <1 cm diam 5
5. Mature asci not turning blue in Melzer's solution, ascospores reticulate 2–3 µm high, known only from Italy..... *R. peregrina*
5.' Mature asci weakly amyloid, ascospores truncate with warts 3–3.5 µm high, known only from native habitats in Australia 6
6. Asci 230–300 µm in length, ascospores 22–25 µm diam..... *R. truncata*
6.' Asci 300–350 µm in length, ascospores 30–39 µm diam..... *R. verrucosa*

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ORCID

Nattapol Kraisitudomsook http://orcid.org/0000-0001-6514-2366
Rosanne A. Healy http://orcid.org/0000-0001-7616-0092
Alija B. Mujic http://orcid.org/0000-0002-5810-5521
Donald H. Pfister http://orcid.org/0000-0002-9018-8646
Matthew E. Smith http://orcid.org/0000-0002-0878-0932

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