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Two new species of *Phaeohelotium* (*Leotiomyces: Helotiaceae*) from Chile and their putative ectomycorrhizal status

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Abstract: Species of the genus *Phaeohelotium* (*Leotiomyces: Helotiaceae*) are cup fungi that grow on decaying wood, leaves, litter, and directly on soil. Northern Hemisphere species are primarily found on litter and wood, whereas in the Southern Hemisphere the genus includes a mix of saprotrophs as well as taxa that grow on soil in association with ectomycorrhizal trees. The diversity of this genus has not been fully explored in southern South America. Here we describe two species from Chile, *Phaeohelotium maiusaurantium* sp. nov. and *Ph. pallidum* sp. nov., found on soil in Patagonian *Nothofagaceae*-dominated forests. We present macro- and micromorphological descriptions, illustrations, and molecular phylogenetic analyses. The two new species are placed in *Phaeohelotium* with high support in our 15-locus phylogeny as well as phylogenetic reconstructions based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene. Our ITS phylogeny places both *Ph. maiusaurantium* and *Ph. pallidum* in a well-supported subclade that includes ectomycorrhizal root tip samples from Australasia. Similar species can be separated from these new taxa based on morphological characteristics, biogeography, substrate, and sequence data. In addition, two unnamed species from Chilean *Nothofagaceae* forests (*Phaeohelotium* sp. 1 and *Phaeohelotium* sp. 2) are documented from scant collections and sequence data and await description until more material becomes available.

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INTRODUCTION

Leotiomyces (*Ascomycota: Pezizomycotina*) is a diverse group of fungi that are taxonomically and systematically challenging. This class contains approximately 12 orders and roughly 60 families of which multiple lineages remain unassigned (Quandt & Haelewaters 2021). The genus *Phaeohelotium* (*Helotiales: Helotiaceae*) was erected to accommodate species that did not fit well with the generic concept of *Helotium* based on the anatomy of the ectal excipulum and color of ascospores (Kanouse 1935, Dennis 1981, Gamundí & Messuti 2006). *Helotium* was replaced by *Hymenoscyphus* on nomenclatural grounds (Dennis 1964). *Phaeohelotium* currently comprises taxa that have been transferred from as many as 16 different genera, including *Discinella*, *Helotium*, *Peziza*, and others (Kanouse 1935, Dennis 1971, Svrček & Matheis 1979, Baral *et al.* 2013, Wijayawardene *et al.* 2022). The type species is *Ph. flavum* (= *Ph. monticola*), which was first found on a decaying log in Harbor

Springs, Michigan, USA (Kanouse 1935, Dennis 1964, Dumont 1981, Baral *et al.* 2013).

The trophic mode for species in this genus has yet to be determined, but ascomata of many species are found on rotten wood, twigs, and leaves suggesting a saprotrophic ecology. However, close matches to ectomycorrhizal (ECM) root tip sequences along with observations of ascoma-production on bare soil suggest that some species may be associates of *Eucalyptus* (*Myrtaceae*) and *Nothofagus* (*Nothofagaceae*) (Baral *et al.* 2013, Tedersoo & Smith 2013).

Five species of *Phaeohelotium* have thus far been reported in South America. These are *Ph. castaneum*, *Ph. flavum*, *Ph. luteum*, *Ph. nothofagi*, and *Ph. recurvum* from Argentina, Brazil, and Chile (Gamundí & Messuti 2006). All of these have been recorded from plant substrates. New collections of *Phaeohelotium* species were made from 2012 to 2019 as part of a larger project to survey fungal biodiversity in Patagonian *Nothofagaceae*-dominated forests, with a special focus on ECM

fungi (Truong *et al.* 2017a). Sequence data analysis identified four putatively ECM *Phaeohelotium* species that were collected directly on soil. The objective of this paper is to describe two of these species, *Phaeohelotium maiusaurantium* and *Ph. pallidum* spp. nov., to compare them to all previously documented South American *Phaeohelotium* species, and to provide ITS-based and multi-locus molecular phylogenetic analyses to determine their placement within *Helotiaceae*.

MATERIALS AND METHODS

Morphological study

Fresh specimens were collected during expeditions to *Nothofagaceae* forests across Chile between 2012 and 2019 (Truong *et al.* 2017a, Nouhra *et al.* 2021). We searched for cup fungi exposed on the soil or fruiting below the leaf litter by gently removing the litter with rakes or by hand (Kraisitudomsook *et al.* 2020). Specimens were photographed *in situ* and in the field laboratory. Macroscopic features were described from fresh specimens using terminology for ascomata outlined in Weber *et al.* (1997). Collections of fresh ascomata were air-dried or dried in a forced air dryer for 48 h at 40 °C. Micromorphological features of dried specimens were examined with an Olympus BX43 (Olympus, Center Valley, Pennsylvania) compound microscope. Separate mounts of fungal tissue were made in deionized (DI) water, tap water, 3 % KOH, and Melzer's solution. At least 20 individual ascospores and other structures were measured at 400–1 000× magnification for each collection after having been rehydrated in 3 % KOH for 20 min. Outlying measurements observed in less than 5 % of the measured population are indicated in parentheses. Histological preparation was done as follows: dried samples were initially rehydrated in 3 % KOH for 20 minutes before being placed in a Formal-Fix Concentrate (Eprexia, Kalamazoo, Michigan) at a dilution of 2 mL Formal-Fix concentrate to 8 mL dH₂O for 24–48 h before proceeding with the fixative steps. Samples were formalin fixed and paraffin embedded using a Sakura Tissue-Tek VIP5 automated tissue processor (Sakura Finetek, Torrance, California) and a Tissue-Tek TEC II embedding center (Sakura Finetek, Torrance, California) as follows: samples were placed in a formalin solution for 5 min, a 70 % alcohol solution for 15 min, a 95 % alcohol solution for 40 min, a 100 % alcohol for 40 min, a xylene solution for 30 min, and a paraffin solution for 50 min. All steps were done while agitating, a pressure vacuum engaged, and at 40 °C, except for the paraffin stage which was at 58 °C. A Leica ST5010 Autostainer XL (Leica Biosystems, Wetzlar, Germany) was used for hematoxylin & eosin staining of samples before sectioning on a Jung BioCut 2030 Rotary Microtome (Leica Biosystems, Wetzlar, Germany) at a thickness of 5 µm at the University of Colorado Cancer Center Research Histology Services. Fungal collections are deposited in the following herbaria: FH (Farlow Herbarium at Harvard University), FLAS (Florida Museum of Natural History Fungarium), NY (New York Botanical Garden), and SGO (Museo Nacional de Historia Natural de Chile) (Thiers 2022).

Molecular protocols

DNA was extracted from ascomata using the Extract-N-Amp Plant kit (Sigma-Aldrich, St. Louis, Missouri) or using a modified CTAB extraction protocol utilizing an initial phenol/chloroform step (Gardes & Bruns 1993). We amplified the following loci:

nuclear small and large nuclear ribosomal subunits (SSU and LSU), internal transcribed spacer region of the ribosomal DNA (ITS), the mitochondrial locus MS456 containing the gene *MCM7*, and the RNA polymerase II largest and second-largest subunits (*rpb1* and *rpb2*). Primer combinations were as follows: NS1/NS4 for SSU (White *et al.* 1990), ITS1F/ITS4 for ITS (White *et al.* 1990, Gardes & Bruns 1993), LR0R/LR5 for LSU (Vilgalys & Hester 1990, Hopple 1994), *Mcm7*-709for/*Mcm7*-1348rev for *MCM7* (Schmitt *et al.* 2009), RPB1-Af/RPB1-Cr for *rpb1* (Stiller & Hall 1997, Matheny *et al.* 2002), and RPB2-f5F/RPB2-7cR for *rpb2* (Liu *et al.* 1999). All 25-µL PCR reactions were conducted on a C1000 Touch Thermal Cycler (Bio Rad, Hercules, California) and consisted of 2.5 µL of 10× Takara Ex Taq buffer (Takara Bio, Shiga, Japan), 1.25 µL of each 10 µM primer, 0.2 µL of Takara Ex Taq, up to 18.8 µL of sterile distilled water, and 1–2 µL of DNA extract. PCR protocols followed those outlined in the original publications. PCR products were visualized on 1.5 % agarose gels stained with ethidium bromide. Amplicons were sequenced by GENEWIZ (South Plainfield, New Jersey). Raw sequence reads were assembled and then edited using FinchTV 1.4 (Geospiza Inc., Seattle, Washington). Newly generated sequences (Table 1) were used as queries for BLAST searches against NCBI GenBank's standard *nr/nt* nucleotide database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify closest relatives.

Phylogenetic analyses

First, we used T-BAS v. 2.1 (Carbone *et al.* 2019) and the “Place Unknowns” tool to place newly generated *Phaeohelotium* sequences onto the 15-locus *Leotiomycetes*-wide tree from Haelewaters *et al.* (2021), named “*Leotiomycetes* v1.” Seven FASTA files with unaligned SSU, LSU, ITS, *MCM7*, *rpb1*, and *rpb2* sequences of *Phaeohelotium* were uploaded to the T-BAS interface. We selected the *de novo* option for the RAXML phylogenetic analysis, with GTRGAMMA as substitution model, 500 bootstrap replicates, and *Neurospora crassa* and *Xylaria hypoxylon* (*Sordariomycetes*) as outgroup taxa.

Next, we constructed two ITS datasets: one to place the genus *Phaeohelotium* among related genera and the second to place the newly generated *Phaeohelotium* sequences among other species in the genus. The first ITS dataset included 106 taxa belonging to *Helotiaceae* and *Tricladiaceae* (outgroup) (Table 2). We also included *Bulgariella pulla* (currently classified as *Leotiomycetes incertae sedis*, Iturriaga *et al.* 2017), *Connersia rilstonii* and *Pleuroascus nicholsonii* (*Pleuroascaceae*), and *Roesleria subterranea* (*Roesleriaceae*, Baral 2016) because these taxa were found to be most closely related to *Helotiaceae* and *Tricladiaceae* by Haelewaters *et al.* (2021). Sequences were aligned using MUSCLE v. 3.7 (Edgar 2004), available on the Cipres Science Gateway v. 3.3 (Miller *et al.* 2010). Sequences were trimmed at the conserved motifs 5'–CATT–3' (3' end of the SSU) and 5'–GACCT(CAAA...)–3' (5' end of the LSU) (Dentinger *et al.* 2011). Because the two spacers (ITS1, ITS2) and 5.8S have different rates of evolution, we treated them as individual partitions in the phylogenetic analysis. Maximum likelihood (ML) inference was performed using IQ-TREE v. 1.6.7 from the command line (Nguyen *et al.* 2015) under partitioned models (Chernomor *et al.* 2016). Nucleotide substitution models were selected under the Akaike information criterion (AIC) with the help of the built-in program ModelFinder (Kalyaanamoorthy *et al.* 2017). Ultrafast bootstrap analysis was implemented with 1 000 replicates (Hoang *et al.* 2017).

Table 1. Fungarium and GenBank accession numbers for studied collections.

Species	Collector's label	Herbarium accession	SSU	ITS	LSU	MCM7	rpb1	rpb2
<i>Phaeohelotium maiusaurantium</i>	DHP-CH-74	FH 00284861	–	OP868959	–	–	–	–
	DHP-CH-80	FH 00284867	–	OP868958	–	–	–	–
	DHP-CH-93	FH 00284876	–	OP868960	–	–	–	–
	MES-2905	FLAS-F-65460	OP951346	OP868961	OP962215	–	–	–
	MES-2976 ^T	FLAS-F-65526	OP951346	OP868953	OP962216	–	–	–
	MES-2925	FLAS-F-65476	–	OP868951	–	OP957112	OP957114	OP957116
<i>Phaeohelotium pallidum</i>	MES-1068 ^T	FLAS-F-63072	–	KY462415	OP962212	–	–	–
	MES-2850	FLAS-F-65411	–	OP868956	OP962213	–	–	–
	MES-2852	FLAS-F-65413	–	OP868955	OP962211	–	–	–
	MES-3308	Specimen destroyed	–	OP868954	–	–	–	–
	MES-3573	FLAS-F-66003	–	OP868950	–	–	–	–
	MES-3574	FLAS-F-66004	–	OP868952	–	OP957113	OP957115	OP957117
<i>Phaeohelotium</i> sp. 1	MES-2358	FLAS-F-65016	–	MH930302	–	–	–	–
<i>Phaeohelotium</i> sp. 2	MES-2650	FLAS-F-65244	–	MH930289	–	–	–	–
	MES-2851	FLAS-F-65412	–	OP868957	OP962214	–	–	–

^TStands for holotype specimen. Accession numbers of sequences generated during this study are in boldface.

Table 2. Species included in the ITS-based phylogenetic analyses to place the genus *Phaeohelotium* among related genera (placement) and to place the newly described species among other species of *Phaeohelotium* (genus). For each species, the following information is shown: GenBank accession number, DNA isolate/voucher information, type status (type species, ex-type specimen), dataset (placement or genus), and source reference. Accession numbers of sequences generated during this study are in boldface.

Species	Accession number	Isolate/voucher	Country	Note	Dataset	Reference
<i>Brunaudia phormigena</i>	KF727423	PDD:75309	New Zealand	type species	placement	P.R. Johnston & D. Park unpubl. data
<i>Bulgariella pulla</i>	KU845537	TL 2011	Norway	type species	placement	Iturriaga <i>et al.</i> (2017)
	MH578505	PDD:111518 / ICMP:22812	New Zealand	type species	placement	P.R. Johnston & D. Park unpubl. data
	KJ704848	DHP-06-607	USA	type species	placement	Iturriaga <i>et al.</i> (2017)
	KU845540	DHP 15-215	Chile	type species	placement	Iturriaga <i>et al.</i> (2017)
<i>Connersia rilstonii</i>	KJ755499	CBS:537.74	Canada	ex-type	placement	Malloch <i>et al.</i> (2016)
<i>Cudoniella acicularis</i>	DQ202512	CBS:100273	Denmark		placement	N. Boonyuen <i>et al.</i> unpubl. data
	DQ202513	CBS:430.94	India	ex-type	placement	N. Boonyuen <i>et al.</i> unpubl. data
<i>Cudoniella indica</i>	DQ202505	SS 708	N/A		placement	N. Boonyuen <i>et al.</i> unpubl. data
	DQ202505	SS 708	N/A		placement	N. Boonyuen <i>et al.</i> unpubl. data
<i>Dicephalospora albolutea</i>	MK425601	HMAS 279693	China	ex-type	genus, placement	Zheng & Zhuang (2019)
<i>Dicephalospora rufocornea</i>	AB926055	TNS:F-40024	Japan	type species	genus, placement	Zhao <i>et al.</i> (2016)
	KU668565	Zhuang 10106	China	type species	genus, placement	Zhuang <i>et al.</i> (2016)
<i>Dicephalospora sessilis</i>	MK584947	MFLU:18-1823	China	ex-type	genus, placement	Ekanayaka <i>et al.</i> (2019)
<i>Endoscypha perforans</i>	KF727424	PDD:102231	New Zealand	type species	placement	P.R. Johnston & D. Park unpubl. data
<i>Graddonina coracina</i>	JQ256423	ILLS:60491	USA	type species	placement	Hustad <i>et al.</i> (2011)
<i>Helicodendron tubulosum</i>	EF029195	ICMP:14615	New Zealand		placement	J.A. Cooper unpubl. data
<i>Helicodendron westerdijkiae</i>	EF029196	ICMP:14616	New Zealand		placement	J.A. Cooper unpubl. data
<i>Helotiaceae</i> sp.	MH682236	ICMP:22540	New Zealand		placement	P.R. Johnston & D. Park unpubl. data
<i>Hymenoscyphus albidoides</i>	KF188722	HMAS 264140	China	ex-type	placement	Zheng & Zhuang (2014)

Table 2. (Continued).

Species	Accession number	Isolate/voucher	Country	Note	Dataset	Reference
<i>Hymenoscyphus aurantiacus</i>	NR_154907	HMAS 264143	China	ex-type	placement	Zheng & Zhuang (2015)
<i>Hymenoscyphus caudatus</i>	AY348578	HMAS 82063	China		placement	Zhang & Zhuang (2004)
	KF188730	HMAS 264150	China		placement	Zheng & Zhuang (2014)
	AB926065	TNS:F-40056	Japan		placement	Zhao et al. (2016)
<i>Hymenoscyphus equiseti</i>	UDB038358	TAAM:194261	Russia	ex-type	placement	Lebeuf et al. (2021)
	OL679972	D. Haelew. F-1493c	Sweden		placement	Lebeuf et al. (2021)
<i>Hymenoscyphus fraxineus</i>	PRJNA297734 (NCBI genome)	CBS:133217	Luxembourg		placement	C. Sambles et al. unpubl. data
<i>Hymenoscyphus fraxineus</i> [as <i>H. pseudoalbidus</i>]	GU586904	ZT:Myc 2022	Switzerland	ex-type	placement	Queloz et al. (2011)
	GU586933	CBS:650.92	Germany	type species	placement	Queloz et al. (2011)
	AB926057	TNS:F-44644	Japan	type species	placement	Zhao et al. (2016)
<i>Hymenoscyphus ginkgonis</i>	NR_119669	KUS F51352	South Korea	ex-type	placement	Han & Shin (2008)
<i>Hymenoscyphus haasticus</i>	NR_137108	PDD:57577 / ICMP:19598	New Zealand	ex-type	placement	Johnston & Park (2013)
<i>“Hymenoscyphus” imberbis</i>	KC411993	H.B. 6797	Germany		placement	Baral et al. (2013)
<i>“Hymenoscyphus” cf. imberbis</i>	OL679974	D. Haelew. F-262	Germany		placement	Lebeuf et al. (2021)
<i>Hymenoscyphus infarciens</i>	PRJNA297733 (NCBI genome)	CBS:122016	France		placement	C. Sambles et al. unpubl. data
<i>Hymenoscyphus kiko</i>	NR_137110	PDD:82964 / ICMP:19613	New Zealand	ex-type	placement	Johnston & Park (2013)
<i>Hymenoscyphus koreanus</i>	KP068057	KUS F52847_01	South Korea	ex-type	placement	Gross & Han (2015)
<i>Hymenoscyphus macrodiscus</i>	KJ472296	HMAS:264158	China	ex-type	placement	Zheng & Zhuang (2015)
<i>Hymenoscyphus macroguttatus</i>	DQ431179	H.B. 7034	Spain		placement	Baral et al. (2007)
<i>Hymenoscyphus</i> as <i>“menthae”</i>	AB926063	TNS:F-40052	Japan		placement	Zhao et al. (2016)
<i>Hymenoscyphus menthae</i>	KM114537	H.B. 5846	Liechtenstein		placement	Gross et al. (2015)
<i>Hymenoscyphus occultus</i>	KP068058	CBS:139469	South Korea	ex-type	placement	Gross & Han (2015)
<i>Hymenoscyphus ohakune</i>	NR_137109	ICMP:19601	New Zealand	ex-type	placement	Johnston & Park (2013)
<i>Hymenoscyphus pusillus</i>	MH476516	HMC 21525	Poland	ex-type	placement	Kowalski & Bilański (2019)
<i>Hymenoscyphus qinghaiensis</i>	KJ472297	HMAS:264175	China	ex-type	placement	Zheng & Zhuang (2015)
<i>Hymenoscyphus repandus</i>	PRJNA297738 (NCBI genome)	CBS:341.76	Germany		placement	C. Sambles et al. unpubl. data
<i>Hymenoscyphus salicellus</i>	PRJNA297737 (NCBI genome)	CBS:111550	The Netherlands		placement	C. Sambles et al. unpubl. data
<i>Hymenoscyphus scutula</i>	KC481695	CBS:480.97	USA		placement	Hamelin et al. (2013)
	AB926114	TNS:F-17507	Japan		placement	Zhao et al. (2016)
<i>Hymenoscyphus tamaricis</i>	DQ431167	br020	Spain		placement	Baral et al. (2007)
<i>Hymenoscyphus tetrasporus</i>	KJ472302	HMAS:266592	China	ex-type	placement	Zheng & Zhuang (2015)
<i>“Hymenoscyphus” varicosporoides</i>	(JGI genome)	Hymvar1	USA		placement	Grigoriev et al. (2014)
	AB481291	FC-2038	Japan		placement	Hosoya et al. (2010)
<i>Hymenoscyphus waikaia</i>	KC164666	PDD:66379	New Zealand		placement	Johnston & Park (2013)
	KC164667	PDD:102886	New Zealand	ex-type	placement	Johnston & Park (2013)

Table 2. (Continued).

Species	Accession number	Isolate/voucher	Country	Note	Dataset	Reference
<i>Hymenoscyphus yui</i>	KJ472303	HMAS:266595	China	ex-type	placement	Zheng & Zhuang (2015)
<i>Hymenotorrendiella dingleyae</i>	MH578484	ICMP:22793	New Zealand		placement	P.R. Johnston & D. Park unpubl. data
<i>Hymenotorrendiella eucalypti</i>	MH578483	ICMP:22792	New Zealand	type species	placement	P.R. Johnston & D. Park unpubl. data
<i>Mycofalcella calcarata</i>	KC834065	CCM F-10289	UK	ex-type	placement	Baschien <i>et al.</i> (2013)
<i>Phaeohelotium confusum</i>	MH921873	PDD:112244	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
	MH578507	PDD:111536	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
	MH578519	PDD:111537	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
<i>Phaeohelotium confusum</i> [as <i>Discinella confusa</i>]	OL653012	PDD:112680	New Zealand		genus, placement	J.A. Cooper & P.R. Johnston unpubl. data
<i>Phaeohelotium confusum</i> [as <i>Discinella terrestris</i>]	GU222294	PDD:89062	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
<i>“Phaeohelotium” epiphyllum</i>	AB926061	TNS:F-40042	Japan		placement	Zhao <i>et al.</i> (2016)
	KT876976	H.B. 9911	Germany		placement	H.-O. Baral & G. Marson unpubl. data
<i>Phaeohelotium geogenum</i>	MF908475	UBC:F33074	Canada		genus	M.L. Berbee <i>et al.</i> unpubl. data
	KC411992	H.B. 7222A	Germany		genus, placement	Baral <i>et al.</i> (2013)
<i>Phaeohelotium flavum</i> [as <i>monticola</i>]	KC411991	H.B. 8612	Germany	type species	genus, placement	Baral <i>et al.</i> (2013)
<i>Phaeohelotium maiusaurantium</i>	OP868960	FH 00284876	Chile		genus, placement	This paper
	KY462393	FH 00284882	Chile		genus	Truong <i>et al.</i> (2017a)
	OP868951	FLAS-F-65476	Chile		genus, placement	This paper
	OP868961	FLAS-F-65460	Chile		genus, placement	This paper
	OP868953	FLAS-F-65526	Chile	ex-type	genus, placement	This paper
	OP868958	FH 00284867	Chile		genus, placement	This paper
	OP868959	FH 00284861	Chile		placement	This paper
<i>Phaeohelotium pallidum</i>	OP868956	FLAS-F-65411	Chile		genus, placement	This paper
	KY462415	FLAS-F-63072	Chile	ex-type	genus, placement	This paper
	OP868955	FLAS-F-65413	Chile		genus, placement	This paper
	OP868954	MES-3308	Chile		genus, placement	This paper
	OP868950	FLAS-F-66003	Chile		genus, placement	This paper
	OP868952	FLAS-F-66004	Chile		genus, placement	This paper
<i>Phaeohelotium pateriforme</i> complex	MK932825	PDD:112176 / ICMP:23087	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
	MK932826	PDD:94748 / ICMP:23370	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
	MK932832	PDD:116634 / ICMP:23365	New Zealand		placement	P.R. Johnston & D. Park unpubl. data
<i>Phaeohelotium</i> sp.	MK932818	ICMP:23361	New Zealand		placement	P.R. Johnston & D. Park unpubl. data
<i>Phaeohelotium</i> sp. 1	OP868962	FLAS-F-65016	Chile		genus, placement	This paper
<i>Phaeohelotium</i> sp. 2	MH930289	FLAS-F-65244	Chile		genus, placement	This paper
	OP868957	FLAS-F-65412	Chile		genus, placement	This paper
<i>Phaeohelotium succineoguttulatum</i>	KC411989	AH7643	Spain		placement	Baral <i>et al.</i> (2013)

Table 2. (Continued).

Species	Accession number	Isolate/voucher	Country	Note	Dataset	Reference
<i>Phaeohelotium tasmanicum</i> complex	MK932819	PDD:111541 / ICMP:23082	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
	MK932821	PDD:116635 / ICMP:23372	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
	MK932823	PDD:111926 / ICMP:23083	New Zealand		genus, placement	P.R. Johnston & D. Park unpubl. data
<i>Phaeohelotium undulatum</i>	KC411988	AH7337	Spain		placement	Baral et al. (2013)
<i>Phaeohelotium cf. undulatum</i>	MK432759	PDD:105499	New Zealand		genus, placement	J.A. Cooper et al. unpubl. data
<i>Pleuroascus nicholsonii</i>	KJ755519	CBS:345.73	USA	ex-type, type species	placement	Malloch et al. (2016)
<i>Roesleria subterranea</i>	EF060298	CBS:407.51	Italy	type species	placement	Kirchmair et al. (2008)
	AB628057	TNS:F-38701	Japan	type species	placement	Y. Degawa et al. unpubl. data
<i>Spirosphaera floriformis</i>	HQ696658	CBS:402.52	The Netherlands	ex-type	placement	Voglmayr et al. (2011)
<i>Tricladium obesum</i>	KC834068	CCM F-14598	Czech Republic	ex-type	placement	Baschien et al. (2013)
<i>Tricladium splendens</i>	AY204635	CCM F-16599	Czech Republic	type species	placement	Baschien et al. (2006)
Uncultured Ascomycota ECM root tip ex <i>Eucalyptus delegatensis</i>	JF960619	clone BH2125R	Australia		genus	Horton (2011)
Uncultured fungus ECM root tip ex <i>Eucalyptus delegatensis</i>	JF960772	clone BH1255R	Australia		genus	Horton (2011)
	JF960769	clone BH3140F	Australia		genus	Horton (2011)
Uncultured fungus ECM root tip ex <i>Eucalyptus regnans</i>	FN298678	H004x_L3399Xaf	Australia		genus, placement	Tedersoo et al. (2009)
Uncultured fungus ECM root tip ex <i>Nothofagus cunninghamii</i>	FN298750	H71_L3609s	Australia		genus, placement	Tedersoo et al. (2009)
	FN298679	H004y_L3677	Australia		genus, placement	Tedersoo et al. (2009)
	FN298677	H004z_L3620	Australia		genus	Tedersoo et al. (2009)
Uncultured fungus ECM root tip ex <i>Pomaderris apetala</i>	FN298689	H010x_L3161b	Australia		genus, placement	Tedersoo et al. (2009)
Uncultured <i>Helotiales</i> ECM root tip ex <i>Eucalyptus delegatensis</i>	JF960780	clone BH3615R	Australia		genus	Horton (2011)
<i>Zalerion varium</i>	AF169303	ATCC:28788	USA	type species	placement	Bill et al. (1999)

Our second ITS dataset included 43 taxa, all within the genus *Phaeohelotium*, and with *Dicephalospora* spp. as outgroup (Table 2). In this analysis, we included uncultured ectomycorrhizal root tip sequences as well as the type species *Ph. flavum* (as *Ph. monticola*), for which a single ITS sequence is available in the National Center for Biotechnology Information (NCBI) GenBank database (acc. no. KC411991, Baral et al. 2013). As above, sequences were aligned using MUSCLE v. 3.7 (Edgar 2004) and trimmed at the conserved motifs *sensu* Dentinger et al. (2011). The selection of nucleotide substitution models with ModelFinder v. 1.6.7 (Kalyaanamoorthy et al. 2017) was done under the Akaike information criterion corrected for small sample size (AICc). Maximum likelihood (ML) was performed under partitioned models using IQ-TREE (Nguyen et al. 2015, Chernomor et al. 2016, Hoang et al. 2017). Visualization of

phylogenetic reconstructions with bootstrap (BS) values was done in FigTree v. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). A link to the final alignments and unedited trees is available here: https://github.com/Quandt-Mycology-Lab/Lab_Codes_and_workflows/tree/master/Phaeohelotium_paper.

RESULTS

Nucleotide alignment datasets and phylogenetic analyses

Placement onto the *Leotiomycetes* v1 tree in T-BAS reveals the position of three of our samples in *Phaeohelotium* in family *Helotiaceae*, in a well-supported clade with *Phaeohelotium geogenum* (Fig. 1). *Phaeohelotium* is retrieved (ML bootstrap =

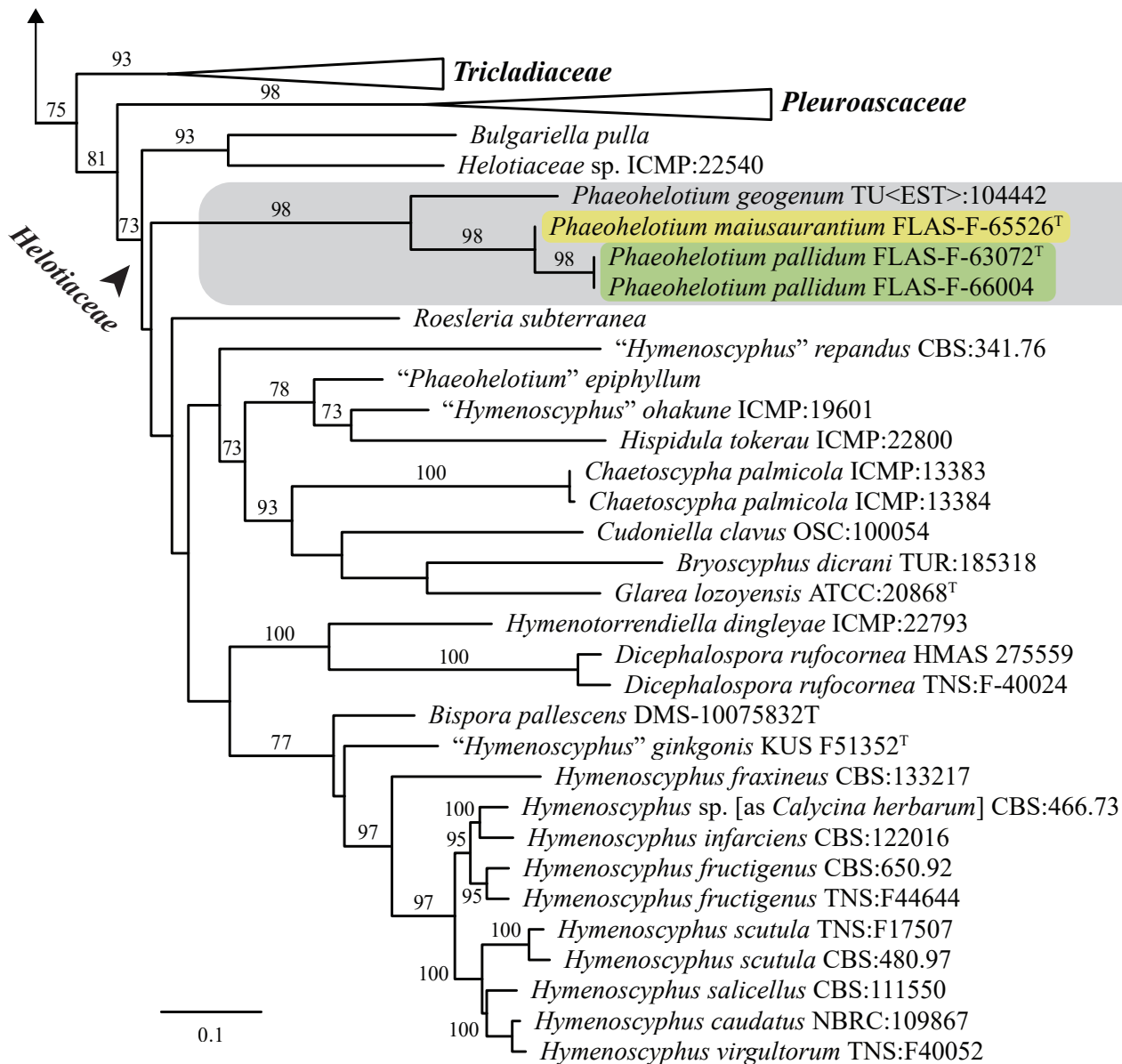


Fig. 1. Excerpt of the best-scoring ML tree ($-\ln L = 685940.513110$), showing the placement of *Phaeohelotium* species in the 15-locus *Leotiomycetes* v1 tree in T-BAS. The topology is the result of a RAxML analysis with 500 bootstrap replicates. For each node, the ML bootstrap support (≥ 70 %) is presented above or below the branch leading to that node. *Phaeohelotium* highlighted in gray, new species highlighted in yellow and green, ^T indicating ex-type sequences.

81 %) among *Pleuroascaceae*, *Bulgariella pulla*, *Roesleriaceae*, and *Helotiaceae*. The relationship of these five clades to one another is unresolved. Our first ITS dataset (Fig. 2) includes 106 isolates and 628 characters, of which 287 are constant and 270 are parsimony-informative. The following models were selected by ModelFinder (AIC): TIM2+F+G4 for ITS1, TIM3e+I+G4 for 5.8S, and SYM+I+G4 for ITS2. The phylogenetic reconstruction of this ITS dataset results in the placement of *Phaeohelotium pallidum* as sister to *Phaeohelotium* sp. 2 with maximum BS support. The clade (*Ph. pallidum*, *Ph. sp. 2*) is retrieved as sister to *Ph. maiusaurantium* (BS = 91 %) (Fig. 2). *Connersia* and *Pleuroascus* are resolved in a clade with maximum support, placed sister to a subclade of *Phaeohelotium* with *Ph. flavum* (type species), *Ph. geogenum*, *Ph. pateriforme* complex, and *Ph. tasmanicum* complex. The *Connersia*–*Pleuroascus*–*Phaeohelotium* clade is sister to a clade with *Hymenoscyphus* isolates belonging the “*Hymenoscyphus*” 1 clade *sensu* Lebeuf *et al.* (2021: fig. 22), although with low support (BS = 69 %).

Our second, genus-level ITS (ITS1 + 5.8S + ITS2) dataset includes 43 isolates and 226 + 158 + 162 characters, of which 113 + 143 + 49 are constant and 77 + 9 + 85 are parsimony-informative (Fig. 3). The following models were selected by ModelFinder (AICc): SYM+G4 for ITS1, TIM3e+I for 5.8S, and TIM2e+G4 for ITS2. The phylogenetic reconstruction of this dataset recovers two intrageneric subclades of *Phaeohelotium* (Fig. 3): the first with saprotrophic taxa *Ph. geogenum*, *Ph. monticola*, *Ph. pateriforme*, and *Ph. tasmanicum*; and the second with *Phaeohelotium* spp. 1 and 2, *Ph. confusum*, *Ph. cf. undulatum*, the two newly described species from Chile *Ph. maiusaurantium* and *Ph. pallidum*, and ECM root tip sequences associated with multiple ECM tree host tree genera across three families (*Eucalyptus* in *Myrtaceae*, *Nothofagus* in *Nothofagaceae*, *Pomaderris* in *Rhamnaceae*). Support for these subclades is moderate to high (BS = 95 % for the putatively saprotrophic subclade, BS = 76 % for the putatively ECM clade). *Phaeohelotium maiusaurantium* is placed as sister to an ECM

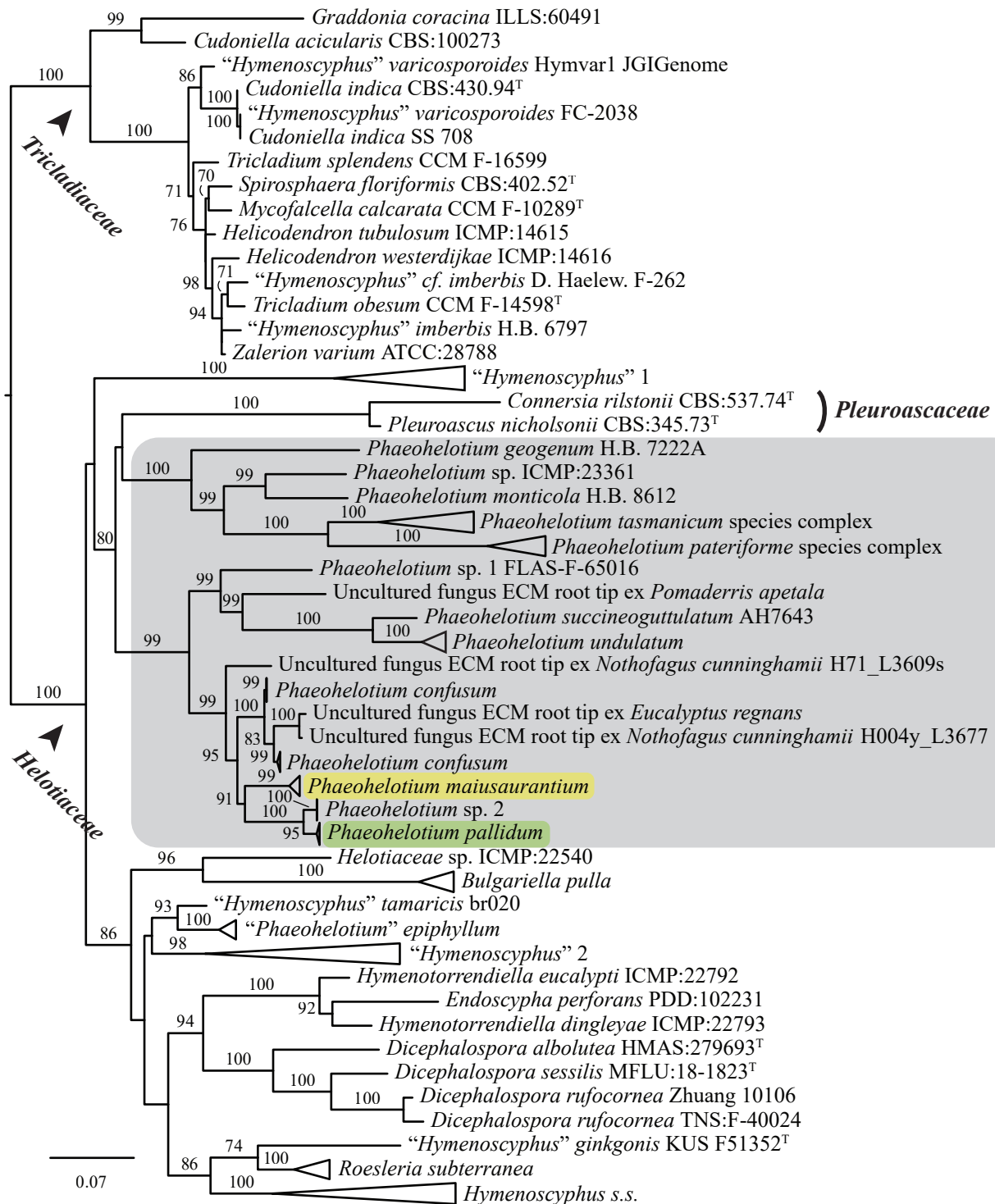


Fig. 2. Phylogeny of *Helotiaceae* and *Tricladaceae* (outgroup), reconstructed from an ITS dataset. The consensus tree topology (-lnL = 8626.682) is the result of ML inference performed with IQ-TREE. For each node, the ML bootstrap support ($\geq 70\%$) is presented above or in front of the branch leading to that node. “*Hymenoscyphus*” 1 and 2 annotations *sensu* Lebeuf et al. (2021: fig. 22), *Phaeohelotium* highlighted in gray, new species highlighted in yellow and green, † indicating ex-type sequences.

root tip sequence associated with *Nothofagus cunninghamii*, whereas *Ph. pallidum* is sister to an undescribed Chilean species, *Phaeohelotium* sp. 2.

Taxonomy

Leotiomycetes O.E. Erikss. & Winka, *Myconet* **1**: 7. 1997.
Helotiales Nannf. ex Korf & Lizoň, *Mycotaxon* **75**: 501. 2000.
Helotiaceae Rehm, *Rabenhorst’s Kryptogamen-Flora, Pilze* -

Ascomyceten **1**: 647. 1886.

Phaeohelotium Kanouse, *Pap. Mich. Acad. Sci.* **20**: 75. 1935.

Type species: Phaeohelotium flavum Kanouse, *Pap. Michigan Acad. Sci.* **20**: 75. 1935.

Synonyms: Helotium monticola Berk., *Grevillea* **4**: 1. 1875.
Phaeohelotium monticola (Berk.) Dennis, *Persoonia* **3**: 54. 1964.
Hymenoscyphus monticola (Berk.) Baral, *Fl. Medit.* **15**: 67. 2005.

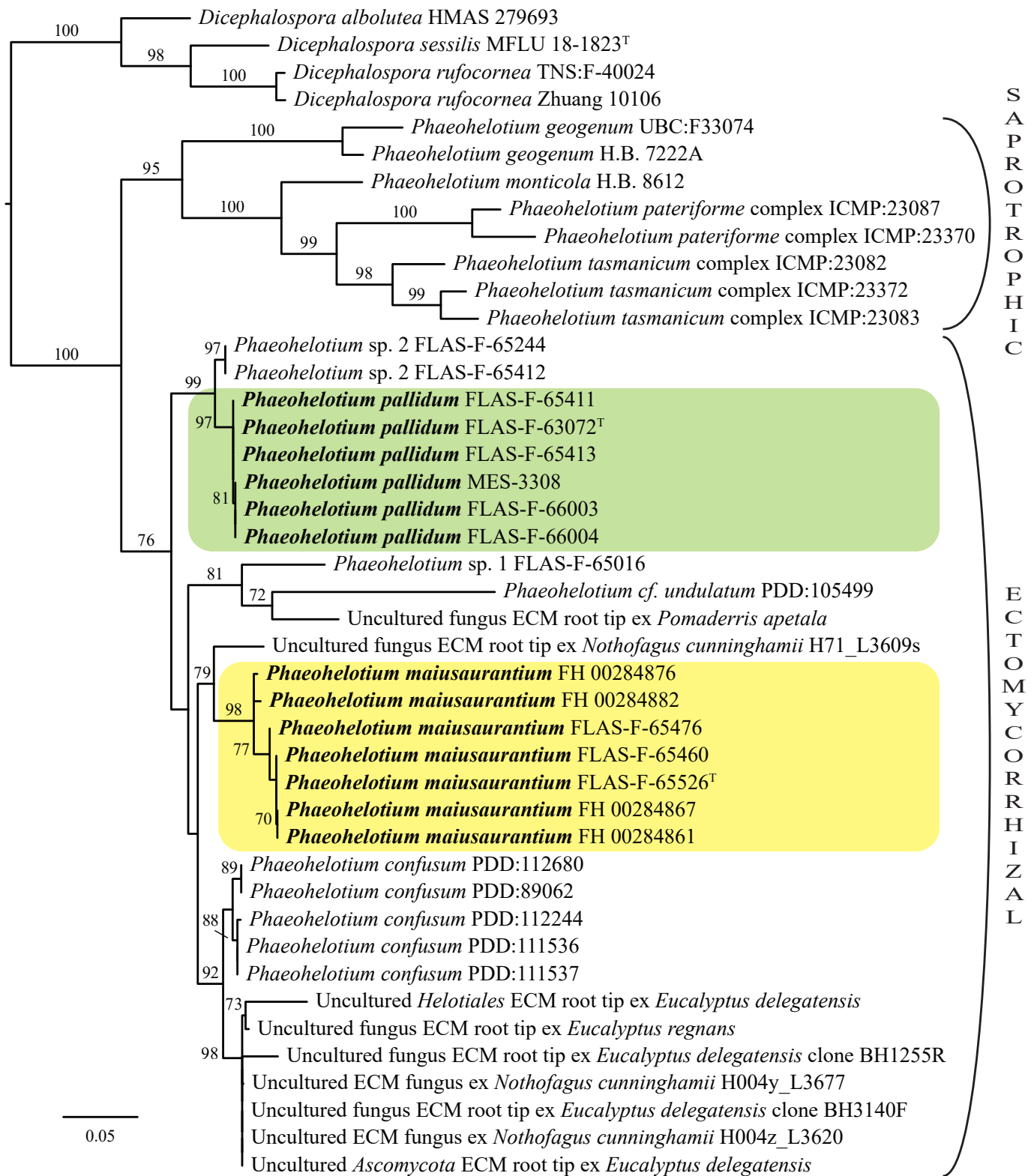


Fig. 3. Phylogeny of *Phaeohelotium* and *Dicephalospora* (outgroup), reconstructed from an ITS dataset. The consensus tree topology (-lnL = -3437.109) is the result of ML inference performed with IQ-TREE. For each node, the ML bootstrap support ($\geq 70\%$) is presented above or in front of the branch leading to that node. ^T indicating ex-type sequences, *Phaeohelotium maiusaurantium* sp. nov. highlighted in yellow, *Ph. pallidum* sp. nov. highlighted in green.

Phaeohelotium maiusaurantium A. Grupe, A. Weier, C.A. Quandt & M.E. Sm., *sp. nov.* Index Fungorum IF 553521. Fig. 4.

Etymology: Referring to the relatively large (maius) orange (aurantium) apothecia.

Diagnosis: Differs from other southern South American species by the color (light orange) and size (3–6 mm diam.) of its apothecia, and the size of its asci (171–208 × 8–10 μm) and ascospores (14–20 × 6–8 μm). Also differs from *Ph. nothofagi* in the amyloidity of its ascus apex, and from *Ph. castaneum* and *Ph. nothofagi* in its ECM lifestyle.

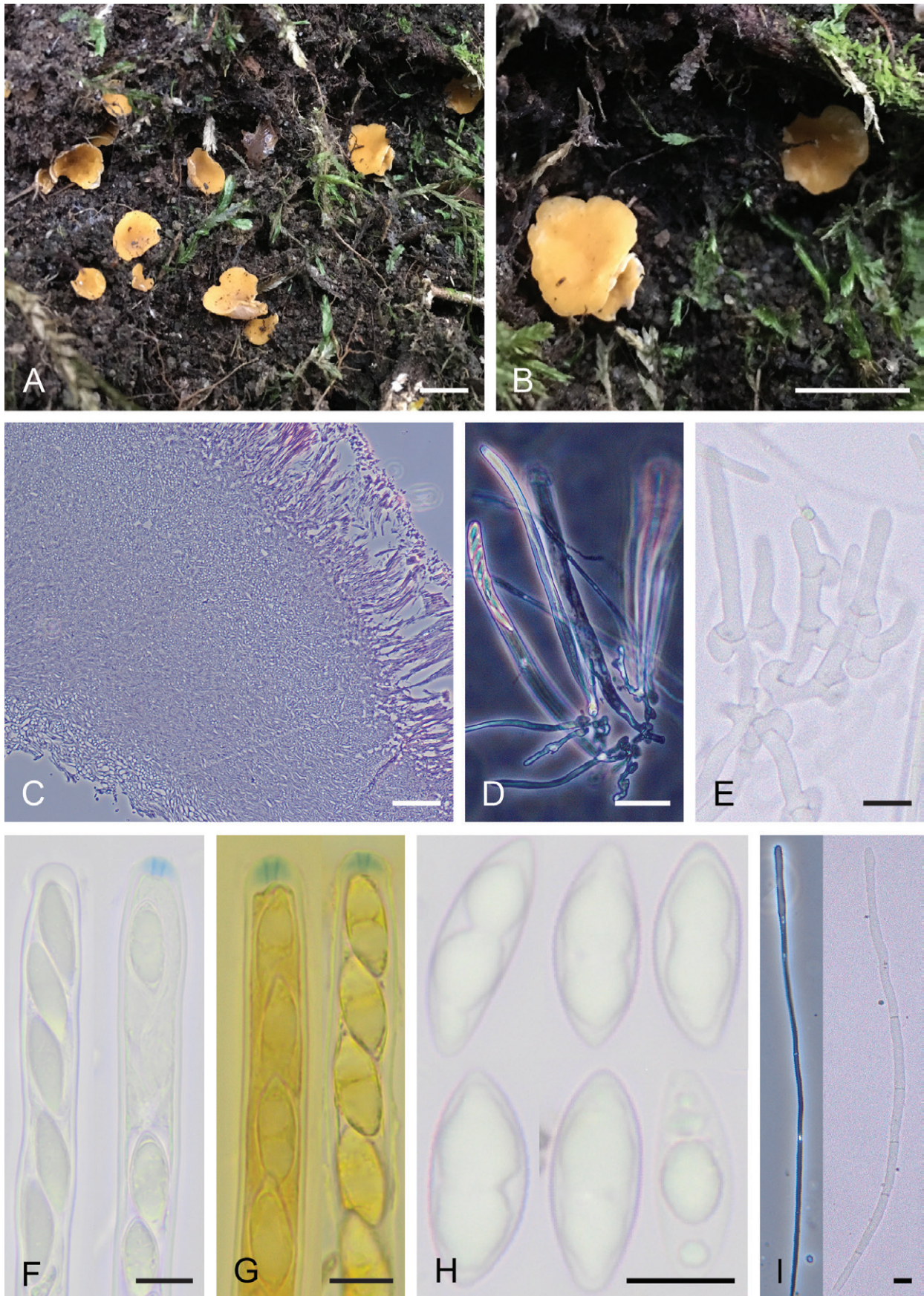


Fig. 4. Morphological characteristics of *Phaeohelotium maiusaurantium*. **A.** Field habit in Vicente Perez Rosales National Park, Chile (FLAS-F-65526, isotype). **B.** Close-up of apothecia (FLAS-F-65526, isotype). **C.** Microtome section, stained with hematoxylin & eosin, of an apothecium showing the mixture of *textura intricata* and loosely interwoven hyphae of the *ectal excipulum* and the *medullary excipulum* (FLAS-F-65476). **D, E.** Developing asci arising from a series of repeating croziers (FLAS-F-65460). **F.** Amyloid ascus apical ring and eight fusiform ascospores (FLAS-F-65460). **G.** Close up of an ascus and the *Hymenoscyphus*-type amyloid ascus apical ring (FLAS-F-65460). **H.** Smooth, fusiform ascospores, each with two large, oil drops that fused in the dead state (FLAS-F-65460). **I.** Multiseptate, filiform paraphysis (FLAS-F-65476). Scale bars: A, B = 5 mm; C = 100 μ m; D = 45 μ m; E–H = 10 μ m, I = 5 μ m.

Typus: Chile, Los Lagos Region, Vicente Perez Rosales National Park, 930 m a.s.l., in *Nothofagus dombeyi* forests, on soil, 18 Apr. 2017, M.E. Smith & R. Healy MES-2976 (**holotype** SGO), (**isotype** FLAS-F-65526). GenBank accession nos. OP951346 (SSU), OP868953 (ITS), OP962216 (LSU).

Description: Apothecia scattered or gregarious in small groups, never fasciculate from a common stipe, 3–6 mm diam. When fresh, initially concave, becoming slightly convex with age, slightly gelatinous consistency, light orange verging on pale orange externally, margin thin, smooth to minutely downy; stipe distinct but buried in substrate, concolorous with the flanks of the apothecia, 1–3 × 1.0 mm. Asci 8-spored with blunt apex, gradually tapering to the base, arising from a series of repeating croziers; (151–)171–208(–223) × 8–10(–12) µm; hyaline in DI water and KOH, amyloid apical ring in Melzer's reagent without KOH pretreatment, of the *Hymenoscyphus*-type, inner part of the ring staining strongly, commonly extending laterally, remaining wall inamyloid. Ascospores fusoid to fusiform with obtuse to acute poles, without ornamentation, containing two large guttules that fuse in the dead state; (13–)14–20(–21) × (5–)6–8(–9) µm; hyaline in DI water and KOH, uncolored in Melzer's reagent. Paraphyses filiform reaching a slightly enlarged rounded apex, generally non-branching, multiseptate, lacking vacuolar bodies in the rehydrated condition; 2–3 µm diam. at apex; hyaline in DI water and KOH, inamyloid in Melzer's reagent. Ectal excipulum of *textura prismatica* at the margins of the hymenium, 74–85 µm thick, a mixture of *textura intricata* and loosely interwoven hyphae below the margins of the disc to the stipe, 97–220 µm thick, cells (6–)8–24(–35) × (3–)6–10(–12) µm, slightly gelatinized. Medullary excipulum of *textura intricata*, 51–416 µm thick, cells 5–11(–19) × (2–)4–8 µm, slightly gelatinized. Subhymenium of *textura intricata*, 38–97 µm thick, cells (4–)6–8(–27) × 3–9 µm, non-gelatinized.

Habitat and distribution: Growing in the Andes mountains of south-central Chile, during the fall. On exposed soil within native *Nothofagus dombeyi* forests.

Additional specimens examined: Chile, Los Lagos Region, Vicente Perez Rosales National Park, 930 m a.s.l., in *Nothofagus dombeyi* forests, on soil, 24 Mar. 2008, D.H. Pfister & M.E. Smith DHP-CH-74 (FH 00284861); *ibid.*, 22 Mar. 2008, D.H. Pfister & M.E. Smith DHP-CH-80 (FH 00284867); *ibid.*, 27 Mar. 2008, D.H. Pfister & M.E. Smith DHP-CH-93 (FH 00284876); *ibid.*, 27 Mar. 2008, D.H. Pfister & M.E. Smith DHP-CH-99 (FH 00284882); *ibid.*, 17 Apr. 2017, R. Healy MES-2905 (FLAS-F-65460); *ibid.*, 17 Apr. 2017, R. Healy & M.E. Smith MES-2925 (FLAS-F-65476).

Notes: *Phaeohelotium maiusaurantium* is morphologically similar to other southern South American species of *Phaeohelotium* (*Ph. castaneum*, *Ph. nothofagi*, *Ph. pallidum* sp. nov. and *Ph. recurvum*). *Phaeohelotium maiusaurantium* can be distinguished morphologically from *Ph. pallidum* by the color of the apothecia (light orange in *Ph. maiusaurantium* vs light gray in *Ph. pallidum*), longer asci (171–208 µm vs 160–175 µm), and longer ascospores (14–20 µm vs 12–15 µm) (Table 3). Both *Ph. maiusaurantium* and *Ph. pallidum* putatively share the same ECM tree host genus (*Nothofagus*). *Phaeohelotium maiusaurantium* can be separated from the other three species (*Ph. castaneum*, *Ph. nothofagi*, *Ph. recurvum*) by substratum and morphological characteristics. *Phaeohelotium castaneum*, *Ph. nothofagi*, and *Ph. recurvum* are saprotrophs and grow directly on fallen leaves, twigs, and rotten wood. Morphologically, *Ph. maiusaurantium* can be differentiated

from *P. castaneum* by its larger apothecia (3–6 mm diam. in *Ph. maiusaurantium* vs 0.5–1.5 mm diam in *P. castaneum*), the color of the apothecia (light orange vs dirty chestnut), its substantially larger asci (171–208 × 8–10 µm vs 63–96 × 4.8–6.4 µm), and larger ascospores (14–20 × 6–8 µm vs 8–12.8 × 3.2–4.8 µm) (Table 3). In comparison to *Ph. nothofagi*, *Ph. maiusaurantium* has light orange-colored apothecia (vs cream or light yellow), and longer and amyloid asci (171–208 µm vs 116–145 µm and inamyloid) (Table 3). Finally, compared to the holotype of *Ph. recurvum*, *Ph. maiusaurantium* has longer asci (171–208 µm vs 115 µm in *Ph. recurvum*) and larger ascospores (14–20 × 6–8 µm vs 10–12.5 × 4–4.5 µm in *Ph. recurvum*) (Table 3). The collections of *Ph. recurvum* from Tierra del Fuego (Gamundí & Romero 1998) have even smaller asci (75–77 × 4.5–5.4 µm) and slightly shorter ascospores (9–10 × 3.6–4.5 µm) compared to the holotype as described by Dennis (1958). Sequence data are needed to discern whether the Argentinian and Tasmanian material of *Ph. recurvum* represents the same species.

Phaeohelotium pallidum A. Grupe, A. Weier, C.A. Quandt & M.E. Sm., **sp. nov.** Index Fungorum IF 553527. Fig. 5.

Etymology: Referring to the pallid color of fresh apothecia.

Diagnosis: Differs from other southern South American species by the color (light gray) and size (2–4 mm diam.) of its apothecia, and the size of its asci (160–175 × 9–11 µm) and ascospores (12–15 × 6–7 µm). Also differs from *Ph. nothofagi* in the amyloidity of its ascus apex, and from *Ph. castaneum* and *Ph. nothofagi* in its ECM lifestyle.

Typus: Chile, Los Lagos Region, Puyehue National Park, ~1 000 m a.s.l., in deep bamboo patches with *Nothofagus dombeyi*, 5 May 2015, R. Healy MES-1068 (**holotype** SGO), (**isotype** FLAS-F-63072). GenBank accession nos. OP951345 (SSU), KY462415 (ITS), OP962212 (LSU).

Description: Apothecia scattered or gregarious in small groups, never fasciculate from a common stipe, 2–4 mm diam when fresh, initially concave, becoming convex with age, slightly gelatinous consistency, light gray, margin thin, smooth to minutely downy; stipe distinct but buried in substrate, concolorous with the flanks of the apothecia, 1–2 × 0.6 mm. Asci 8-spored with blunt apex, gradually tapering to the base, arising from a series of repeating croziers; (153–)160–175(–185) × 9–11(–12) µm; hyaline in DI water and KOH, amyloid apical ring in Melzer's reagent without KOH pretreatment, of the *Hymenoscyphus*-type, inner part of the ring staining strongly, commonly extending laterally, remaining wall inamyloid. Ascospores fusiform with obtuse to acute poles, unornamented, containing two large guttules that fuse in the dead state; 12–15 × 6–7(–8) µm; hyaline in DI water and KOH, uncolored in Melzer's reagent. Paraphyses filiform reaching a slightly enlarged rounded apex, generally non-branching, septate, lacking vacuolar bodies in rehydrated condition; 2–3 µm diam at apex; hyaline in DI water and KOH, inamyloid in Melzer's reagent. Ectal excipulum a mixture of *textura prismatica* at the margins of the disc, 24–67 µm thick, cells 5–23(–31) × 4–9 µm, a mixture of *textura intricata* and loosely interwoven hyphae below the flanks of the hymenium to the stipe, 65–180 µm thick, slightly gelatinized. Medullary excipulum of *textura intricata*, 87–275 µm thick, cells 6–24 × 5–9(–16) µm, slightly gelatinized. Subhymenium of *textura intricata*, 51–73 µm thick, cells (4–)6–23 × (3–)5–9(–14) µm, non-gelatinized.

Table 3. Comparative morphology of species in the genus *Phaeohelotium*.

Species	Ascus length × width (µm)	Ascospore length × width (µm)	Amyloid apex	Substrate and location	Reference	Sequences in GenBank
<i>asiaticum</i>	90–100 × 10–13	18–24(–26) × 5–6	Amyloid	On decorticated branchlet, Japan	Korf (1959)	No
<i>baileyianum</i> ¹	(140–)160–210(–225) × (8–)9–10(–12)	(12–)14–20(–23) × (5.5–)6–8(–9)	Amyloid	Under <i>Eucalyptus</i> , Australia (Tasmania)	Baral et al. (2013)	No
<i>carneum</i>	80–90 × 6–7	12–15 × 3.5–5	Amyloid	On twigs and wood debris submerged in water, Britain and Ireland and Switzerland	Miśkiewicz (2000)	No
<i>carpinicola</i>	85–105 × 10–12	12–16 × 4–5	Amyloid	With <i>Carpinus betulus</i> and <i>Vaccinium myrtillus</i> , Germany	Arendholz 1979	No
<i>castaneum</i>	63–96 × 4.8–6.4	8–12.8 × 3.2–4.8	Amyloid	On a submerged twig, Argentina	Gamundí (1962)	No
<i>confusum</i>	(140–)160–210(–225) × 8–9.5(–10)	(11.5–)13–17(–19) × (5.5–)6–7(–7.5)	Amyloid	On <i>Nothofagus</i> litter or on soil under <i>Nothofagus</i> , New Zealand	Baral et al. (2013)	Yes
<i>conicola</i>	85–90 × 12–15	15–19 × 4–6.5	Amyloid	On wood of a spruce cone, Czech Republic	Svrček (1984)	No
<i>flavum</i> ²	100–125 × 8–9	14–16 × 4–5	Amyloid	On a decaying log, USA	Kanouse (1935)	No
<i>fluviatile</i>	80–100 × 6–9	7.5–10 × 3.5–4.5	Amyloid	On fallen log of <i>Abies</i> sp. in a stream, India	Raitviir & Sharma (1984)	No
<i>fulvidulum</i>	120–140 × 10–12	14–20 × 3–4	Amyloid	Rotten leaves of grasses or sedges, France	Boudier (1907)	No
<i>geogenum</i>	125–150 × 10–12	25–30 × 4.5–5	Amyloid	On moist, heavy soil, thick fallen oak branches, decorticated wood, Switzerland	Svrček & Matheis (1979)	Yes
<i>hylocomii</i>	50–60 × 6–6.5	9–11 × 3.5–4	Amyloid	<i>Hylocomium splendens</i> stems, Estonia	Leenurm et al. (2000)	No
<i>lilacinum</i>	125 × 10–12	14–20 × 5–8	Amyloid	On wet rotten wood, UK	Dennis (1981)	No
<i>luteum</i>	120–140 × 9–11	(16–)18–22(–24) × (4–)5–6(–8)	N/A	On unknown substrate in subtropical rainforests, Brazil	Dennis (1960)	No
<i>maiusaurantium</i>	(151–)171–208(–223) × 8–10(–12)	(13–)14–20(–21) × (5–)6–8(–9)	Amyloid	On soil in <i>Nothofagus dombeyi</i> forests, Chile	This paper	Yes
<i>melleoflavum</i>	50–55 × 4.5–5.5	7–9 × 1.5–2	Amyloid	On decorticated branch of <i>Populus nigra</i> , Czech Republic	Svrček (1992)	No
<i>monticola</i> ²	95–100 × 7–10	12–18 × 4–5	Amyloid	On decorticated wood, USA	Dennis (1964)	Yes
<i>nobile</i>	120–150 × 8–12	8–15	Inamyloid	On wet rotting branches of Oak, Czech Republic	Velenovský (1934)	No
<i>nothofagi</i>	116–145 × 8.5–10	(14.5–)18.5–20.5(–21) × (4–)5–6	Inamyloid	On fallen leaves of <i>Nothofagus dombeyi</i> , Argentina	Gamundí & Messuti (2006)	No
<i>pallidelilacinum</i>	95–125 × 8–12	(7.5–)8.5–12.5 × 4–5	Amyloid	On rotten wood of <i>Pinus mugo</i> , Slovakia	Svrček (1992)	No
<i>pallidum</i>	(153–)160–175(–185) × 9–11(–12)	12–15 × 6–7(–8)	Amyloid	On soil with <i>Nothofagus dombeyi</i> , Chile	This paper	Yes
<i>pani</i>	95–100 × 6–7	8–12 × 3–3.5	Inamyloid	On a decaying <i>Populus</i> sp. twig, Czech Republic	Svrček (1984)	No
<i>pateriforme</i>	170–180 × 9–10	23–28 × 4–5	Amyloid	On decorticated wood and dead sticks, Australia (Tasmania)	Dennis (1958)	Yes
<i>purpureum</i>	40–50 × 4.5–5	5–8 × 1	Inamyloid	On dried up branches of <i>Pinus sylvestris</i> , UK	Dennis (1974)	No
<i>readeri</i>	135–150 × (8–)9–11(–12)	(11–)13–16(–18) × 6.8–8.3	Inamyloid	N/A	Baral et al. (2013)	No

Table 3. (Continued).

Species	Ascus length × width (µm)	Ascospore length × width (µm)	Amyloid apex	Substrate and location	Reference	Sequences in GenBank
<i>recurvum</i>	115 × 9	10–12.5 × 4–4.5	Amyloid	On moss and rotten wood, Australia (Tasmania)	Dennis (1958)	No
<i>rubropurpurascens</i>	50–60 × 4–6	7–10 × 2.5–4	Amyloid	On leaves, Czech Republic	Svrček (1976)	No
<i>samaricola</i>	80–100 × 7–9	8–13 × 3–4	Amyloid	On fallen <i>Acer pseudoplatanus</i> leaves, Czech Republic	Svrček (1983)	No
<i>subcarneum</i>	80–90 × 6–7	12–15 × 3.5–5	Amyloid	On twigs and wood debris submerged in water, Britain and Ireland and Switzerland	Miśkiewicz (2000)	No
<i>succineoguttulatum</i>	160–250 × (9–)10–11(–12)	(12–)14–20(–24) × (6.2–)7–9.5(–10)	Inamyloid	On soil under <i>Eucalyptus globulus</i> , Spain	Baral <i>et al.</i> (2013)	Yes
<i>tasmanicum</i>	N/A	11–18 × 3–4.5	N/A	On dead wood, Australia (Tasmania)	Rodway (1925)	Yes
<i>terrestre</i>	100–110 × 9–12	12–15.5 × 4–5	Inamyloid	On loamy damp ground, Czech Republic	Svrček (1987)	No
<i>undulatum</i>	160–250 × (9–)10–11(–12)	(12–)14–20(–24) × (6.2–)7–9.5(–10)	Amyloid	On soil under <i>Eucalyptus globulus</i> , Spain	Baral <i>et al.</i> (2013)	Yes

¹*Phaeohelotium baileyianum* was introduced by Baral *et al.* (2013) as a new name for *Discinella terrestris*.

²*Phaeohelotium monticola* was synonymized with the type species *Ph. flavum* (Dennis 1964, Dumont 1981: 354). Nevertheless, both names are presented here, with their characteristics as reported by Dennis (1964) and Kanouse (1935), respectively.

Habitat and distribution: Growing in the coastal forests and Andes mountains of south-central Chile in the fall and spring seasons. On exposed soil, typically under leaf litter in mature *Nothofagus dombeyi* forests.

Additional specimens examined: Chile, Los Lagos Region, Puyehue National Park, 930 m a.s.l., on soil beneath *Nothofagus dombeyi*, 14 Apr. 2017, C. Truong MES-2850 (FLAS-F-65411); *ibid.*, 14 Apr. 2017, C. Truong MES-2852 (FLAS-F-65413); Los Rios, Parque Nacional Alerce Costero, on soil beneath *Nothofagus alpina* and *N. dombeyi*, 13 Oct. 2019, M. Caiafa & M.E. Smith MES-3573 (FLAS-F-66003); *ibid.*, 13 Oct. 2019, P. Sandoval MES-3574 (FLAS-F-66004); Los Rios, Villarica National Park, 10 May 2019, M. Caiafa & M.E. Smith MES-3308 (specimen destroyed during fieldwork, only photos and sequences available).

Notes: Like *Ph. maiusaurantium*, *Ph. pallidum* can be distinguished from previously recorded southern South American species of *Phaeohelotium* (*Ph. castaneum*, *Ph. nothofagi*, *Ph. recurvum*) based on substratum (ECM vs saprotrophic) and morphological characteristics. *Phaeohelotium pallidum* differs morphologically from *Ph. castaneum* in apothecial color (light gray in *Ph. pallidum* vs dirty chestnut in *Ph. castaneum*), ascus size (160–175 × 9–11 µm in *Ph. pallidum* vs 63–96 × 4.8–6.4 µm in *Ph. castaneum*), and ascospore size (12–15 × 6–7 µm in *Ph. pallidum* vs 8–12.8 × 3.2–4.8 µm in *Ph. castaneum*). Compared to *Ph. nothofagi*, *Ph. pallidum* has light gray apothecia (vs cream or pale yellow in *Ph. nothofagi*), and larger and amyloid asci (160–175 × 9–11 µm in *Ph. pallidum* vs 116–145 × 8.5–10 µm and inamyloid in *Ph. nothofagi*) (Table 3). Finally, compared to the holotype of *Ph. recurvum*, *Ph. pallidum* has longer asci (160–175 µm vs 115 µm in *Ph. recurvum*) and larger ascospores (12–15 × 6–7 µm vs 10–12.5 × 4–4.5 µm in *Ph. recurvum*) (Table 3).

Phaeohelotium pallidum is phylogenetically most closely related to an undescribed taxon, *Phaeohelotium* sp. 2 (Fig. 2). The phylogenetically most closely related described species to

both *Ph. pallidum* and *Ph. maiusaurantium* is *Ph. confusum* (Figs 2, 3). However, *Ph. pallidum* and *Ph. confusum* only share 92.11–95.64 % identity, and *Ph. maiusaurantium* and *Ph. confusum* share 91.83–95.02 % identity. In addition, *Ph. confusum* is exclusively found under *Nothofagus* in New Zealand (Baral *et al.* 2013). For *Ph. baileyianum* (synonym *Discinella terrestris*), which is morphologically similar to *Ph. confusum*, no sequence data are currently available. *Phaeohelotium maiusaurantium* and *Ph. pallidum* differ from *Ph. baileyianum* in their always hyaline ascospores (vs often brown in *Ph. baileyianum*). In addition, *Ph. baileyianum* occurs under *Eucalyptus* with records in Australia and Tasmania (Baral *et al.* 2013).

Phaeohelotium pateriforme (Cooke) P.R. Johnst., **comb. nov.** Index Fungorum IF 553544.

Basionym: *Helotium pateriforme* Cooke [as “*pateraeforme*”], *Grevillea* **11**: 102. 1883.

Synonyms: *Peziza pateriformis* Berk., in Hooker, *Bot. Antarct. Voy., III, Fl. Tasmania* **2**: 276. 1859. *Nom. illegit.*, Art. 53.1, non *Peziza pateriformis* Durieu & Lév., *Ann. Sci. Nat. Bot. (sér. 3)* **9**: 140. 1848.]

Calycina pateriformis (Berk.) Kuntze, *Revis. gen. pl.* (Leipzig) **3**: 448. 1898.

Phaeohelotium tasmanicum (Rodway) P.R. Johnst., **comb. nov.** Index Fungorum IF 553546.

Basionym: *Helotium tasmanicum* Rodway, *Pap. & Proc. Roy. Soc. Tasmania* **1920**: 155. 1921.

Notes: These two new combinations are made based on phylogenetic evidence. Johnston (2019) presented an ITS phylogeny incorporating *Phaeohelotium* isolates from Australia and New Zealand. Ten isolates of *Helotium pateriforme* and five isolates of *Helotium tasmanicum* were placed within the genus *Phaeohelotium*, with maximum support. Our ITS phylogenies,

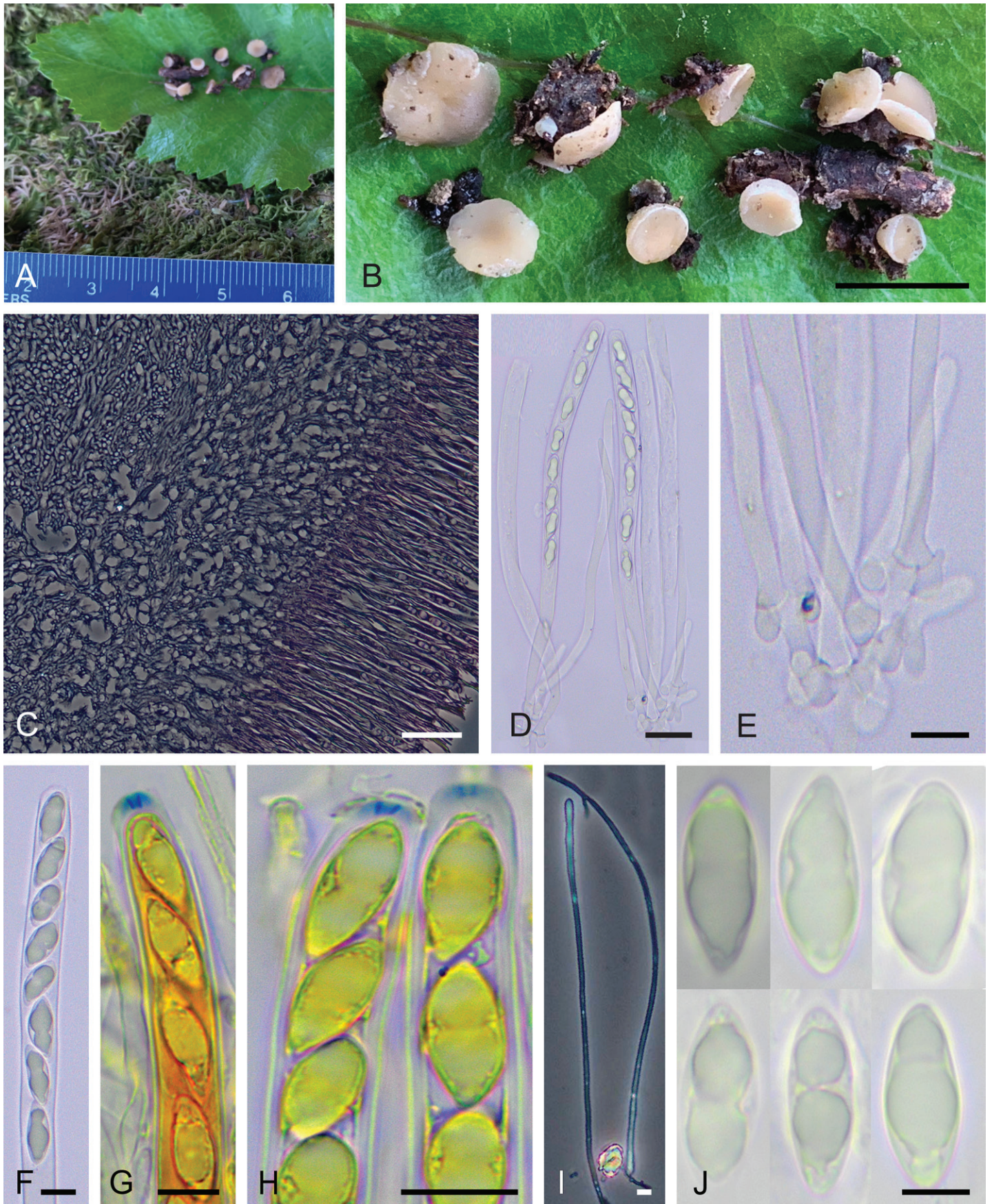


Fig. 5. Morphological characteristics of *Phaeohelotium pallidum*. **A.** Collection of apothecia from Puyehue National Park, Chile (FLAS-F-66004). **B.** Close-up of apothecia (FLAS-F-66004). **C.** Microtome section, stained with hematoxylin & eosin, of an apothecium showing organization of the subhymenium and medullary excipulum layers (FLAS-F-63072, isotype). **D.** Developing ascus arising from a series of repeating croziers (FLAS-F-63072, isotype). **E.** Close-up of the series of repeating croziers (FLAS-F-63072, isotype). **F.** A single ascus with eight fusiform ascospores (FLAS-F-63072, isotype). **G, H.** Close-up of the amyloid apical ascus ring (FLAS-F-63072, isotype). **I.** Two multiseptated, filiform paraphyses (FLAS-F-63072, isotype). **J.** Smooth, fusiform ascospores, each with two large, oil drops that fused in the dead state (FLAS-F-63072, isotype). Scale bars: B = 5 mm; C = 50 μ m; D = 20 μ m; E–H = 10 μ m; I–J = 5 μ m.

incorporating additional *Phaeohelotium* isolates and species compared to Johnston (2019), found the same result: *Helotium pateriforme* and *H. tasmanicum* are retrieved within *Phaeohelotium* with maximum support, positioned closely to the type species, *Ph. flavum*, as part of the saprotrophic subclade (Figs 2, 3). These species are also collected on wood and both taxa are to be referred to as species complexes as per Johnston (2019).

Additional materials examined

Phaeohelotium sp. 1

Specimens examined: Chile, Magallanes, Magallanes National Park, *Nothofagus pumilio* forest, on soil under moss layer, 3 Apr. 2017, A. Mujic MES-2358 (FLAS-F-65016).

Phaeohelotium sp. 2

Specimen examined: Chile, Los Lagos, Puyehue National Park, *Podocarpus nuyigena* forest with *Nothofagus dombeyi*, on bare soil and heavily decayed wood, 3 Apr. 2017, C. Truong MES-2851 (FLAS-F-65412); *ibid.*, 10 Apr. 2017, C. Truong MES-2650 (FLAS-F-65244).

DISCUSSION

Our phylogenetic analyses show that *Phaeohelotium* in the sense of Baral *et al.* (2013) is a polyphyletic genus (Figs 1, 2). *Phaeohelotium epiphyllum* and *Ph. tamaricis* are consistently phylogenetically distinct from the other members of the genus including the type species *Ph. flavum* (Dennis 1958, 1964, Baral *et al.* 2013). In addition, in our 15-locus phylogeny, *Ph. flavum* is phylogenetically distinct from all other *Phaeohelotium* sequences (unedited tree available through GitHub) but this is likely due to lack of sequence data; only a single ITS sequence is available for *Ph. flavum* whereas for the other *Phaeohelotium* isolates sequences of three to five loci are included in this phylogeny.

Here we reveal for the first time the likely divergence in ecological strategy (ECM vs saprotrophic) within the genus *Phaeohelotium*. Our ITS phylogenies (Figs 2, 3) show two supported subclades. One of these lineages is comprised of taxa that are only collected from decaying wood and litter, while the second one includes species that are collected from the soil in forests with ECM plants as well as sequences directly from ECM root tips. This suggests genetic divergence between the two guilds. It might be that the ECM subclade represents a different genus altogether, although currently we do not have enough data to make such a taxonomic decision. To resolve this, more species need to be incorporated in phylogenetic analyses to confirm or reject this hypothesis. In addition, sequences of more loci need to be generated to decipher the position of *Phaeohelotium* with regard to the closely related *Connersia* and *Pleuroascus* (Fig. 2, Johnston 2019, Lebeuf *et al.* 2021: fig. 22), and careful morphological study of original materials may unlock more taxonomically valuable characteristics. We advocate for further molecular investigation of *Phaeohelotium sensu lato* by sampling more taxa and sequencing more loci to test the monophyly of this genus.

The type species of the genus, *Ph. flavum*, was collected from decorticated wood in Michigan, USA (Kanouse 1935,

Dennis 1964). Baral *et al.* (2013) used an ITS sequence from a German specimen of *Ph. flavum* [as *Ph. monticola*], collected from a *Fagus sylvatica* trunk. *Phaeohelotium maiusaurantium* and *Ph. pallidum* from Chile, *Ph. succineoguttulatum* and *Ph. undulatum* from Spain, and *Ph. confusum* (= *Discinella confusa*) from New Zealand all grow directly on soil, and the ITS sequences from isolates form a well-supported subclade with ECM root tip sequences (Figs 2, 3). The ectomycorrhizal guild can be assigned to closely related species at low taxonomic levels (such as genus) (Tedersoo *et al.* 2010, Zanne *et al.* 2020) and ECM clades rarely or never revert to saprotrophy because they typically lose important carbohydrate-degrading enzymes upon the transition to the ECM lifestyle (Tedersoo & Smith 2013, Kohler *et al.* 2015). These patterns further support the inference that the taxa in this subclade form ECM associations. The other *Phaeohelotium* subclade (*Ph. flavum*, *Ph. geogenum*, *Ph. pateriforme* species complex, *Ph. tasmanicum* species complex) exhibits high to maximum support, and these species have an apparent saprotrophic lifestyle having been collected from fallen decomposing wood, rotting litter, and seeds and cupules (Dennis 1958, 1964, Baral *et al.* 2013, Johnston 2019). In addition to their trophic differences, species in these subclades also differ in their ascus apex morphology and excipular anatomy. As noted by Johnston (2019), species in the subclade of saprotrophs possess weakly amyloid pores, with two lines restricted to the inner half of the ascus wall. This characteristic differentiates them from species in the putatively ECM subclade, where the amyloid reaction of the pore is present throughout the ascus wall or restricted to the outer portion of the wall. These two subclades are also morphologically separated based on the excipular cell walls, which are gelatinized in the ECM subclade and non-gelatinized among the saprotrophic taxa.

Whether these subclades differ in ultimate ascospore coloration is uncertain based on our current sampling. Most species in the genus have ascospores that are hyaline, darkening with age (Kanouse 1954, Dennis 1981, Johnston 2019). Dennis (1981) suggested that this is “probably not an essential character.” Indeed, species seem to vary in the timing of darkening as well as in whether darkening occurs at all (Gamundí & Messuti 2006, Johnston 2019, this paper). In *Ph. confusum*, *Ph. luteum*, *Ph. maiusaurantium*, *Ph. pallidum*, and *Ph. recurvum*, brown ascospores have never been observed (Gamundí & Messuti 2006, Baral *et al.* 2013, this paper). *Phaeohelotium baileyanaum*, on the other hand, has brown ascospores prior to release from the ascus (Johnston 2019). Based on formal descriptions of taxa and their inferred trophic modes, we hypothesize that species with permanently hyaline ascospores and those with ascospores browning with age occur in both the ECM (Baral *et al.* 2013, this paper) and the saprotrophic subclades (Dennis 1981, Gamundí *et al.* 2004, Gamundí & Messuti 2006).

The recognition of *Ph. maiusaurantium* and *Ph. pallidum* as two new species from Chile and the two new combinations (*Ph. pateriforme*, *Ph. tasmanicum*) brings the total number of formally described species in the genus to 32 (Index Fungorum 2022, Table 3). However, sequence data are available for only nine described species within the genus. Note that *Hymenoscyphus epiphyllum* (synonym *Ph. epiphyllum*) and *Hymenoscyphus tamaricis* (synonym *Ph. tamaricis*) are not considered members of the genus based on molecular phylogenetic data (Figs 1, 2). We also generated sequences for two undescribed species of *Phaeohelotium*, but not enough material was available to warrant formal description as per the guidelines by Aime *et al.* (2021). The ecological strategy of

Ph. maiusaurantium and *Ph. pallidum* may be ECM; both species grow directly on soil, they are closely related to ECM root tip sequences from New Zealand and Tasmania, and preliminary data revealed *Phaeohelotium*-like sequences from South American ECM root tip amplicon data (A. Mujic, pers. comm.). If confirmed, this would be another example of independent evolution of the ECM lifestyle from non-mycorrhizal ancestors (Tedersoo & Smith 2013). Fieldwork should be directed to collect ascomata of *Phaeohelotium* and root tip samples under likely candidate tree associates in both Patagonia and Australasia. Given the significant ecological and genetic changes involved in switching from a free-living saprotrophic state to an ECM symbiont, this may warrant the erection of a new genus to accommodate the *Phaeohelotium* ECM subclade.

With regard to the biogeography of the ECM subclade, that ECM root tip samples collected in Australasia are retrieved in a clade with Chilean and Australasian collections is a phylogenetic signal of their shared sympatric ancestry from part of the Gondwana supercontinent. Similarly, Truong *et al.* (2017b) found that *Amanita* species in southern South America were grouped with relatives in Australia in a 34.5-M-yr-old clade, corresponding to the fragmentation of South American, Australian, and Antarctic Plates. The discovery of *Ph. succineoguttulatum* and *Ph. undulatum* in Spain associated with *Eucalyptus* is certainly due to the exportation of *Eucalyptus* species to the Northern Hemisphere for silviculture. The introduction of non-native ECM fungi has been documented in other lineages (Giachini *et al.* 2000, Díez 2005, Hynson *et al.* 2013, Hayward *et al.* 2015, Kraisitudomsook *et al.* 2019). If the *Phaeohelotium* ECM subclade indeed evolved from a saprotrophic lineage, then the presence of the ECM species *Ph. maiusaurantium* and *Ph. pallidum* in Chile and the saprotrophic species *Ph. castaneum* and *Ph. nothofagi* in Argentina hints at travel of the ECM lineage potentially migrating along the southern edge of Gondwanaland. This would be possible given the fossil record of *Nothofagus* trees in Antarctica and the extant members of *Nothofagus* in Chile, Australia, and New Zealand (Linder & Crisp 1995, Heads 2006). In other *Helotiales* lineages with a similar geographic pattern, such as *Cyttaria*, it is theorized that co-migration with host plants, long-distance dispersal events, or vicariance are responsible for the current observed distribution (Johnston 2006, Peterson *et al.* 2010). Whether the geographic pattern we observe in the putatively ECM subclade is due to a combination of these factors remains an outstanding question for these species of *Phaeohelotium*. We note that there is 100 % shared identity between an ITS sequence of *Phaeohelotium* sp. from New Zealand (ICMP:23361) included in our tree and that of a collection from Chile (DHP-CH-11B, GenBank acc. no. KY462387), perhaps providing further evidence for the Chilean–Australasian link.

From what we know about *Phaeohelotium* thus far, saprotrophic taxa occur in both the Northern and Southern Hemispheres. We do not know, however, where they are most species-rich. It does appear that all ECM species are restricted to the Southern Hemisphere, except for the Spanish species that are documented with introduced *Eucalyptus* (Baral *et al.* 2013), as pointed out above. This suggests that the ECM lifestyle in *Phaeohelotium* may have evolved in the Southern Hemisphere. This phenomenon of unique taxa that evolved the ECM symbiosis with plants in the Southern Hemisphere is known in two genera of *Basidiomycota*, *Austropaxillus* (Skrede *et al.* 2011) and *Descolea* (Kuhar *et al.* 2017). However, the ECM

lineage within *Phaeohelotium* may be the first known group of *Ascomycota* to have evolved the ECM symbiosis in the Southern Hemisphere (Tedersoo & Smith 2013).

With the formal description of *Ph. maiusaurantium* and *Ph. pallidum*, we add to the growing recognition of high undescribed diversity in *Leotiomycetes*. As previously acknowledged (*e.g.*, Truong *et al.* 2017a, Cazabonne *et al.* 2022), continued fieldwork will prove important to accumulate fresh *Phaeohelotium* collections for sequencing and deposition in fungaria. This will undoubtedly result in the discovery of more diversity in the genus and help to determine whether a new genus should be recognized to accommodate species in the ECM subclade. In addition, we hope that future work will elucidate the origins of the switch to the ECM lifestyle within *Phaeohelotium* through utilizing comparative genomics between species with different trophic modes.

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Data availability statement: All final alignments and unedited trees are available through GitHub: https://github.com/Quandt-Mycology-Lab/Lab_Codes_and_workflows/tree/master/Phaeohelotium_paper.

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