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Re-examination of the Southern Hemisphere truffle genus *Amylascus* (*Pezizaceae, Ascomycota*) and characterization of the sister genus *Nothoamylascus* gen. nov.

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Abstract *Amylascus* is a genus of ectomycorrhizal truffles within *Pezizaceae* that is known from Australia and contains only two described species, *A. herbertianus* and *A. tasmanicus*. Species of *Amylascus* are closely related to truffles (*Pachyphlodes*, *Luteoamylascus*) and cup fungi (*Plicariella*) from the Northern Hemisphere. Here we reevaluate the species diversity of *Amylascus* and related taxa from southern South America and Australia based on new morphological and molecular data. We identify previously undocumented diversity and morphological variability in ascospore color, ascospore ornamentation, hymenial construction, epithecium structure and the amyloid reaction of the ascus in Melzer's reagent. We redescribe two *Amylascus* species from Australia and describe seven new *Amylascus* species, five from South America and two from Australia. This is the first report of *Amylascus* species from South America. We also describe the new South American genus *Nothoamylascus* as sister lineage to the *Pachyphlodes-Amylascus-Luteoamylascus* clade (including *Amylascus*, *Luteoamylascus*, *Pachyphlodes*, and *Plicariella*). We obtained ITS sequences of mitotic spore mats from *Nothoamylascus erubescens* gen. & sp. nov. and four of the seven newly described *Amylascus* species, providing the first evidence of mitotic spore mats in *Amylascus*. Additional ITS sequences from mitotic spore mats reveal the presence of nine additional undescribed *Amylascus* and one *Nothoamylascus* species that do not correspond to any sampled ascomata. We also identify three additional undescribed *Amylascus* species based on environmental sequences from the feces of two ground-dwelling bird species from Chile, *Scelorchilus rubecula* and *Pteroptochos tarnii*. Our results indicate that ascomata from *Amylascus* and *Nothoamylascus* species are rarely collected, but molecular data from ectomycorrhizal roots and mitotic spore mats indicate that these species are probably common and widespread in southern South America. Finally, we present a time-calibrated phylogeny that is consistent with a late Gondwanan distribution. The time since the most recent common ancestor of: 1) the family *Pezizaceae* had a mean of 276 Ma (217–337 HPD); 2) the *Amylascus-Pachyphlodes-Nothoamylascus-Luteoamylascus* clade had a mean of 79 Ma (60–100 HPD); and 3) the *Amylascus-Pachyphlodes* clade had a mean of 50 Ma (38–62 HPD). The crown age of *Pachyphlodes* had a mean of 39 Ma (25–42 HPD) and *Amylascus* had a mean age of 28 Ma (20–37 HPD), falling near the Eocene-Oligocene boundary and the onset of the Antarctic glaciation (c. 35 Ma).

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INTRODUCTION

Amylascus was described from Australia based on *Hydnobolites herbertianus* (Cribb 1957). Trappe (1971) transferred *H. herbertianus* to his newly proposed genus *Amylascus* as *A. herbertianus* and designated it as the type species. Trappe later transferred a second Australian species, *Terfezia tasmanicus* (Rodway 1926), to *Amylascus* based on morphological similarities between the two species (Trappe 1975). The genus name refers to the amyloid reaction of asci in Melzer's reagent. These truffle fruiting bodies are characterized by ascomata with a basal tuft of hyphae, a peridium (excipulum) of large cells (~100 µm diam), a gleba with meandering veins composed of an epithecium similar in cellular construction to the excipulum, asci randomly distributed in the gleba, and hyaline to yellowish globose ornamented ascospores. *Amylascus herbertianus* has spiny ascospores and is easily distinguished from *A. tasmanicus*, in which the ascospores and spines are obscured by a perispore. The perispore of *A. tasmanicus* is similar to those in some species of *Pachyphlodes* in Corda (1854) in which a thin layer of material, similar in composition to the spines, develops and spreads from the spine tips to form

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a covering (perispore) over the ascospore, leaving spine-length space between the perispore and the ascospore wall. Hence, in this lineage of fungi, the perispore is part of the ascospore ornamentation, and not a membrane or gelatinous sheath (Healy et al. 2018). Due to the prominent basal tuft of hyphae and the excipular and epithecial structures that are similar to *Genea* species, Trappe (1975) originally considered *Amylascus* within the family *Geneaceae* (= *Pyronemataceae*). However, molecular phylogenetic studies have since shown that *Amylascus* is a member of *Pezizaceae*, and closely related to the Northern Hemisphere ectomycorrhizal genera *Pachyphlodes*, *Plicariella* (= *Scabropezia*) and *Luteoamylascus* (Læssøe & Hansen 2007, Cabero et al. 2016). Australian *Amylascus* species have been collected in forests dominated by *Eucalyptus* spp. and other ectomycorrhizal *Myrtaceae* and are assumed to form ectomycorrhizas (Tedersoo et al. 2010). ITS sequences from ectomycorrhizal root tips collected in South American *Nothofagaceae* forests showed high phylogenetic similarity to sequences from Australian *Amylascus* specimens, suggesting that *Amylascus* may have a wider geographic and host range than previously documented. Data from ectomycorrhizal root tips also showed that *Amylascus* species were among the most detected ectomycorrhizal taxa in South American *Nothofagus* forests, and that some of the fungi in this group can form mycorrhizae with multiple species of *Nothofagaceae* (Nouhra et al. 2013 as *Pachyphloeus* spp., Truong et al. 2017).

Studies of asexual mitotic spore mats of *Gyromitra*, *Hydnobolites*, *Morchella*, *Pachyphlodes*, *Peziza* s.lat., *Plicariella*, *Ruhlandiella* and *Tuber* showed that integrating DNA sequences from mitotic spore mats broadly expands the known range of many taxa and often reveals the presence of previously undescribed taxa (Urban et al. 2004, Healy et al. 2013, Carris et al. 2015, Kraisitudomsook et al. 2019, Pfister et al. 2022a, b). Accordingly, we included DNA sequences from mitotic spore mats as well as ascomata of *Amylascus* to more fully assess the diversity and distribution of these and related taxa.

Here we provide the first revisionary systematic study of *Amylascus* in over 40 years. This is based on recent and historical collections, morphological assessments, and multi-locus phylogenetic reconstructions. Our goal was to generate a resolved phylogeny for Southern Hemisphere *Amylascus* species and to determine their relationship to Northern Hemisphere species of the genera *Pachyphlodes*, *Plicariella* and *Luteoamylascus.* We integrate these data to estimate species and clade divergence times to better understand the historical biogeographic patterns of these truffle fungi, and to reassess species hypotheses and the phylogeographic structuring of this clade. We also integrate environmental sequence data from mitotic spore mat collections, ectomycorrhizal root surveys, and bird feces microbiome analysis to improve our understanding of the diversity, distribution, and ecology of these fungi. We hypothesized that we would be able to resolve new taxa through multi-locus phylogenetics, which would support morphological characteristics. We also hypothesized that divergence time estimations would uncover deep phylogenetic splits that were consistent with plate tectonic movement and support the hypothesis that *Amylascus* has an ancient temperate Gondwanan origin. We expected that the inclusion of environmental sequence data would expand both the known diversity of *Amylascus* species and the range of species, as has been found in other groups of fungi (Bonito et al. 2010, Healy et al. 2013, Kraisitudomsook et al. 2019).

MATERIALS AND METHODS

Field collections and fungarium specimens

Forty-nine ascomata and mitotic spore mats of *Amylascus* and related taxa were collected in *Nothofagaceae* forests of Patagonia (Chile and Argentina) during expeditions in 2001, 2008, and 2015–2022. Eight ascomata from Australia were collected during expeditions in 1988, 1992, and 1996 or studied from the collections of the New York Botanical Garden (NYBG), Royal Botanic Gardens Victoria (MEL), the Tasmanian Museum and Art Gallery (HO), and Oregon State University (OSC). Fresh material was collected by searching on the soil surface or under leaf litter and soil with the aid of a hand rake. All collections were stored in plastic boxes to be photographed and processed within 12 h. Clean dried material from fresh or fungarium collections was stored into Cetyl Trimethyl Ammonium Bromide lysis buffer (CTAB) (Gardes & Bruns 1993) or an alkaline extraction solution (Vandepol et al. 2020) for DNA extraction. Voucher specimens were dried over low heat, stored with silica gel in plastic bags, and accessioned into the Herbario del Museo Botánico de Córdoba (CORD), the Florida Museum of Natural History of the University of Florida (FLAS), the Museo Nacional de Historia Natural de Chile (SGO), and Oregon State University (OSC).

Morphological definitions and analyses

Hand sections of dried voucher specimens were mounted in deionized water, 3 % KOH, or Melzer's reagent, and viewed with a Zeiss Axio Imager A2 compound microscope (Carl Zeiss, Oberkochen, Germany). Bright-field and differential interference contrast (DIC) images were captured with an Axiocam 305 camera using Zen Pro v. 3.1 software (Carl Zeiss, Oberkochen, Germany). In some cases multiple images were stacked using Helicon Focus v. 8.0.4 Pro (Helicon Soft Ltd 2000, Kharkiv, Ukraine).

Specimens were prepared for scanning electron microscopy (SEM) following the methods of Healy et al. (2018). Scanning electron micrographs were captured with a Hitachi S3500N scanning electron microscope (Hitachi High-Technologies in America, Schaumburg, IL, USA) at the University Imaging Center, University of Minnesota (St. Paul, MN, USA) at 10 kV. Spores were examined with light microscopy and dimensions ($n \geq 20$) were measured in tap water whereas all other features (asci, excipulum, paraphyses, glebal hyphae, basal hyphae) were measured in 3 % KOH to re-inflate these structures, which remain collapsed or folded in water mounts. Measurements were performed on CZI images with Zen Pro v. 3.1. Spore measurements excluded ornamentation and extreme measurements (unusually small or unusually large values) are shown in parentheses outside the reported size ranges. All microscopy images were taken from material mounted in 3 % KOH with bright-field settings unless otherwise noted. For some images, brightness and contrast were improved and background debris removed using Photoshop CS5 v. 12.1 (Adobe Systems Incorporated, San Jose, CA, USA).

Definitions for morphological terminology are provided below with references:

- Ectal excipulum – the outer layer of tissue of an ascomycete ascocarp, part of the peridium (Korf 1973).
- Epithecium – the sterile tissue that results from an overgrowth of branching paraphyses above the hymenium in a truffle. This layer acts as a covering over the asci. The cellular structure of the epithecium may range from parallel hyphae to a *textura intricata*, to a *textura angularis,* to a *textura angularis* intergraded with a *textura globulosa*, similar to that of the ectal excipulum (Korf 1973).
- Gleba – the inner tissue of a truffle. Pezizales truffle glebal tissues consist of asci, ascogenous tissue, sterile tissue, and may include canals or other open spaces, distinct from the excipulum (Trappe et al. 2009).
- Hymenium – the layer of asci and paraphyses in a palisade (Korf 1973).

– Medullary excipulum – the layer of peridial tissue subtending the ectal excipulum but morphologically distinct from it, consisting of cells that are smaller, and/or differently shaped than those of the ectal excipulum (Korf 1973).
– Mitotic spore mat – masses of hyphae with mitotic spores that develop on soil or woodland debris. Also known as anamorphs or asexual spore mats (Healy et al. 2013).
– Oleiferous hyphae – hyphae with lipid content that is visible during microscopy, distinguished by their refractive appearance and rich yellow to orange color, sometimes referred to as gloeoplerous hyphae in other fungal groups (Montecchi & Sarasini 2000).
– Perispore – a thin layer of material, similar in composition to the spines on the ascospore, which covers the spine tips to form a covering over the ascospore, leaving spine-length space between the perispore and the ascospore wall. The perispore may entirely or partially surround the ascospore (Healy et al. 2018).
– Ptychothecium – a truffle (e.g., an enclosed ascocarp that is typically hypogeous) that has hollow or stuffed canals (spaces above the fertile tissue filled with parallel to loosely interwoven hyphae) in the gleba (Weber et al. 1997).
– *Textura angularis* – a type of fungal tissue composed of angular, more-or-less isodiametric cells that are tightly packed, forming no intercellular spaces (Korf 1973).
– *Textura globulosa* – a type of fungal tissue composed of rounded cells that are spherical to oblong, forming intercellular spaces (Korf 1973).
– *Textura intricata* – a type of fungal tissue composed of interwoven hyphae (Korf 1973).

DNA extraction, amplification and sequencing

DNA extraction from specimens stored in 2 % CTAB was performed using a modified CTAB extraction method (Gardes & Bruns 1993). This method was used to obtain single copy genes (*EF1*α, *rpb1*, *rpb2*). To obtain ribosomal genes from scant materials such as mitotic spore mats, rapid extractions were performed from material stored in alkaline extraction buffer following the methods of Vandepol et al. (2020). The ITS1-5.8S-ITS2 (ITS) region of nrDNA (ITS) was amplified with primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990); the 28S (LSU) region of nrDNA with primers LROR (Hopple Jr. & Vilgalys 1994) and LR5 (Vilgalys & Hester 1990); translation elongation factor 1-α (*EF1*α) with primers 983f and 2218r (Rehner & Buckley 2005); the largest subunit of RNA polymerase (*rpb1*) with primers Af and Cr (Matheny et al. 2002); and the second largest subunit of RNA polymerase (*rpb2*) with primers P5f, Pb7f (forward) and P7r, bRPB2-7r2, and P11Ar (reverse) (Hansen et al. 2005, Liu et al. 1999). Published primers failed to amplify the *rpb2* for some species. To create primers, *Pachyphlodes rpb2* sequences were aligned with *Pezizales* sequences downloaded from GenBank and the software package Primer3 (Untergasser et al. 2007) was used to select primers that were optimized to amplify species of *Pachyphlodes*. Pachy-3f (5' AATACGAACCCTCAAGGT 3') anneals approximately 100 nucleotides (nt) downstream from the RPB2-5f priming site and Pachy-3r (5' CAAGTGTGCGATCGTCATAC 3') anneals approximately 250 nt upstream of the RPB2-11Ar priming site (Liu et al. 1999). Amplicons of the Pachy-3f and Pachy-3r primer pair are c. 1400 nt. Ribosomal DNA regions (ITS and LSU) were amplified with Taq DNA Polymerase (New England Biolabs Inc., Ipswich, Massachusetts) and single-copy genes (*EF1*α, *rpb1*, and *rpb2)* with Phusion Hot Start Flex DNA Polymerase (New England Biolabs Inc.), Hotstar high fidelity Taq kit (Qiagen, Venlo, Netherlands), or REdExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St. Louis, USA) following manufacturer recommendations. Thermocycler conditions followed those of Hansen et al. (2005). Successful amplification was detected by gel electrophoresis of 1.5 % agarose gels stained with SYBR Green I (Molecular Probes, Eugene, Oregon). Amplicons were cleaned with EXO (Exonuclease I) and SAP (shrimp alkaline phosphatase) enzymes (Werle et al. 1994). Sanger sequencing was performed by Genewiz (South Plainfield, NJ, USA) or Eurofins Genomics (Louisville, KY, USA). Chromatograms were manually checked for quality, edited where necessary, and low-quality ends trimmed in Geneious Pro v. 5.6.7 (Drummond et al. 2012).

Phylogenetic analyses

To assemble datasets, we downloaded sequences based on publications that included sequences from *Amylascus* and related species (Cabero et al. 2016, Caiafa et al. 2021, Hansen et al. 2001, 2005, Nouhra et al. 2013). Our newly generated sequences of ITS, 28S, *EF1*α, *rpb1* and *rpb2* were used as queries for BLAST searches (NCBI) to find any additional similar sequences to include in our analyses. Datasets for each locus were assembled individually and aligned in MAFFT v. 7.471 (Katoh & Toh 2010). Alignments were manually improved in SeAL v. 2.0a11 (Rambaut 2007). Maximum likelihood (ML) analyses were performed for each individual locus with RAxML-HPC2 v. 8.2.12 (Stamatakis 2014) using the GTRCAT substitution model with 1 000 bootstrap replicates. The resulting best tree for each alignment was visualized in FigTree v. 1.2.4 (Rambaut 2009) and checked for any supported incongruence. Since many ITS sequences were available from environmental sources (e.g., ECM root tips, mitotic spore mats, bird fecal samples), we analyzed the ITS region separately from the other loci. Statistical support for ML was considered when bootstrap values were ≥ 70 %. The 28S, *EF1α*, *rpb1*, and *rpb2* alignments were concatenated by hand and analyzed using both Maximum Likelihood (RAxML, as above) with 1 000 bootstrap replicates and through Bayesian inference (MB) with Mr. Bayes v. 3.2.7 (Huelsenbeck & Ronquist 2001). Models for partitions of the full matrix were chosen as described below.

For Bayesian analysis, PartitionFinder 2 was implemented using the greedy algorithm (Lanfear et al. 2017) to fit the best model of substitution for each partition. Partitions were specified for each nucleotide position of the *rpb1*, *rpb2*, and *EF1*α genes in the concatenated alignment, and for each of the two introns in *rpb1*, two introns in *rpb2*, and three introns in *EF1*α. Models selected for each partition were: 1) GTR+I+G for 28S, *EF1*α, and *rpb2*; 2) GTR+G for *rpb1* position 3; 3) HKY+I+G for *EF1*α introns 1, 2, 3, *rpb1* intron 1, and *rpb2* intron 2; 4) GTR+I for *rpb2* intron 1; 5) HKY for *rpb1* position 1, 2 and; 6) K80+I for *rpb1* intron 2. MrBayes was run in parallel with four Markov Chain Monte Carlo chains running for 20 million generations and trees sampled every 1 000 generations. The first 25 % of generations was discarded as burn-in and stationarity was evaluated based on the standard deviation of split frequency (≥ 0.01) and mixing behavior of the chains in Tracer (Rambaut & Drummond 2007) to ensure that coverage was adequate. Posterior probabilities ≥ 95 % were considered as significant support. Phylogenetic trees were visualized in FigTree v. 1.4.2 and edited in Adobe Illustrator v. 15.1.0 (Adobe Systems, Inc., San Jose, CA). The ITS tree was midpoint rooted, because our intention for the ITS analysis was to delimit species within our focus genera (*Amylascus* and *Nothoamylascus*) using the entire ITS sequence, rather than to infer the direction of evolutionary change. The multi-locus tree was rooted with outgroup sequences from *Ruhlandiella* spp. and *Phylloscypha phyllogena* (*Pezizaceae*) based on results from Hansen et al. (2005). All analyses were run on the Cyberinfrastructure for Phylogenetic Research Science Gateway (CIPRES) v. 3.3 (Miller et al. 2010). Alignments are available on the Open Science Framework (OSF)

Table 1 Taxa included in multi-locus alignment, including the country where collected, collector number and/or herbarium number, whether the source of sequence was an ascoma or spore mat, the GenBank accession number for each locus, and the reference(s) for GenBank accession numbers. Type specimens (holotype or isotype) are in **bold**.

Taxon	Locality	Type/Collector number/ Herbarium of deposit	Lifestage	GenBank accession numbers				Reference
				28S	*EF1α*	*rpb2*	*rpb1*	
Amylascus cineraceus	**Chile**	**MES-1770/FLAS-F-64534**	**ascoma**	**OQ270081**	**OQ191355**	**OQ230425**	**OQ230400**	**Truong et al. (2017), this publication**
Amylascus domingueziae	**Argentina**	**JT26237/CORD4235**	**ascoma**	**KJ775856**	**–**	**OQ230426**	**OQ230401**	**this publication**
Amylascus fuscosporus	**Argentina**	**JT26240/CORD4234**	**ascoma**	**KJ775857**	**OQ191356**	**OQ230423**	**OQ230402**	**this publication**
Amylascus hallingii	**Australia**	**AQ794771/NY1491200**	**ascoma**	**KJ775858**	**OQ191359**	**OQ230422**	**OQ230404**	**this publication**
Amylascus herbertianus	Australia	JT37768/OSC, FLAS-F-69588	ascoma	MT461369	OQ191361	OQ230427	OQ230398	this publication
Amylascus procerus	**Argentina**	**JT26241/CORD4233**	**ascoma**	**OQ270082**	**OQ191357**	**OQ230424**	**OQ230399**	**this publication**
Amylascus tasmanicus	Australia	JT18084/C, OSC, FLAS-F-70963	ascoma	AF335113	–	AY500465	OQ230397	Hansen et al. (2001, 2005), this publication
	Australia	JT18208/OSC, FLAS-F-70964	ascoma	MT461368	OQ191360	–	OQ230396	this publication
Amylascus verus	**Argentina**	**JT26238/CORD4248**	**ascoma**	**JN121353**	**OQ191358**	**OQ230421**	**OQ230403**	**Healy et al. (2013), this publication**
Luteoamylascus aculeatus	**Spain**	**AH43987**	**ascoma**	**KT318376**	**–**	**KT318379**	**–**	**Cabero et al. (2016)**
Nothoamylascus erubescens	Chile	MES-1651/FLAS-F-64664	spore mat	MT461372	OQ191362	OQ230419	OQ230394	this publication
	Argentina	MES-2158/FLAS-F-64773	spore mat	MT461371	OQ191364	OQ230420	–	this publication
	Argentina	MES-574/DUKE	spore mat	JX414175	–	OQ230418	OQ230393	Healy et al. (2013), this publication
	Argentina	**JT26170/CORD**	**ascoma**	**OQ270083**	**OQ191363**	**–**	**–**	**this publication**
Pachyphlodes annagardnerae	USA	RH1034/MIN:925696	spore mat	KJ775836	OQ191354	OQ230428	OQ230410	this publication
Pachyphlodes austro-oregonensis	**USA**	**SOC775/OSC112205**	**ascoma**	**OQ270084**	**OQ191353**	**OQ230430**	**–**	**this publication**
Pachyphlodes carnea	USA	JT12818/OSC	ascoma	KT899976	OQ191337	OQ934081	–	this publication
	USA	JT8005/OSC43593	ascoma	AY500544	OQ191338	AY500466	OQ230415	this publication
Pachyphlodes citrina	Italy	JRWL2197/OSC	ascoma	EU543196	OQ191350	OQ230433	OQ230406	Healy et al. (2009), this publication
	USA	RH1016/FLAS-F-66245	ascoma	OQ270085	OQ191349	OQ230432	OQ230405	this publication
Pachyphlodes conglomerata	Italy	Macchioni1860/OSC	ascoma	EU543194	OQ191340	OQ230440	OQ230417	Healy et al. (2009), this publication
Pachyphlodes ligerica	France	RH1843/FLAS-F-62613	ascoma	MT461375	OQ191351	OQ230431	OQ230407	this publication
Pachyphlodes marronina	USA	RH299/HUH258432	ascoma	EU427549	OQ191341	OQ230443	OQ230413	Healy et al. (2009), this publication
Pachyphlodes nemoralis	**UK**	**FLAS-F-59181**	**ascoma**	**JN121362**	**OQ191342**	**OQ230439**	**OQ230411**	**Healy et al. (2013), this publication**
Pachyphlodes oleifera	Spain	CJ00110601NR08/FLAS-F-66168	ascoma	KJ775854	OQ191339	OQ230441	OQ230416	this publication
Pachyphlodes sp. 20	USA	RH1233/FLAS-F-66250	ascoma	KJ775850	OQ191336	OQ230442	OQ230414	this publication
Pachyphlodes sp. 24	Mexico	JT19617/OSC, FLAS-F-70965	ascoma	KJ775843	OQ191344	–	OQ230412	this publication
	Mexico	ITCV1509	ascoma	OQ270086	OQ191345	OQ230437	–	this publication
Pachyphlodes sp. 36	USA	FLAS-F-60761	spore mat	OQ270087	OQ191345	OQ230438	–	this publication
Pachyphlodes thysellii	USA	JT13182/OSC, FLAS-F-70967	ascoma	EU543197	OQ191343	OQ230435	–	Healy et al. (2009), this publication
	USA	RH1007/FLAS-F-66243	ascoma	JN121369	OQ191347	OQ230436	OQ230409	this publication
Pachyphlodes virescens	Mexico	JT32465/OSC, FLAS-F-70962	ascoma	KJ775824	OQ191348	OQ230434	OQ230408	this publication
Phylloscypha phyllogena	USA	RH1440/MIN:956796	ascoma	MZ018885	OQ923305	OQ934082	OQ934084	this publication
Ruhlandiella berolinensis	Spain	SPN1/FLAS-F-62154	ascoma	MG947627	OQ923303	MH155172	MH156172	Kraisitudomsook et al. (2019)
Ruhlandiella patagonica	Chile	MES-2284/FLAS-F-62148	ascoma	MG947619	OQ923304	MH155166	MH156168	Kraisitudomsook et al. (2019)

using the following link: https://osf.io/hmyr7/?view_only=c6d66636eec4470db2ae2a142b780446.

For species delimitation, the complete ITS region of rDNA (ITS1, 5.8S, ITS2) for *Amylascus* and *Nothoamylascus* sequences were trimmed according to Healy et al. (2013). Sequences were clustered into operational taxonomic units (OTUs) at 97 % similarity with 20 % overlap using the 'dirty data' algorithm in Sequencher v. 5.0.1 (Gene Codes Inc., Ann Arbor, MI). Taxa used for the ITS analysis are listed in Table S1.

Divergence times based on our multi-locus alignment were estimated with BEAST v. 2.5.2 (Bouckaert et al. 2019), with 45 additional taxa belonging to representatives across *Pezizomycetes* retrieved from GenBank (Table S2). Concatenated sequences from each species included at least two of the following loci: 28S, *EF1α*, *rpb1*, and *rpb2* sequences. Ambiguously aligned regions were excluded in GBlocks using the least stringent settings (Castresana 2000, Talavera & Castresana 2007). We used a log-normal-distributed clock model, the pure-birth Yule model as tree prior, and selected the most appropriate substitution model (transition/transversion splits) with bModelTest implemented within BEAST. A uniform prior was used for the birthRate and a gamma distribution (alpha = 1, beta = 0.001) for the ucldMean parameters. We used three secondary calibration points with a normal distribution and SD = 1 Ma that were estimated from primary fossil data in Beimforde et al. (2014): 1) the time to the most recent common ancestor (tMRCA) of *Pezizomycetes* (mean = 414 Ma); 2) the tMRCA of *Helvella-*

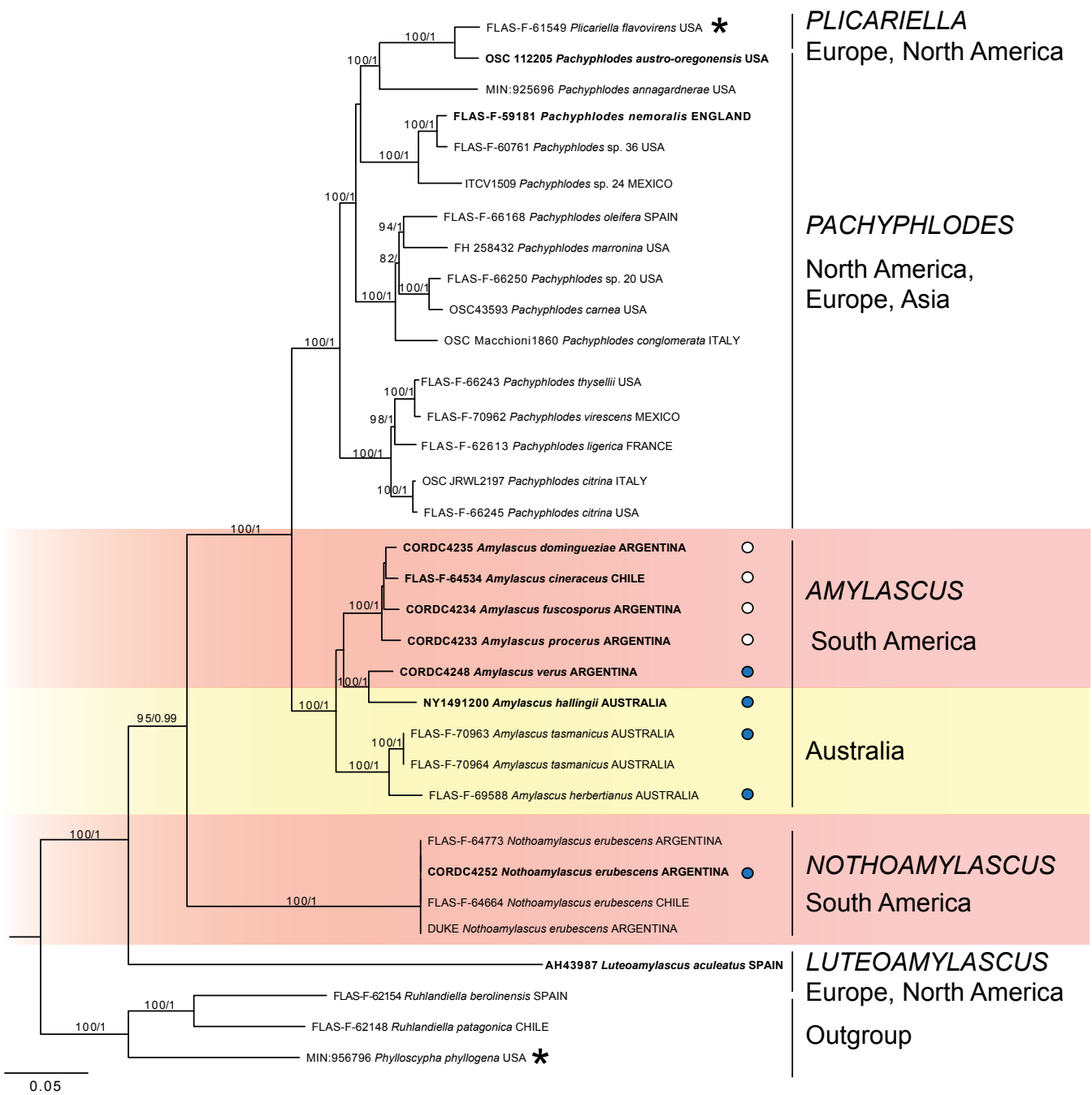

Fig. 1 Best ML phylogram from a RAxML analysis of a concatenated dataset of 28S rDNA, *EF1α*, *rpb1*, and *rpb2* sequences. Branch supports show bootstrap values ≥ 70 % on top, and posterior probability ≥ 0.95 on bottom. Phylogenetic tree rooted with *Ruhlandiella* and *Phylloscypha* (*Pezizaceae*) as outgroup taxa. Text at the terminals provides fungarium accession number, scientific binomial, and country of origin for each collection. Bolded text indicates type specimens. Taxa highlighted in orange are from South America whereas taxa highlighted in yellow are from Australia. Asterisks (*) indicate cupulate, operculate taxa whereas all others are hypogeous taxa without operculate asci. Amyloid reaction of asci indicated by circles to right of boxed species; unfilled circles indicate inamyloid asci whereas blue filled circles indicate amyloid asci.

ceae-Discinaceae-Morchellaceae (mean = 154 Ma); and 3) the tMRCA of *Pyronemataceae* (mean = 79 Ma). We ran four parallel MCMC runs of 100 million generations, sampling every 10 000th state. Runs were evaluated for convergence and chain mixing in Tracer v. 1.7.2 (Rambaut et al. 2018) and combined with Log-Combiner, removing a 10 % burnin, and resampling every 40 000th state. The final tree file included 9 000 states, which were processed with TreeAnnotator, estimating node heights using the 'ca' (common ancestor) method (Heled & Bouckaert 2013), and summarized posterior node data to a maximum-clade-credibility (MCC) tree.

RESULTS

A total of 61 ITS, 20 LSU, 30 *EF1*α, 27 *rpb1*, and 27 *rpb2* sequences were newly generated in this study. Table 1, S1, S2 list GenBank accession numbers as well as relevant voucher information, including country of origin and whether sequences were derived from ascomata, mitotic spore mats, ectomycorrhizal root tips, or environmental samples from bird feces. The ITS dataset consisted of 74 sequences with 687 nucleotide positions (Table S1). The multi-locus phylogenetic analysis consisted of the 28S locus (33 sequences, 827 nucleotide positions), the *EF1*α locus (27 sequences, 1 087 nucleotide positions including three introns), the *rpb1* locus (24 sequences, 758 nucleotide positions including two introns), and the *rpb2* locus (29 sequences, 1 483 nucleotide positions including two introns). For each locus, *A. herbertianus* and *A. tasmanicus* were supported as belonging together in the same clade, *A. cineraceus*, *A. domingueziae*, *A. fuscosporus*, and *A. procerus* were supported as belonging together in the same clade, and *A. hallingii* and *A. verus* were supported as belonging together in the same clade. However, these three clades were inconsistent with respect to their relationships with each other among the four different loci, and these relationships were therefore unresolved. There were no other supported incongruences among the phylogenies from the four different loci (28S, *rpb1*, *rpb2*, *EF1*α) (Fig. S1). The

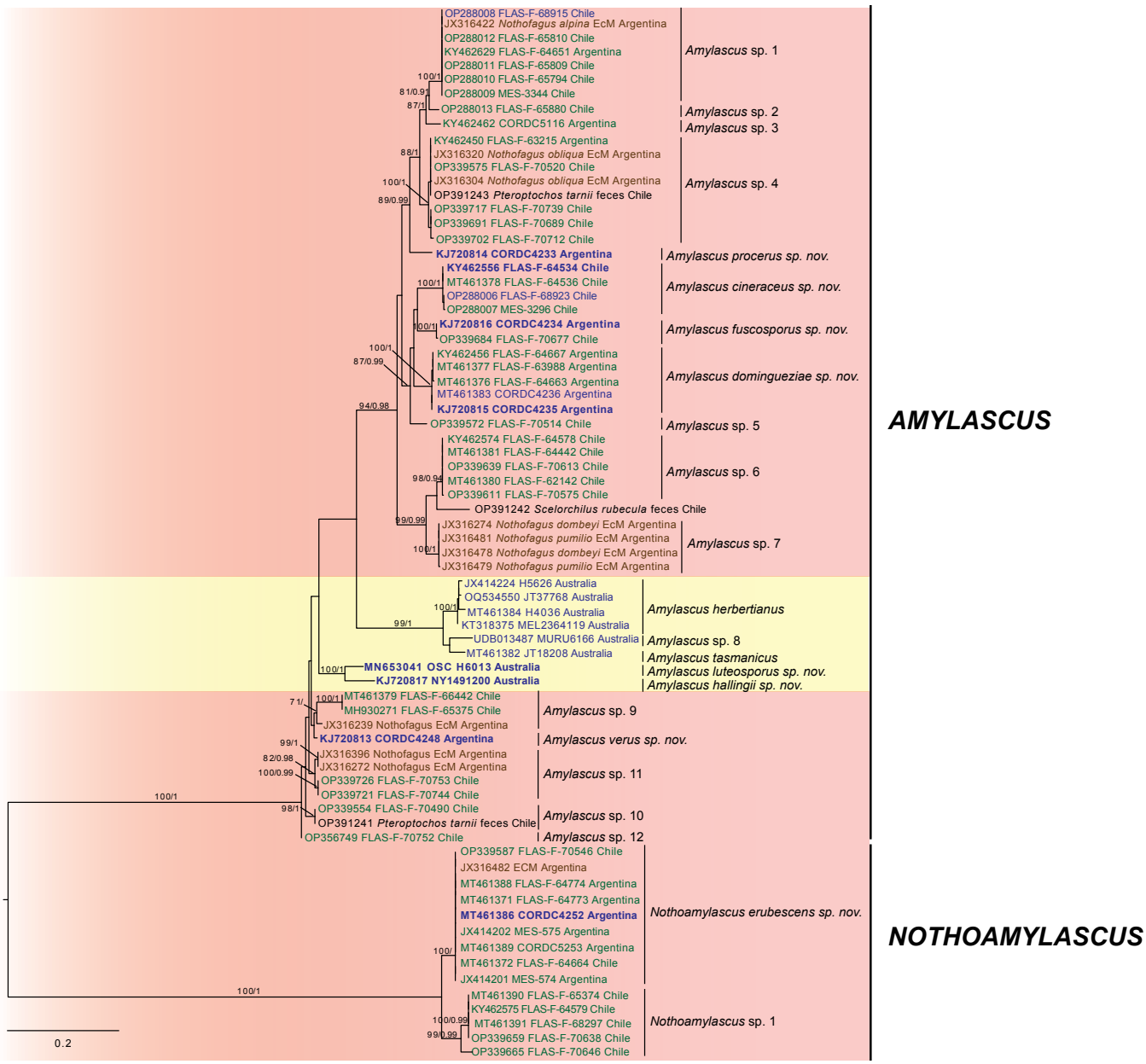

Fig. 2 Best ML phylogram from a RAxML analysis of ITS sequences. Branch supports show bootstrap values ≥ 70 % on top, and posterior probability ≥ 0.95 on bottom. The phylogenetic tree was midpoint rooted. Text at the terminals provides Genbank accession number, fungarium accession number, and country of origin for each collection. Scientific binomials or undescribed species hypotheses are listed to the right of the delimitation lines. Bolded text indicates type collections. Taxa highlighted in orange are from South America whereas taxa highlighted in yellow are from Australia. Text colors indicate the type of material sequences were obtained from: blue from ascomata, green from mitotic spore mats, brown from ectomycorrhizal root tips, and black from bird feces (*Pteroptochos tarnii* or *Scelorchilus rubecula*).

concatenated multi-locus alignment contained 4 058 nucleotide positions. The resulting best ML tree (Fig. 1, Likelihood score = -30084.105324) showed that *Amylascus* species form a monophyletic and well-supported clade with species from Australia and South America. It also showed that *Amylascus* is sister to *Pachyphlodes-Plicariella*, a clade known only from the Northern Hemisphere. Note that *Plicariella* is placed within *Pachyphlodes*, making *Pachyphlodes* non-monophyletic. We intend to combine *Plicariella* into *Pachyphlodes* in a forthcoming phylogenetic treatment of *Pachyphlodes* and will not further discuss the species of *Plicariella* as separate from *Pachyphlodes* here.

A previously unknown lineage with species exclusively from South America, described below as a new genus, *Nothoamylascus*, is well supported as sister to the *Pachyphlodes-Amylascus* clade. The northern temperate *Luteoamylascus* lineage is well-supported as sister to all of the other taxa (*Pachyphlodes-Amylascus-Nothoamylascus*), albeit on a long branch.

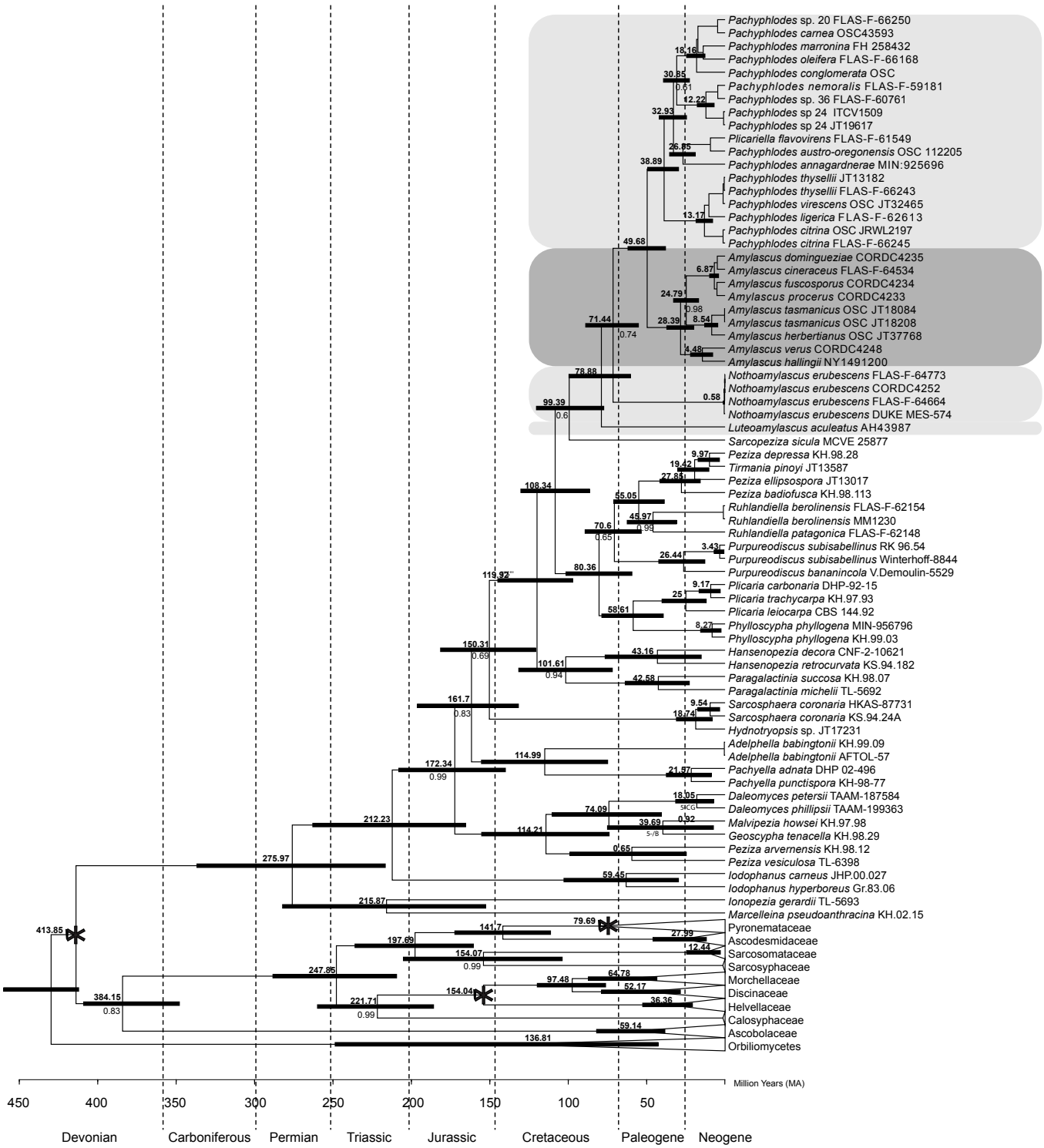

Fig. 3 Chronogram estimation of *Amylascus*, *Luteoamylascus*, *Nothoamylascus*, and *Pachyphlodes* (including *Plicariella*) in a phylogenetic context among other Pezizales fungi obtained from BEAST analysis of 28S rDNA, *EF1α*, *rpb1* and *rpb2* and calibrated with three secondary estimates (black stars) according to Beimforde et al. (2014). Number above the node represents the time to the most recent common ancestor (tMRCA) with a black bar representing the 95 % confidence interval (HPD). All nodes receive maximum Bayesian Posterior Probability unless specified below the node. The genera *Amylascus*, *Luteoamylascus*, *Nothoamylascus*, and *Pachyphlodes* are indicated in shaded boxes with the specimen voucher number and the geographic origin of the collection indicated to the right of the taxon name. The deeply shaded box is the *Amylascus* clade. Orbiliomycetes were used as the outgroup for the analysis. Geological epochs are marked at the bottom.

The three *Amylascus* clades described above had significant support in both ML and MB (Fig. 1) phylogenies, with *A. hallingii* (Australia) being most closely related to *A. verus* (South America), while the other two supported clades are geographically distinct; one being from Australia and the other from South America. The placement of these three clades within *Amylascus* showed incongruences between the ML and MB (Fig. 1) phylogenies, likely as a result of the incongruences among loci described above: *A. herbertianus* and *A. tasmanicus* were sister to the clade of *A. cineraceus*, *A. dominguezia*e, *A. fuscosporus*, and *A. procerus* in the MB topology, while *A. hallingii* and *A. verus* were sister to this clade of four species in the ML topology. We were unable to include *A. luteosporus* (Australia) in the multi-locus analysis because we did not recover the single copy loci from our DNA extract. However, the ITS sequence and morphological characters helped to distinguish this species from other *Amylascus* species (see below).

For species delimitation, we included full ITS sequences (502–606 bp) from 16 ascomata, 43 mitotic spore mats, 11 ectomycorrhizal root tips, and the ITS1 sequences (287–300 bp) from three bird fecal samples and an ascoma of *A. herbertianus* (JT37768) (218 bp), yielding a total of 74 sequences (Table S1).

Our analyses delimited two genera and 12 species from ascomata sequences; two genera and 12 species from mitotic spore mat sequences; two genera and six species from ectomycorrhizal root tip sequences, placed two of the bird feces sequences with mitotic spore mat sequences (*Amylascus* spp. 4 and 10), and placed a third bird feces sequence between *Amylascus* sp. 6 (consisting only of mitotic spore mat sequences) and *Amylascus* sp. 7 (consisting only of ectomycorrhizal root tip sequences). We do not consider this single environmental sequence as enough evidence to delimit a separate species, so we did not designate an OTU number until more data are available. In total, we delimited twenty-one species of *Amylascus* (five from Australia and 16 from South America) as well as two species of *Nothoamylascus* from South America (Fig. 2). However, we only had sufficient mature ascomata to formally describe or re-describe nine species of *Amylascus* (two previously described species and seven species described below) and one species of *Nothoamylascus*.

Phylogenetic analyses of the ITS region resolved the same species as OTU clustering at 97 % sequence similarity with only a few exceptions. Given the potential of short sequences to bias phylogenetic analyses, we clustered the ITS sequences both with and without the short sequences from the bird feces samples. There was one discrepancy observed between the two clustering runs. *Amylascus verus* clustered with *Amylascus* sp. 11 and ECM root tip sequence JX316239 when the shorter sequences were included. When the shorter sequences were excluded, *A. verus* and JX316239 were separated as two different OTUs. The phylogenetic analysis of the ITS sequences resolved *A. verus* as a distinct entity from *Amylascus* sp. 11 and ECM root tip sequence JX316239, so we accepted this delimitation. The ECM root tip sequence JX316239 was weakly supported in the same clade as *Amylascus* sp. 9 based on the ML analysis, so to be conservative we retained these sequences as the same OTU. A second discrepancy occurred between the clustering method and the phylogenetic method where *Amylascus* sp. 10 clustered with *Amylascus* sp. 12 based on clustering. However, this delimitation was not supported by phylogenetic analysis. We opted to separate these two taxa according to our phylogenetic results to avoid a non-monophyletic OTU delimitation.

For the lineage dating analysis, the 28S dataset included 98 sequences and 841 bp positions, the *EF1*α locus had 71 sequences and 748 bp positions, the *rpb1* locus had 59 sequences and 600 bp positions, and the *rpb2* locus had 90 sequences and 490 bp positions. The time-calibrated molecular phylogeny gave an estimated age of the family *Pezizaceae* with a mean of 276 Ma (217–337 HPD) (Fig. 3). The tMRCA of the *Amylascus-Pachyphlodes-Nothoamylascus-Luteoamylascus* clade had a mean of 79 Ma (60–100 HPD) in the Cretaceous, with *Nothoamylascus* starting to diverge around 71 Ma (55–89 HPD). The tMRCA of *Amylascus-Pachyphlodes* had a mean of 50 Ma (38–62 HPD), with a crown age of *Pachyphlodes* with a mean of 39 Ma (25–42 HPD) and 28 Ma for *Amylascus* (20–37 HPD). Diversification between Australian and South American *Amylascus* species began around 25 Ma (17–33 HPD) and up to 14 Ma ago (8–22 HPD) for *A. verus* and *A. hallingii*.

TAXONOMY

Amylascus was first described based on ascomata with a finely verrucose or tomentose exterior, with an epithecium that resembles the excipulum in cellular structure, a gleba with irregularly arranged asci (not in a hymenium), and ellipsoid to subglobose amyloid asci with hyaline to yellow-brown ascospores. During this study we discovered new *Amylascus* species with morphological features that do not fit well with this original generic description. Here we provide revised descriptions of the two described species of *Amylascus*, and we emend the genus description to encompass the morphological diversity found in the taxa we describe here. We describe seven new *Amylascus* species and propose the new genus *Nothoamylascus* based on *Nothoamylascus erubescens* sp. nov.

It is notable that for many of the taxa described here, only a single or a few mature ascomata collections exist in fungaria. Although this situation is not ideal for taxonomic studies, we have been unable to recover additional ascomata for these taxa despite extensive targeted searches in southern South American *Nothofagaceae* forests over several years. Here we opt to describe all *Amylascus* and *Nothoamylascus* species for which mature ascomata are available to ensure that other scientists are aware of these rare fungi and to enable communication about their biology and diversity. We also note that some of these fungi are widespread and common as mitotic spore mats fruiting on soil, as ascospores in the feces of Patagonian birds (*Pteroptochos tarnii* and *Scelorchilus rubecula*), or as ectomycorrhizas with *Nothofagaceae* trees (Fig. 2).

Amylascus Trappe, ***emend.*** Healy & M.E. Sm.

Ascoma a lobed to folded ptychothecium, up to 30 mm diam, hypogeous to epigeous, pink to gray or brown, sometimes with violaceous or yellowish tones, usually hairless except for a basal tuft of hyphae or rarely tomentose, with a finely to coarsely warted exterior. Warts pyramidal, varying in height across the ascoma, with higher warts in the protected crevices of the folds. *Excipulum* clearly separated into an ectal excipulum and medullary excipulum, although the medullary excipulum may be thin and look similar to the subhymenium in the gleba. Ectal excipulum composed of *textura angularis* or *textura globulosa*, with large rounded to slightly angular cells. The walls of the outermost cells are usually pigmented. Medullary excipulum of *textura intricata*. Oleiferous hyphae present in some species, not observed in others. Glebal tissues ranging from hymenial with a palisade of asci and paraphyses to disorganized with asci randomly distributed throughout the gleba. Paraphyses either indistinguishable from glebal hyphae or present as an irregular palisade among the asci, they may be the same length as asci or may exceed the asci in length, they may terminate as free ends or be interwoven above the asci to form an epithecium. *Epithecium* absent or constructed of paraphyses that exceed the asci and are parallel to loosely interwoven, or form a tissue of *textura angularis*, similar and sometimes indistinguishable

from the ectal excipular tissue. *Asci* cylindrical, clavate, ovate or nearly globose, with croziers at the base, and four to eight ascospores in an uniseriate, irregularly biseriate, or irregular arrangement. *Ascospores* globose, hyaline, yellowish, or dark brown; containing one large oil droplet that almost entirely fills the spore, ornamented with free spines or with ornaments encompassed by a perispore. Trophic status: Ectomycorrhizal with *Nothofagaceae* in South America and found in wet sclerophyll and montane forests in Australia where ectomycorrhizal hosts include species of *Nothofagaceae*, *Myrtaceae*, and perhaps other plant lineages.

Distribution — Known only from the Southern Hemisphere.

Type. Amylascus herbertianus (J.W. Cribb) Trappe, Trans. Brit. Mycol. Soc. 57(1): 89. 1971.

Amylascus herbertianus (J.W. Cribb) Trappe, Trans. Brit. Mycol. Soc. 57(1): 89. 1971. ***emend.*** R.A. Healy — MycoBank MB 308680; Fig. 4

Basionym. Hydnobolites herbertianus J.W. Cribb, Pap. Dept. Bot. Univ. Queensland 3: 151. 1958 '1957'.

Typus. Australia, Queensland, Mt. Glorious, 25 Apr. 1955 *J.W. Cribb* (holotype BRI AQ0642725, not examined).

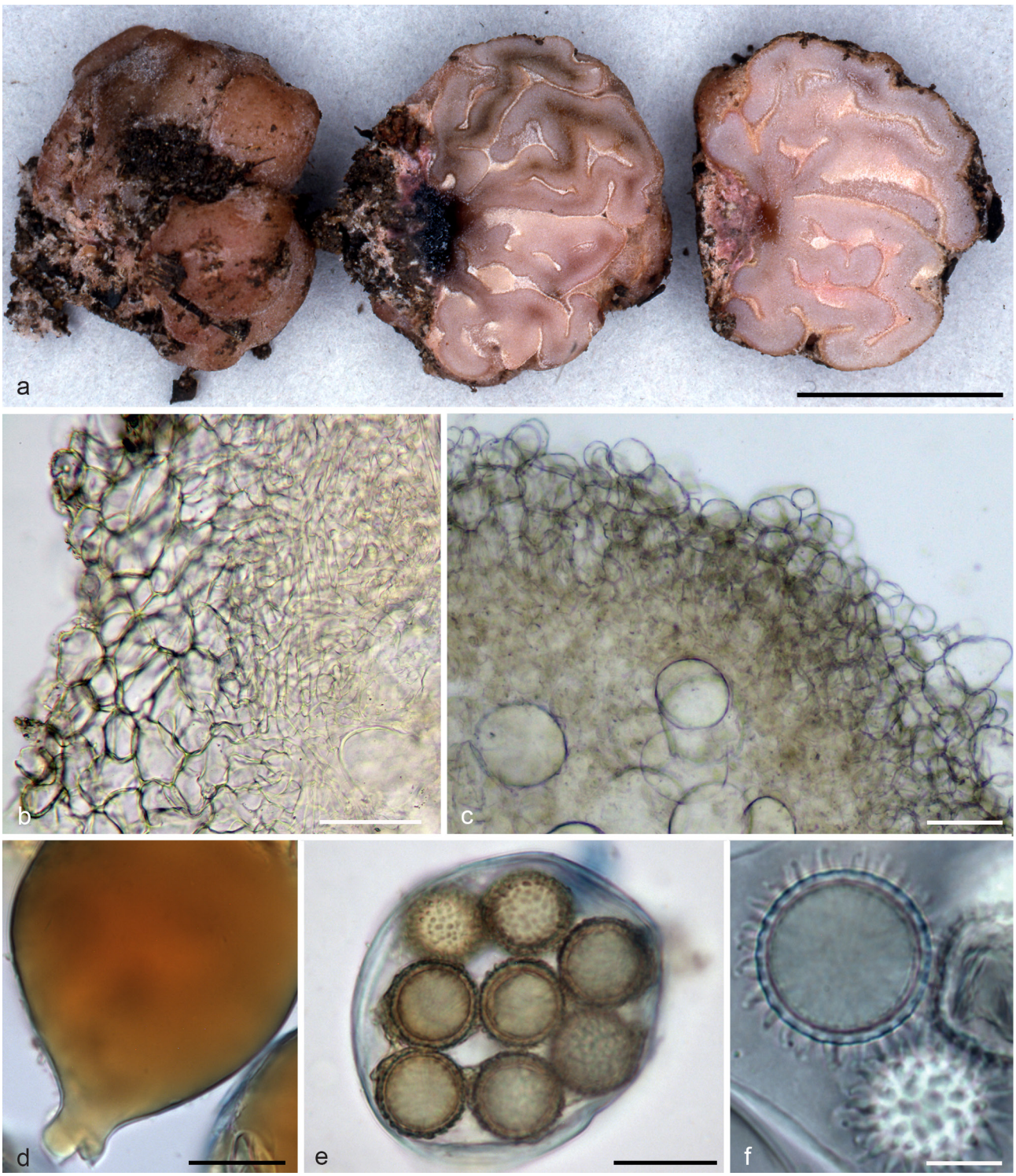

Fig. 4 *Amylascus herbertianus* (all FLAS-F-69586 except in e). a. Fresh ascomata halves showing lobed, verrucose excipular surface on left and convoluted gleba of veins lined with empty canals that open at the surface; b. cross section of excipulum; c. cross section of epithecium; d. young ascus in Melzer's reagent showing crozier at base; e. amyloid ascus with spores (Melzer's reagent) (FLAS-F-69588); f. ascospores with free spines. — Scale bars: a = 1 cm; b, c = 50 µm; d, e = 25 µm; f = 10 µm.

Ascomata subglobose, lobate, plicate, up to 20 mm diam, with basal tuft of hyphae. Outer surface verrucose, tomentose, when fresh light pinkish brown excipulum (immature), becoming grayish pink brown (mature), drying to reddish brown. Gleba light pink (immature), grayish pink (mature), labyrinthine with meandering white veins lining empty canals, canals opening at various points to the outer surface. Odor mild. *Excipulum* 300–400 µm thick, ectal excipulum composed of a *textura globulosa* (cells closest to the surface) intergraded with a *textura angularis* with cells (10–)15–60(–80) µm diam, walls yellow, ~1 µm thick. A sparse tomentum of hyaline, branching hyphae develop from the surface cells, and are smooth to rough, and 4–16 µm diam. Ectal excipulum distinctly delimited from the medullary excipulum, 22–51 µm thick, composed of *textura*

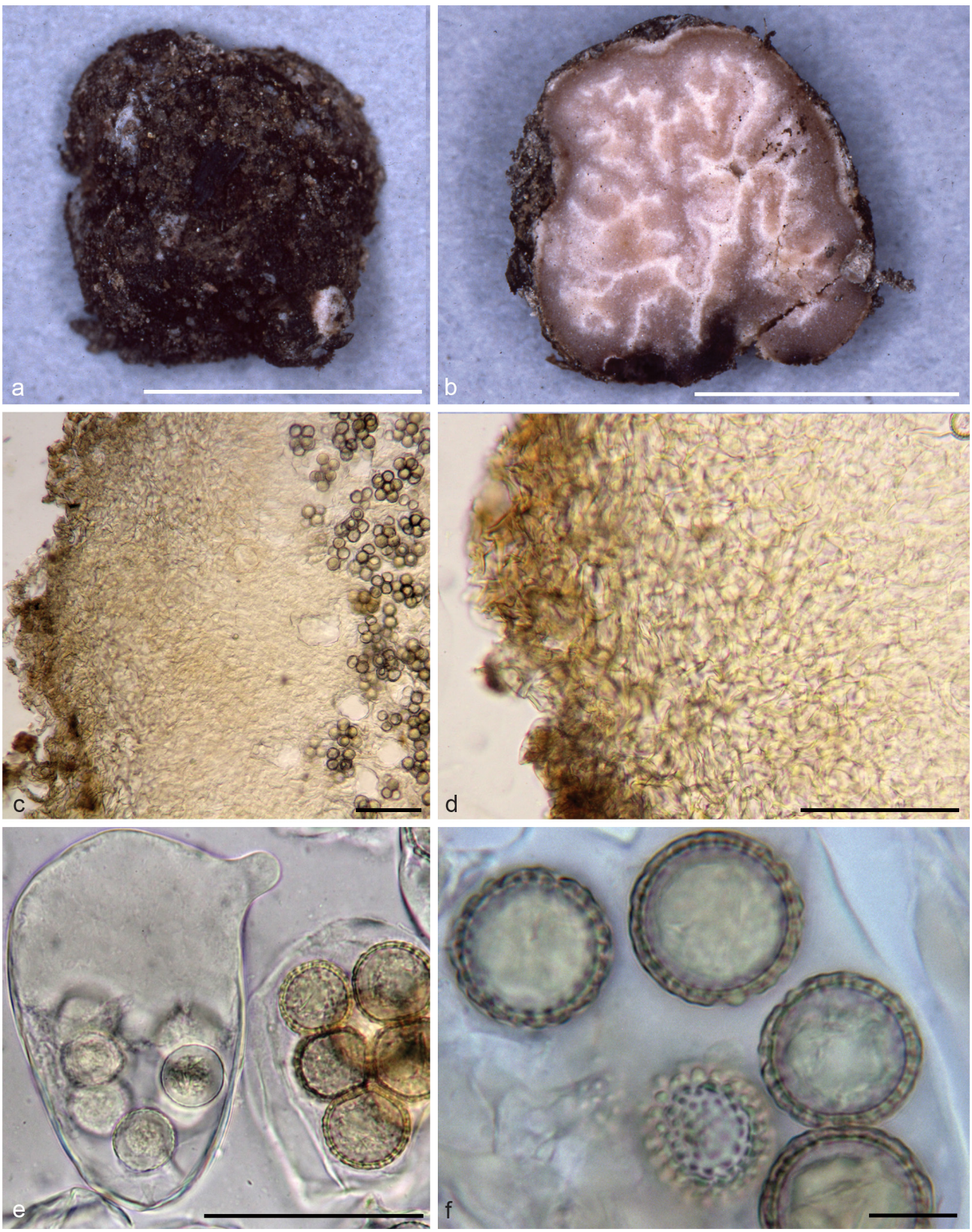

Fig. 5 *Amylascus tasmanicus* (FLAS-F-70964). a. Fresh ascomata showing excipulum; b. ascoma showing convoluted, infolded gleba; c–f. *A. tasmanicus* (HO39875, holotype); c. section through ascoma showing excipulum and gleba with asci and ascospores; d. partial cross section of ascoma; e. asci, immature on left showing pedicel, mature on right showing light brown ascospores with perispore; f. ascospores showing spines and perispore. — Scale bars: a, b = 1 cm; c, d = 100 µm; e = 50 µm; f = 10 µm.

intricata with a mixture of even hyphae 5–9 µm diam, and hyphae that are slightly swollen up to 12 µm. Oleiferous hyphae not observed. Glebal hyphae composed of *textura intricata*, hyphae hyaline, 8–10 µm diam, occasionally slightly inflated to 12 µm; similar to hyphae of the medullary excipulum but intermixed with asci (no organized hymenium). *Epithecium* similar to ectal excipulum, composed of *textura globulosa* (cells closest to surface) intergraded with a *textura angularis*. *Paraphyses* not recognizable. *Asci* subglobose to obovoid, 65–100 × 50–75 µm, with short pedicel 3–12 × 5–9 µm ending in a crozier, walls thin when immature and 1–2 µm thick at maturity, the entire wall evenly amyloid in Melzer's reagent, with (4–)5–8 ascospores irregularly arranged in the ascus. *Ascospores* globose, almost entirely filled with one large oil droplet at maturity, hyaline, or yellow, light brown in Melzer's reagent, ornamented with course to narrow spines, (15–)17–22(–23) µm, Q = 1, av. 19 µm, walls ~1 µm, spines 2–3 × 0.3–1 µm, spaced 1–2 µm apart, weakly cyanophilic in cotton blue, no perispore.

Distribution & Ecology — Australia, ascomata hypogeous in wet sclerophyll forests with *Eucalyptus* species in autumn (Feb.–Apr.).

Ascomata collections examined. Australia, paratype Mt. Glorious, SE Queensland, under leaf mould, light rain forest, 19 Feb. 1955, *J.W. Cribb Gilkey #859* (OSC 34836); ibid., 9 Feb. 1955, *JT2095* (OSC 34757); New South Wales, Barrington Tops National Park, along Mount Allyn Rd, with *Casuarina* sp., *Eucalyptus* sp., 28 Apr. 1992, *N. Bougher & M.A. Castellano H5626* (OSC; FLAS-F-69586); New South Wales, New England National Park, wet sclerophyll forest, 7 Mar. 2008, *M. Danks* (MEL2364119); Queensland, Bald Mtn. Road, Atherton District, 4 May 1988, *M.A. Castellano H4036* (OSC, FLAS-F-69587); west of Brisbane, J.C. Slaughter Falls parking area, uphill slope along east Ithaca Creek trail, under *Eucalyptus grandis*, *Syncarpia* sp., and *Acacia incinnata*, 24 July 2017, *E.M. Castellano JT37768* (OSC, FLAS-F-69588).

Mitotic spore mats unknown.

Notes — This species was first described by Cribb (1957, as *Hydnobolites herbertianus*) and additional morphological information was provided by Trappe (1971). Here we provide additional information based on recently collected specimens from Queensland and New South Wales. An SEM image of an ascospore from a specimen identified by Cribb (OSC34757) is shown in Cabero et al. (2016). *Amylascus herbertianus* is easily distinguished from all other known Australian species by its spiny ascospores that lack a perispore.

Amylascus tasmanicus (Rodway) Trappe, Trans. Brit. Mycol. Soc. 65(3): 498. 1975 — MycoBank MB 308681; Fig. 5

Basionym. *Terfezia tasmanica* Rodway, Pap. & Proc. Roy. Soc. Tasmania 1925: 167. 1926 '1925'.

Typus. Australia, Tasmania, Cascades, May 1925, *L. Rodway*, *s.n.* Tasmanian Herbarium Hobart 39875 (holotype HO, examined).

Ascomata irregular, up to 30 mm diam, with furrows and ridges, with basal tuft of hyphae. Outer surface obscurely verrucose and tomentose (?), chestnut brown. Gleba pink with white veins, of folded to convoluted tissues intermixed with labyrinthine canals that open to the outside in places on the sides and top. Odor mild. *Excipulum* about 400 µm thick, cells poorly reviving, composed of *textura angularis* and *textura globulosa*, cells 30–90 µm diam, outermost cells with light yellowish brown walls. Oleiferous hyphae not observed. Glebal hyphae composed of *textura intricata*, hyphae hyaline, 8–20 µm diam, intermixed with asci (no organized hymenium). *Epithecium* composed of large cells which line the veins and chambers and give rise to hyaline hyphae that partially and loosely fill the chambers. *Paraphyses* not recognizable. *Asci* weakly but evenly amyloid along the entire wall in Melzer's reagent, walls 1–2 µm thick at maturity, ellipsoid to subglobose, 70–100 × 50–65 µm, including stem that is 10–12 × 6–12 µm, arising from croziers, 3–8 ascospores, irregularly biseriate or irregularly arranged in the ascus. *Ascospores* globose, almost entirely filled with one large oil droplet at maturity, hyaline to light yellow, pale yellow brown in Melzer's, 18–24 µm diam excluding ornamentation of rods and cones, Q = 1, av. 23 µm, ornaments 1–2 µm high, spaced 1–2 µm apart, tips embedded in perispore, ornaments weakly cyanophilic in cotton blue, walls 1 µm thick.

Distribution & Ecology — Australia (Tasmania and Victoria), ascomata hypogeous in *Eucalyptus* woodlands in autumn (May).

Other ascomata collections examined. Australia, Victoria, East Gippsland, Alpine National Park, Black Mtn track, 1.1 km east of junction with Cobberas trail, 23 May 1996, under *Eucalyptus pauciflora* and *E. dalrympleana*, *A. Jumpponen JT18084* (C, MEL, OSC); Alpine National Park, Native Cat Track, 0.8 km from Black Mountain Road. Claridge Site #2, 24 May 1996, *A. Jumpponen JT18208* (OSC).

Mitotic spore mats unknown.

Notes — This species is characterized by its dark brown excipulum, weakly amyloid ovoid asci, and light yellow, perispore-covered ascospores. No image of fresh material of the type specimen exists, but images of the ascoma of a recent collection of this species (for which there is an ITS sequence) is shown in Fig. 5. The collections cited above agree with the description by Trappe (1975) except that an obvious tomentum was not observed on the type specimen nor on the sequenced collections, which is why we put a question mark after this descriptor.

Amylascus cineraceus Healy & M.E. Sm., *sp. nov.* — MycoBank MB 847731; Fig. 6

Etymology. The epithet '*cineraceus*' is Latin for ash-colored, referring to the predominantly gray color of the ascomata.

Typus. Chile, Osorno, Puyehue National Park, foothills of Volcan Puyehue, up the road past El Caulle north of Rio Golgol, under *Nothofagus dombeyi*, 7 May 2016, *A. Mujic & M.E. Smith MES-1770* (holotype FLAS-F-64534; isotype SGO).

Ascomata knobby, convoluted, up to 27 × 15.5 mm, sessile, basal tuft of hyphae not observed. Outer surface verrucose with well-spaced conical warts, hairless, yellowish white when young becoming gray with some yellowish white areas when fresh and mature. The yellowish wart tips combined with the underlying gray give the ascomata a greenish cast. Gleba between excipulum and epithecium, dark gray with vinaceous tints, convoluted with irregular canals that come to surface at places on outer surface. Inner surface indistinguishable from outer surface except for position in the ascomata. Odor not detected. *Excipulum* 240–624 µm thick, ectal excipulum with conical warts up to 384 µm high, grayish brown in water and KOH under transmitted light, composed of *textura globulosa* (outermost cells) intergraded with *textura angularis*, cells up to 85 × 71 µm, walls up to 4 µm thick. Large cells of the ectal excipulum grade into smaller cells of the medullary excipulum, about 60–80 µm thick composed of *textura intricata*, hyphae hyaline, 3–11 µm diam at the septum, and inflated up to 30 µm diam. Oleiferous hyphae not observed. *Epithecium* similar in composition to excipulum, 80–440 µm, outer layer with conical warts up to 360 µm high, gray in water and KOH, composed of large rounded cells similar to excipular cells, isodiametric to ovoid, up to 79 × 51 µm, outermost cell walls up to 4 µm thick, inner layer thin, similar to medullary excipulum. Glebal tissue composed of *textura intricata* intermingled with asci (no organized hymenium), hyphae hyaline 3–14 µm diam at septum, not much swollen between septa. *Paraphyses* not recognizable. *Asci* broadly cylindrical to obovate, widest near the base, inamyloid in Melzer's reagent, 243–287 × 35–50 µm, including short pedicel 8–22 × 8–10 µm diam, arising from croziers, with

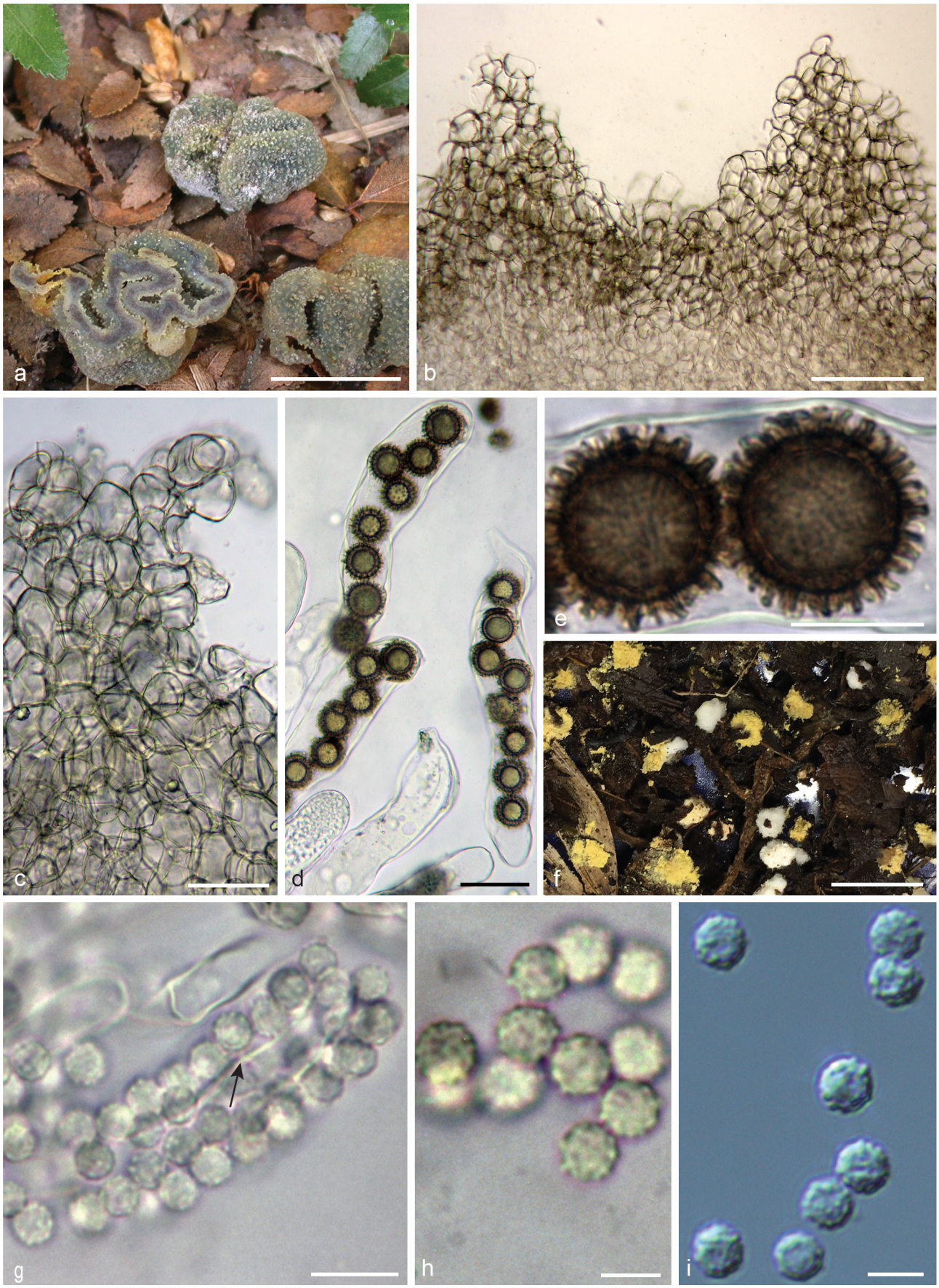

Fig. 6 a–e. *Amylascus cineraceus* ascoma (FLAS-F-64534, holotype), f–i. *A. cineraceus* mitotic spore mat (FLAS-F-64536). a. Fresh ascoma halves showing gleba (lower left) and excipulum (top and right); b. excipular wart; c. epithecial wart; d. ascus and ascospores in KOH; e. ascospores in water; f. mitotic spore mat; g. mitotic spores attached by denticle to conidiophore (arrow); h. mitotic spores in KOH; i. mitotic spores, showing warts. — Scale bars: a, f = 2 cm; b = 100 µm; c, d = 50 µm; e = 20 µm; g = 10 µm; h, i = 5 µm.

(4–)6–8 ascospores arranged uniseriately. *Ascospores* globose, almost entirely filled with one large oil droplet at maturity, dark brown, ornamented with spines, (22–)24–27(–28) µm diam, Q = 1, av. 24.4 µm excluding tapered spines, 2–4(–5) µm high, av. 3.5 µm high, without a perispore covering the spines.

Mitotic spore mats composed of white hyphae with yellow mitospores (mature) that develop in small patches (≤ 1 cm) within soil (shallow hypogeous to ~5 cm) or on the soil surface and on fallen leaves. Subtending hyphae smooth, hyaline to yellow, 5–15 µm diam at septum, loosely interwoven to parallel, not conidiogenous, conidiophores arising from subtending hyphae, smooth, hyaline to yellow, branching, branches of uneven lengths, 6–8.9 µm diam not inflated, conidiogenous hyphae 5.4–7.5 µm diam at the apices. Polyblastic mitotic spores produced synchronously from short denticles (≤ 1 µm long) along conidiophore branches. Mitotic spores globose to ovoid, dry, hydrophobic, warted, 5–6 µm diam, av. 5.1 µm diam excluding warts, warts up to 0.7 µm high × 0.6 µm wide.

Distribution & Ecology — South America (Chile), ascomata hypogeous, mitotic spore mats hypogeous to epigeous in montane *Nothofagaceae* forests, autumn (May).

Other ascomata collections examined. Chile, Valdivia, San Pablo de Tregua Reserve (reserve of the Universidad Austral), hypogeous in soil under *Nothofagus dombeyi* and *Lophozonia obliqua*, 9 May 2019, *M.E. Smith*, *M. Caiafa*, *Y. Maldonado*, *MES-3295* (FLAS-F-68923).

Mitotic spore mat collections examined. Chile, Osorno, Puyehue National Park, foothills of Volcan Puyehue, up the road past El Caulle north of Rio Golgol, hypogeous in soil or on fallen leaves and soil surface under *Nothofagus dombeyi*, 7 May 2016, *R. Healy*, *M.E. Smith*, *A. Mujic MES-1772* (FLAS-F-64536); Valdivia, San Pablo de Tregua Reserve (reserve of the Universidad Austral), on soil under *Nothofagus dombeyi* and *Lophozonia obliqua*, 9 May 2019, *M.E. Smith*, *M. Caiafa*, *Y. Maldonado MES-3296* (specimen destroyed, but photos and DNA sequences available).

Notes — This species is characterized by its predominantly gray ascoma (with warts that are yellow at the tips and therefore give the ascomata a greenish cast) and the combination of the broadly cylindrical inamyloid asci, dark brown and spiny ascospores, an epithecium that resembles the excipulum, and the disorganized hymenium. It is one of the two species of *Amylascus* in which the ascomata and mitotic spore mats were found concurrently. It is most similar to the closely related species *Amylascus fuscosporus* sp. nov. but differs in its larger ascospore size (24.4 µm diam on average for *A. cineraceus* compared to 20.5 µm diam on average for *A. fuscosporus*).

***Amylascus dominguezia*e** Healy, Trappe, & M.E. Sm., *sp. nov*. — MycoBank MB 847732; Fig. 7

Etymology. Epithet is in honor of the esteemed Argentinean mycologist and collector of this species, Dr. Laura Dominguez.

Typus. Argentina, Neuquen, Lanín National Park, Ruta 48A Hua Hum, 3.5 km from the border with Chile, under *Lophozonia alpina*, *L. obliqua*, and *Nothofagus dombeyi*, 25 Apr. 2001, *L. Dominguez JT26237* (holotype CORD-C4235; isotype FLAS-F-69557).

Ascomata irregularly subglobose, convoluted, up to 20 × 11 mm, sessile, with white basal tuft of hyphae. Outer surface with low conical warts, hairless except for basal attachment, pink when young, becoming dark chestnut with reddish vinaceous tints when mature and fresh, reddish brown with light yellowish brown warts when dried. Inner surface indistinguishable from outer surface. Gleba between excipulum and epithecium, dark gray to black with red tints when fresh, convoluted with irregular canals that come to the outer surface in places. Odor indistinct. *Excipulum* 84–300 µm thick with warts up to 288 µm. Ectal excipulum composed of large, rounded, isodiametric to ovoid cells, up to 82.5 × 53 µm, walls 1.65 µm thick, yellowish brown. Medullary excipulum 22–51 µm thick, composed of *textura intricata* that is a mixture of non-inflated hyphae 5–9 µm diam and hyphae slightly inflated up to 13 µm. *Epithecium* similar in structure to excipulum, 128–225 µm thick, warts up to 163 µm high, composed of rounded cells up to 53 × 79 µm; outer cells light brown. Glebal tissue composed of *textura intricata* mingled with asci (no organized hymenium), hyphae hyaline, 6.6–13 µm at the septum, inflated in places up to 16 µm. No oleiferous hyphae observed. *Paraphyses* not recognizable. *Asci* cylindrical to broadly cylindrical, 173–198(–223) × (30–)35–52 µm, arising from croziers, inamyloid, usually with 8 ascospores, but (4–)5–8 spored asci also present, spores uniseriate to irregularly biseriate. *Ascospores* globose, filled with one large oil droplet at maturity, dark brown, further darkening in Melzer's reagent, ornamented with spines, (11–)12.5–14.5(–16.5) µm, Q = 1, av. 13 µm excluding ornamentation of tapered spines 1–1.8 µm high, av. 1.5 µm high, with some spines connected at the base, walls 1.65 µm thick, no perispore.

Mitotic spore mats composed of white hyphae with yellow mitotic spores (mature) that occur in small patches within soil (shallow hypogeous to ~5 cm) or on the soil surface. Subtending hyphae smooth, hyaline, 6.9–9.5 µm diam at septum, conidiophores smooth, hyaline, branching, branches of uneven lengths, 5.4–9.3 µm diam slightly swollen up to 14.9 µm at tips. Polyblastic mitotic spores produced synchronously from denticles (0.7–1 µm long) along conidiophore branches. Mitotic spores globose to ovoid, dry, hydrophobic, warted, 4.8–6.4 µm diam, av. 5.6 µm diam excluding warts, warts 0.5–0.8 µm high, of variable width to 1 µm.

Distribution & Ecology — Southern South America (Argentina), ascomata hypogeous, mitotic spore mats on soil surface in montane forests of *Lophozonia obliqua*, *L. alpina*, and *Nothofagus dombeyi*, autumn (Apr.–May).

Other ascomata collections examined. Argentina, Neuquen, Lanín National Park, Ruta 48A Hua Hum, 3.5 km from the border with Chile, under *Lophozonia obliqua*, *L. alpina*, and *Nothofagus dombeyi* (40 7 920S – 71 38 575W), 25 Apr. 2001, *E. Cázares & L. Dominguez JT26239*, CORD 4236.

Mitotic spore mat collections examined. Argentina, Neuquen, Lanín National Park, on soil in forest of *Nothofagus dombeyi*, 16 May 2015, *R. Healy MES-1295* (FLAS-F-64667, CORD); trail to cascada Chachin, on soil in forest of *Lophozonia alpina*, *L. obliqua*, *Nothofagus dombeyi*, 15 May 2015, *R. Healy MES-1296* (FLAS-F-63988, CORD); north of Lago Lacar about half way between San Martin and the Hua Hum, on soil in forest of *L. alpina* and *L. obliqua*, 18 May 2015, *R. Healy MES-1352*, (FLAS-F-64663).

Notes — This species is characterized by its predominantly reddish brown ascomata, and the combination of inamyloid, broadly cylindrical asci, dark brown, spiny ascospores, an epithecium that resembles the excipulum, and lack of an organized hymenium. This species is most similar to the closely related *A. cineraceus* and *A. fuscosporus*, but the ascospores of *A. dominguezia*e are much smaller (13 µm av.) than those of *A. cineraceus* (24.4 µm av.) or *A. fuscosporus* (20.5 µm av.). Collection JT26239 was a mixed collection. Pieces were sorted by morphology, but measurements were only taken from the holotype (JT26237). Based on morphological similarity, collection JT26245 is likely this species, but we were only able to obtain DNA sequences of a non-target fungus in the Eurotiales from the ascomata.

Amylascus fuscosporus Healy, Castellano & M.E. Sm., *sp. nov.* — MycoBank MB 847733; Fig. 8

Etymology. The epithet is derived from the Latin 'fuscus' (L), meaning brown, in reference to the color of the ascospores.

Typus. Argentina, Neuquen, Lanín National Park, Ruta 48A Hua Hum, 3.5 km from the border with Chile, under *Lophozonia alpina*, *L. obliqua*, and *Nothofagus dombeyi*, 25 Apr. 2001, *M.A. Castellano JT26240* (holotype CORD-C4234; isotype FLAS-F-69555).

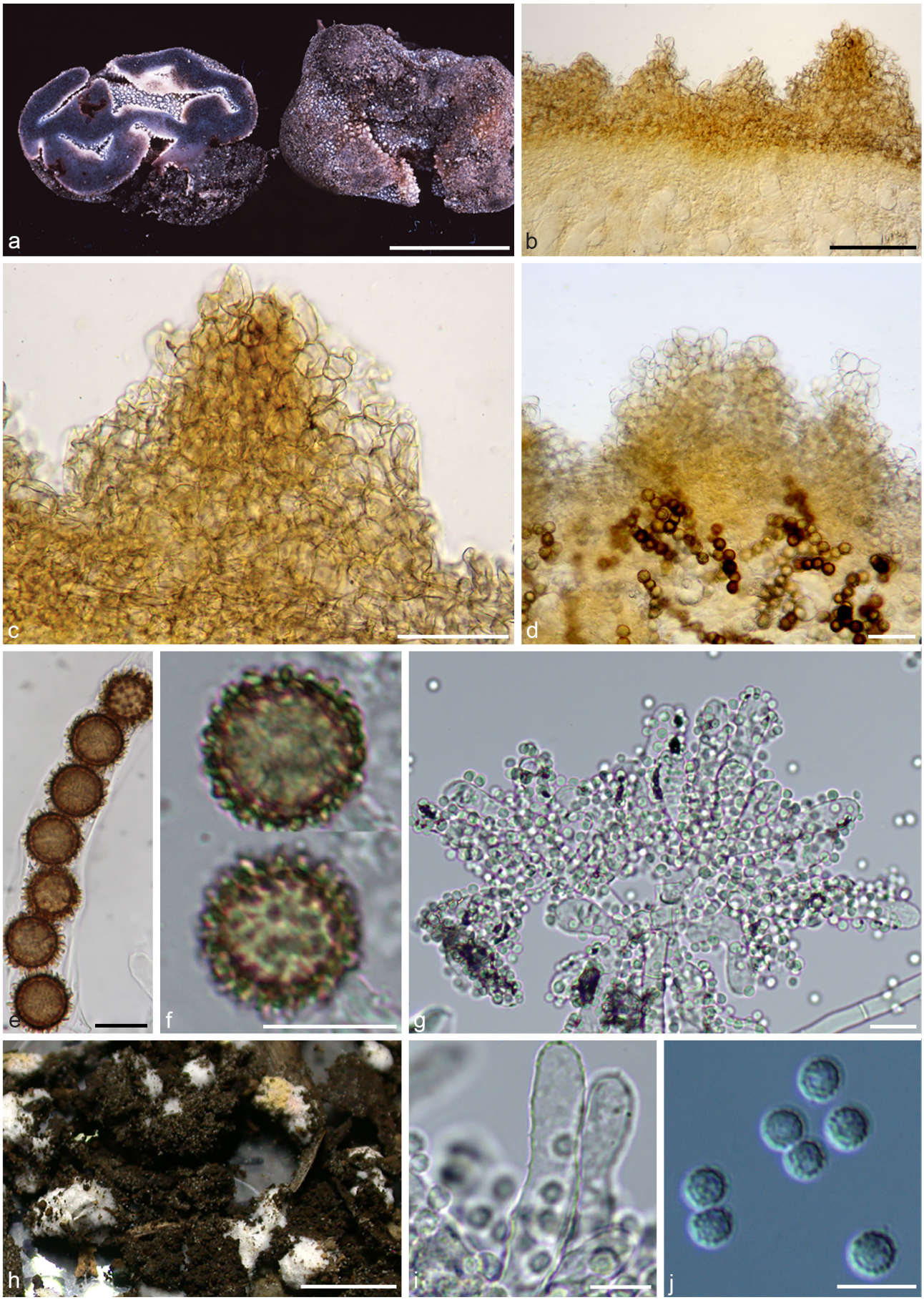

Fig. 7 a–f. *Amylascus dominguezieae* ascoma (CORDC4235, holotype), g–j. *A. dominguezieae* mitotic spore mat (FLAS-F-64667). a. Fresh ascoma showing gleba with epithecial warts (left) and warted excipulum (right); b. cross section through excipulum and part of gleba; c. long section of excipular wart; d. long section through epithecial warts and gleba; e. cylindrical ascus with brown ascospores (water); f. ascospores (water); g. mitotic spore mat conidiophore; h. mitotic spore mat; i. two young conidiophores; j. mitotic spores showing warts. — Scale bars: a = 1 cm; b, d = 50 µm; c = 25 µm; e–g = 20 µm; h = 1 cm; i, j = 10 µm.

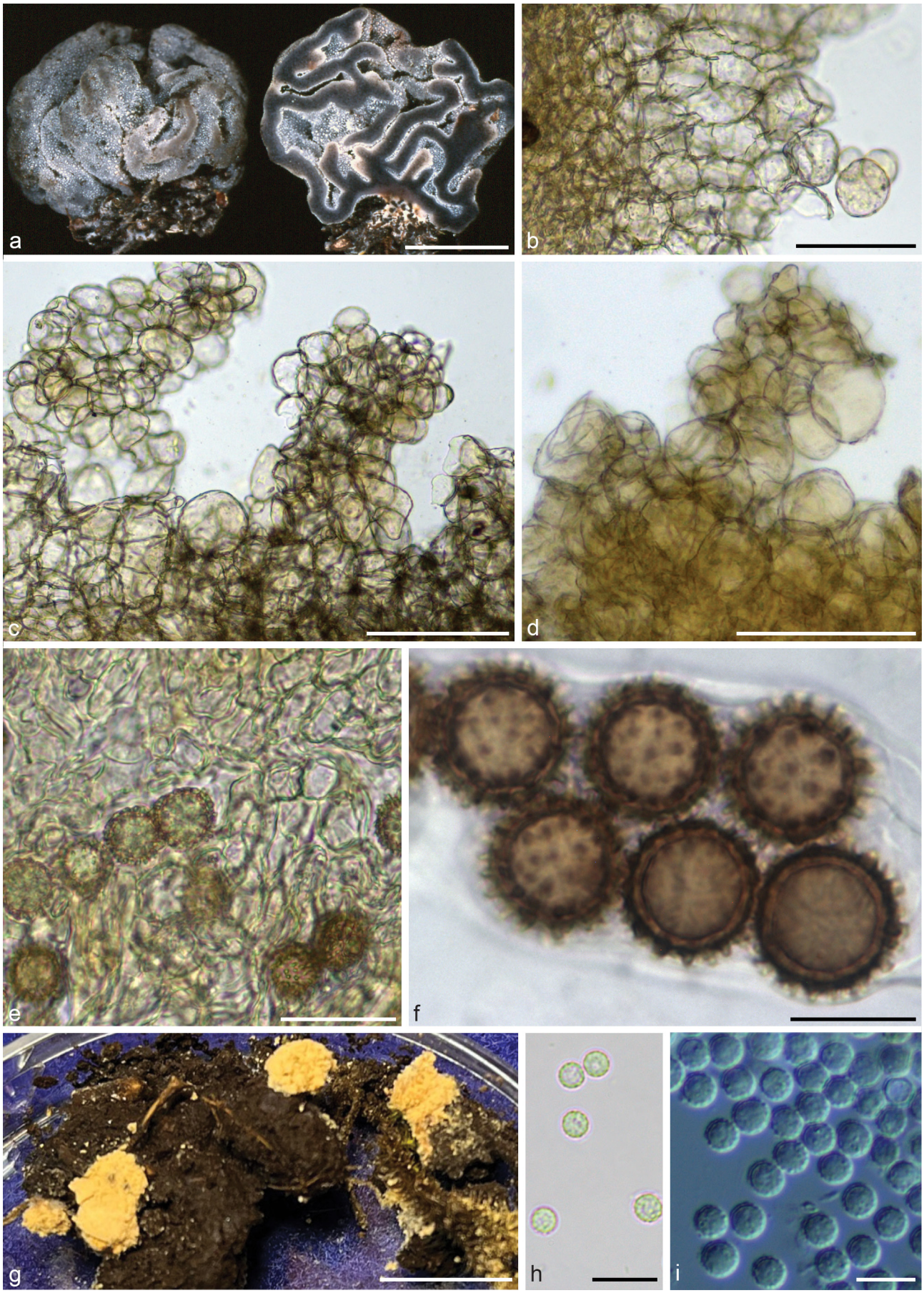

Fig. 8 a–f. *Amylascus fuscosporus* ascoma (CORDC4234, holotype), g–i. *A. fuscosporus* mitotic spore mat (FLAS-F-70677). a. Fresh ascoma halves showing excipulum (left) and gleba with epithecial warts (right); b. long section through excipulum and part of gleba (KOH); c. cross section of excipular wart; d. cross section through epithecial wart; e. gleba; f. ascospores within ascus; g. mitotic spore mat; h. mitotic spores; i. mitotic spores showing warts. — Scale bars: a, g = 1 cm; b–d = 100 µm; e = 50 µm; f = 20 µm; h, i = 10 µm.

Ascoma globose, knobby, deeply convoluted, 26 × 20 mm, sessile, with basal tuft of hyphae, outer surface ornamented with conical warts (3 per mm), hairless except for basal hyphae, outer surface dark gray, but warts paler grayish white when fresh, outer surface brown with light brown warts when dried, basal tuft of hyphae pink and white when fresh, reddish brown when dried. Gleba between excipulum and epithecium, dark gray, convoluted with irregular canals that come to the surface at places on the exterior. Epithecium paler gray but otherwise indistinguishable from excipulum. Odor earthy. *Excipulum* of *textura angularis* intergraded with *textura globulosa*, 148–1150 µm thick, with conical warts up to 900 µm high (highest in the crevices), hyaline to yellowish brown in water and KOH, composed of large, rounded, isodiametric to ovoid cells, up to 82 × 45 µm, walls 1.5 µm thick, medullary excipulum composed of *textura intricata*, 48 µm thick, of hyaline to yellow brown hyphae that intergrade with gleba, cells 4.5–7.6 µm diam at septum, walls 1 µm thick. *Epithecium* similar to excipulum, 304–400 µm thick, with conical warts up to 160 µm high, light brown in water and KOH, composed of large, rounded cells similar to excipular cells, isodiametric to ovoid, up to 80 × 50 µm, outermost walls 3 µm thick. Glebal tissue composed of *textura intricata* with occasional oleiferous hyphae, intermingled with asci (no organized hymenium), hyphae hyaline to yellow, not reviving well in water or KOH, 3.3–7.8 µm diam at septum, some inflated to 12 µm. *Paraphyses* not recognizable. *Asci* cylindrical to broadly cylindrical, with croziers, inamyloid in Melzer's reagent, 130–160 × 40–50 µm, with 4–8 ascospores in uniseriate

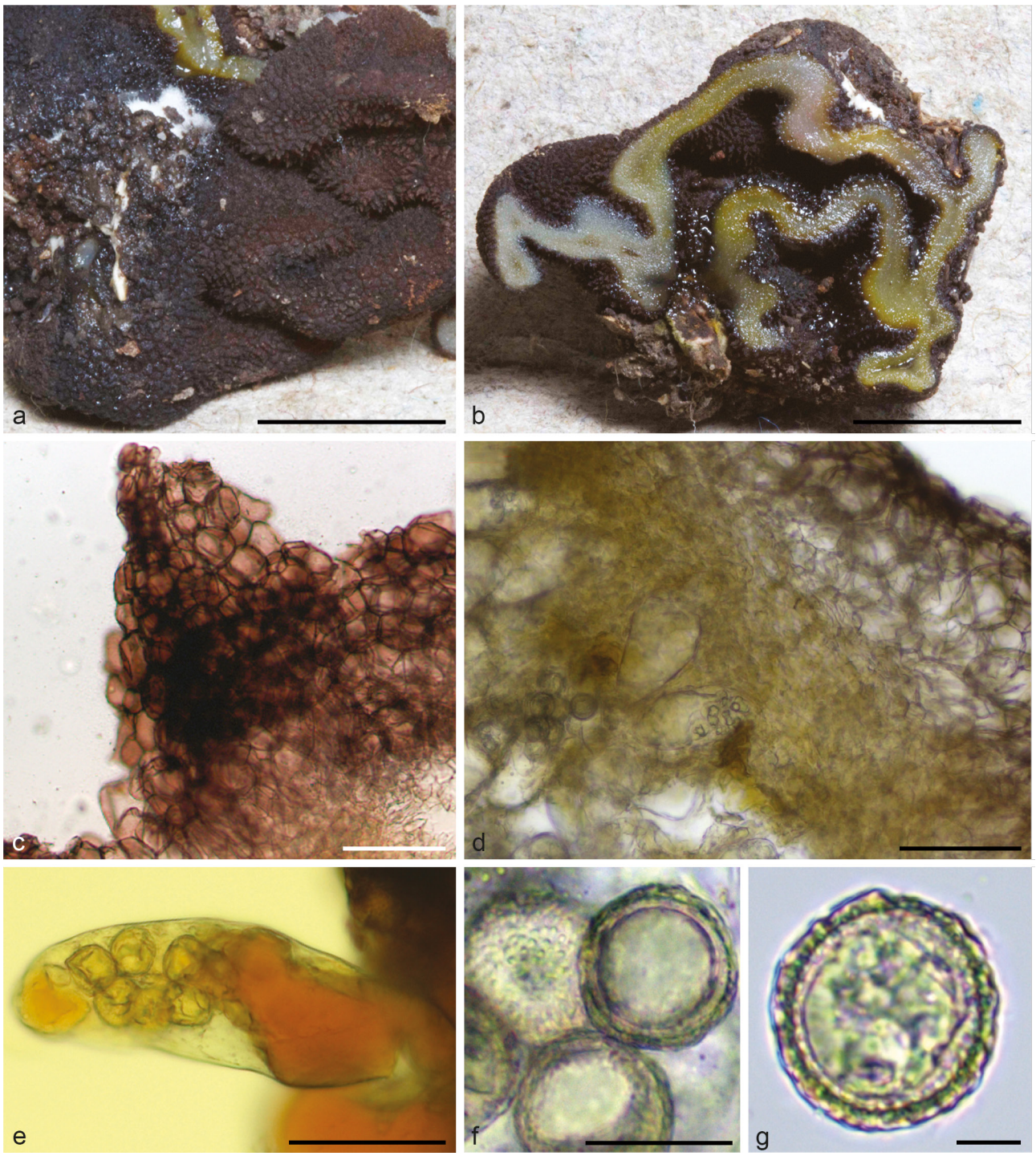

Fig. 9 *Amylascus hallingii* (NYBG1491200, holotype). a. Fresh ascoma half showing excipulum, white basal hyphae and excipular warts; b. fresh ascoma half showing gleba and epithecial warts; c. excipular wart; d. cross section through excipulum and part of gleba; e. amyloid ascus (immature, Melzer's reagent); f. ascospores; g. ascospore showing spines and perispore. — Scale bars: a, b = 0.5 cm; c, d = 100 µm; e = 50 µm; f, g = 20 µm.

to irregularly biseriate arrangement. *Ascospores* globose, filled with one large oil droplet at maturity, dark brown, ornamented with spines, 18–22(–24) µm diam, Q = 1, av. 20.5 µm diam excluding tapered spines, 2–4(–4.5) µm high, av. 3 µm high, wall 1 µm thick, without a perispore covering the spines.

Mitotic spore mats composed of white hyphae with yellow mitotic spores (mature) that develop in small patches on soil surface. Subtending hyphae smooth, hyaline, septate, 5.4–6.7(–7.9) µm diam at septum, parallel, not much branched, with some anastomoses between filaments. Conidiophores smooth, hyaline, branching, branches of uneven lengths. Polyblastic mitotic spores produced synchronously from denticles (≤ 1 µm long) along conidiophore branches. Mitotic spores globose to subglobose, dry, hydrophobic, warted, 5.5–6.2 µm, diam, av. 5.9 µm diam excluding warts, warts up to 0.4 µm high × 0.8 µm wide at base, unevenly distributed along the mitotic spore wall.

Distribution & Ecology — Southern South America (Argentina and Chile), ascomata hypogeous, mitotic spore mats epigeous in montane *Nothofagaceae* forests, autumn (Apr.).

No other ascomata collections known.

Mitotic spore mat collection examined. Chile, Ñuble, Quillón, Cerro Cayumanque (36 42 10S – 72 30 27W), 540 m a.s.l., 11 May 2022, *P. Sandoval MES-4151* (FLAS-F-70677).

Notes — This species is characterized by its predominantly gray ascoma, and the combination of broadly cylindrical inamyloid asci, dark brown spiny ascospores, an epithecium that resembles the excipulum, and a disorganized hymenium. It is most similar to the closely related *A. cineraceus*, but differs in its smaller ascospore size (20.5 µm av. for *A. fuscosporus* vs 24.4 µm av. for *A. cineraceus*).

Amylascus hallingii Healy & M.E. Sm., *sp. nov.* — MycoBank MB 847734; Fig. 9

Etymology. Named after the eminent mycologist Roy Halling, one of the collectors of this species.

Typus. Australia, Queensland, Springbrook National Park, Apple Tree Park, hypogeous in soil of wet sclerophyll woodlands with species of *Eucalyptus*, *Syncarpia*, and *Allocasuarina*, 2 June 2011, *R. Halling*, *N. Fechner & T. Baroni AQ794771* (holotype NY 1491200).

Ascomata oblong, lumpy, folded, convoluted, fresh size not recorded, dry size 15 × 10 × 8 mm, sessile with basal tuft of hyphae, outer surface ornamented with high, tapered warts, hairless except for basal hyphae. Dark reddish brown to black when fresh, basal hyphae white. Gleba between excipulum and epithecium, white to yellowish or greenish, convoluted with irregular canals that come to the surface at places on the outside. Epithecium indistinguishable from excipulum. Odor not recorded. *Excipulum.* 81–600 µm thick, ectal excipulum with conical warts up to 500 µm high, highest in the crevices, reddish brown in water and KOH under transmitted light, composed of isodiametric to rectangular rounded cells up to 110 × 49 µm, outermost cells with thick walls, up to 3 µm, inner cell walls 1.5 µm. *Epithecium* same color and similar structure as excipulum, conical warts up to 326 µm, cells up to 88 × 31 µm. Medullary excipulum composed of *textura intricata*, 57–90 µm thick, intergrading with glebal hyphae, both hyaline to light yellow in water and in KOH. Gleba composed of *textura intricata* intermixed with occasional oleiferous hyphae and asci (no organized hymenium), glebal hyphae 3.5–8 µm diam and oleiferous hyphae 10–13 µm diam, walls 1 µm thick. *Paraphyses* not recognizable. *Asci*, clavate, short pedicellate or pedicel lacking, with croziers, weakly but evenly amyloid, 115–181 × 49–62 µm, with (3–)6–8 ascospores arranged uniseriately to biseriately. *Ascospores* globose, filled with one large oil droplet at maturity, pale to deep yellow, ornamented with short spines, 17.7–20.3(–21.5) µm diam, Q = 1, av. 19.8 µm diam excluding spines, 0.8–1.8 µm, av. 1.3 µm high, enveloped by a perispore.

Distribution & Ecology — Known from a single collection, hypogeous, from wet sclerophyll woods of Australia that includes potential ECM trees *Eucalyptus* and *Allocasuarina*, autumn (early June).

No other ascomata collections known.
Mitotic spore mats unknown.

Notes — This species is characterized by its dark reddish brown excipulum with pronounced warts, weakly amyloid clavate asci, and yellow ascospores with spore ornaments that are encompassed by a perispore. This species is most morphologically similar to *A. luteosporus*, but the asci of *A. hallingii* are shorter and more clavate than those of *A. luteosporus*, there is no organized hymenium in *A. hallingii*, and oleiferous hyphae are more prominent in *A. luteosporus*. In addition, *A. hallingii* has a well-developed epithecium that is similar in structure to the excipulum whereas *A. luteosporus* has a weakly developed epithecium that is comprised of loosely interwoven hyphae.

Amylascus luteosporus Healy, Castellano & M.E. Sm., *sp. nov.* — MycoBank MB 847735; Fig. 10

Etymology. Derived from the Latin '*luteus*' meaning deep yellow in reference to the color of the mature ascospores.

Typus. Australia, Queensland, Mt. Glorious, summit along Mt. Glorious Hwy, D'Aguilar State Forest, 4 May 1992, *M.A. Castellano H6013* (holotype FLAS-F-69585).

Ascomata lobed and furrowed with apical to lateral orifice, up to 15 × 24 mm, with basal tuft of hyphae. Outer surface varying from verrucose to smooth, dark brown, orifice yellow, unexposed furrow surfaces dark green. Gleba of infolded dark green fertile and grayish yellow sterile veins lined by empty canals, sometimes converging at the surface of the ascoma. Odor is faint but pleasant. *Excipulum* 255–542 µm thick, ectal excipulum with conical warts up to 441 µm high, outermost portion brown in water, yellow in KOH, inner portion hyaline to lighter brown than outer portion. Ectal excipulum composed of a *textura angularis* intergraded with *textura globulosa*, comprised of large cells up to 49 × 44 µm, outermost cells with thicker walls than interior cells, outermost cell walls 1.2 µm thick, inner cell walls 0.8 µm thick. Medullary excipulum is of a small-celled *textura angularis* grading to a *textura intricata* towards the gleba. Oleiferous hyphae are prominent, with yellow to orange-brown content, hyphae sometimes swollen, parallel to interwoven among other excipular cells. Gleba composed of disorganized hymenia. *Epithecium* present in some places as parallel to loosely interwoven hyphae, but absent over most of the hymenium, so that there is sometimes space (a canal) between the opposing hymenial surfaces. *Paraphyses* in palisade with asci, mostly similar in length to asci but some are longer than the asci and are loosely interwoven to form a rudimentary epithecium. Some paraphyses branched near the base or up to mid length, hyaline, non- to slightly inflated, 4–8.4 µm at septum, sometimes inflated 5–10 µm. *Asci* cylindrical to broadly cylindrical, with short pedicels ending in croziers, 190–265 × 29–40 µm, pedicels up to 24 µm long × 16 µm diam, weakly but evenly amyloid in Melzer's reagent, with 7–8 ascospores arranged uniseriately to irregularly biseriately. *Ascospores* globose, filled with one large oil droplet at maturity, hyaline to deep yellow in water and KOH, medium brown in Melzer's reagent, ornamented with short spines, 16.5–20.6 µm diam, Q = 1, av. 18.7 µm diam excluding spines, spines 0.8–2.5 µm, av. 1.7 µm high, enveloped by a perispore.

Distribution & Ecology — Australia. Hypogeous in montane forest with *Eucalyptus* species, autumn (Apr.).

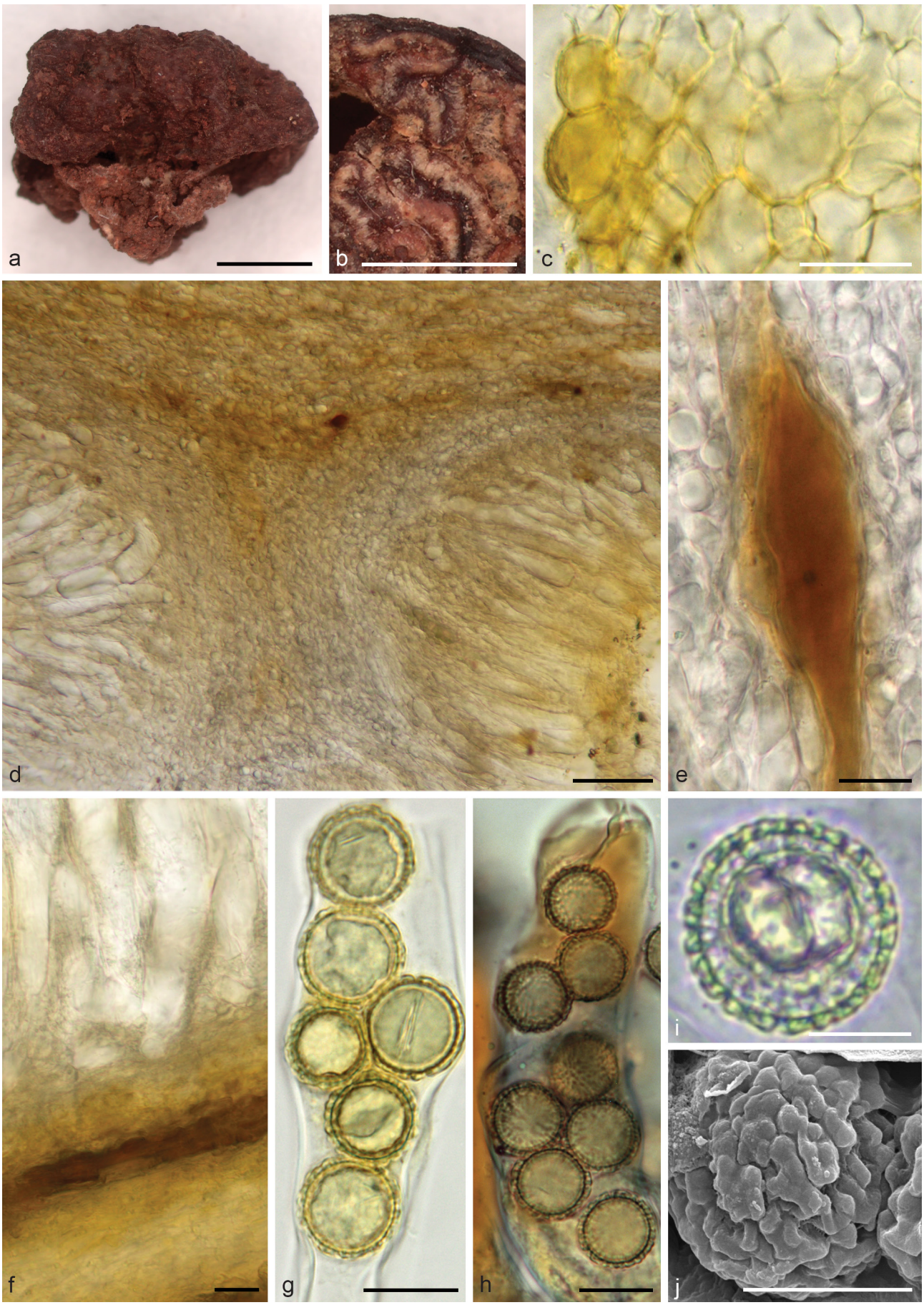

Fig. 10 *Amylascus luteosporus* (FLAS-F-69585, holotype). a. Ascoma exterior (dry); b. gleba of ascoma (dry); c. cross section through excipulum; d. section through gleba showing palisade of paraphyses and asci on each side of the trama; e. oleiferous hypha in medullary excipulum; f. oleiferous hyphae in medullary excipulum; g. ascus with yellow, mature ascospores; h. amyloid ascus and ascospores that are brown in Melzer's reagent; i. ascospore showing spines and perispore; j. SEM of ascospore showing perispore surface. — Scale bars: a, b = 0.5 mm; c = 50 µm; d = 100 µm; f–h = 20 µm; i, j = 10 µm.

No other ascomata collections known.
Mitotic spore mats unknown.

Notes — This species is characterized by its reddish brown excipulum with pronounced warts, abundant oleiferous hyphae in the excipulum, weakly amyloid clavate asci in a loosely organized hymenium, with hymenia clearly separated by veins of *textura intricata*, and yellow ascospores with spore ornaments that are encompassed by a perispore. This species is similar to *A. hallingii* in its DNA sequences, having significant support as sister species, and also has morphological similarities. There is a distinct (though disorganized) hymenium in *A. luteosporus* that is lacking in *A. hallingii*, and the asci of *A. hallingii* are shorter and more clavate in shape than those of *A. luteosporus.* Oleiferous hyphae are more prominent in *A. luteosporus* than in other species of *Amylascus.* In addition, *A. hallingii* has a well-developed epithecium that is similar in structure to the excipulum whereas *A. luteosporus* has a weakly developed epithecium that is comprised of loosely interwoven hyphae. We were unable to obtain the single copy genes necessary to include this species in our multi-locus analysis, but the ITS sequence and morphological characters help to separate this species from all others.

Amylascus procerus Healy, Castellano & M.E. Sm., *sp. nov.* — MycoBank MB 847736; Fig. 11

Etymology. The Latin epithet '*procerus*', means long, and refers to the elongated paraphyses in this species.

Typus. Argentina, Neuquen, Lanín National Park, Ruta 48A Hua Hum, 3.5 km from the border with Chile, under *Lophozonia alpina*, *L. obliqua*, and *Nothofagus dombeyi*, 25 Apr. 2001, *M.A. Castellano JT26241* (holotype CORD-C4233; isotype FLAS-F-69556).

Ascoma irregularly subglobose, folded, 17 × 12.5 mm, sessile, with basal tuft of hyphae, outer surface pinkish brown with white to pinkish brown warts, ~3 per mm, when fresh. Outer surface hairless except for basal tuft of hyphae, basal hyphae cream colored. Gleba convoluted with cavities and irregular canals of various sizes that come to various places at the surface, epithecium mostly suede-like (smooth) and white with underlying yellow layer, but in some places forming brown warts similar

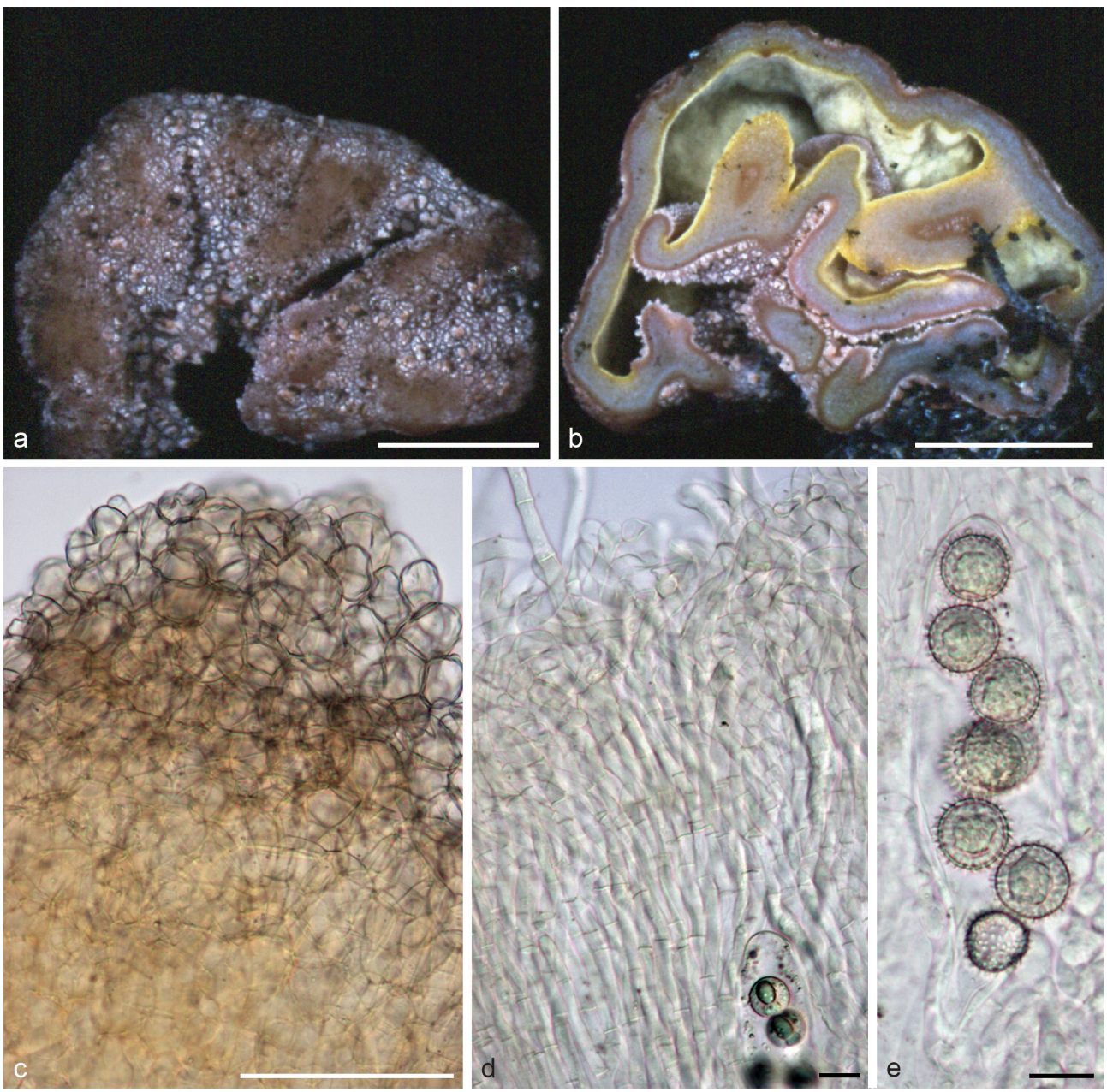

Fig. 11 *Amylascus procerus* (CORDC4233, holotype). a. Fresh ascoma showing excipulum with excipular warts; b. fresh ascoma showing gleba and smooth epithecium; c. excipular wart; d. hymenium showing overgrowth of asci by paraphyses; e. ascus with ascospores (immature). — Scale bars: a, b = 0.5 cm; c = 100 µm; d = 20 µm; f = 10 µm.

to those on the surface. Epithecium lines the cavities and canals. Odor not recorded. *Excipulum* 160–650 µm thick, with conical warts up to 500 µm high in the crevices, but shorter, up to 300 µm on exposed surfaces of excipulum, hyaline to light brown in water and KOH, composed of large isodiametric to ovoid cells, up to 52 × 30 µm, walls 0.8–1 µm thick, medullary excipulum hyaline to lighter brown; of similar-sized cells to ectal excipulum. *Epithecium* hyaline, composed of parallel to interwoven paraphyses, 8 µm diam, even to slightly inflated at tips to 10 µm, exceed ascus by 180–280 µm, tips free or interwoven to form epithecium, in some places similar to excipulum with brown warts up to 200 µm high, composed of isodiametric to oblong rounded cells. Occasional oleiferous hyphae present among asci. *Asci* clavate, with croziers, inamyloid, 240–280 × 40–48 µm, with 4–8 ascospores arranged irregularly biseriately. *Ascospores* globose, filled with one large oil droplet at

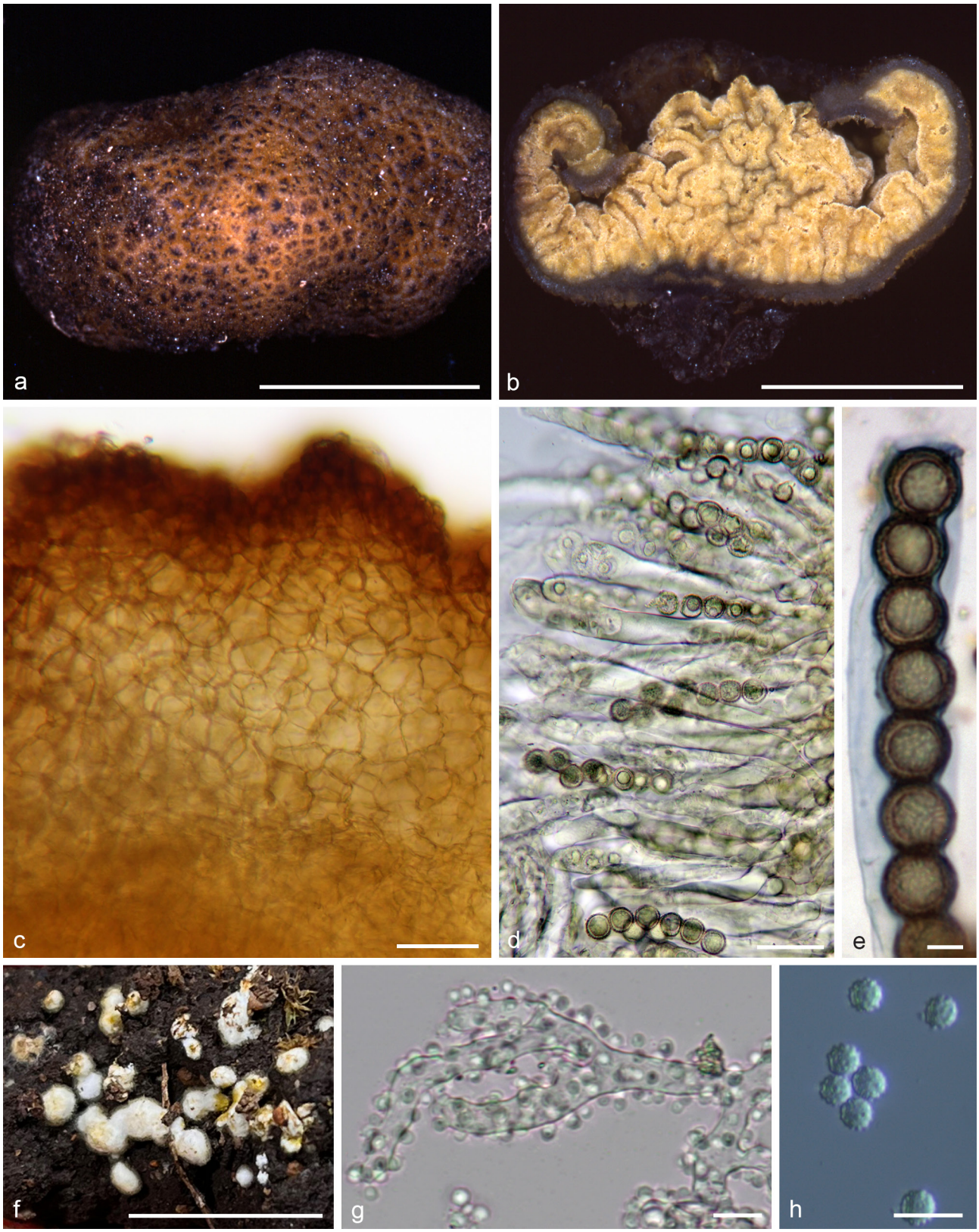

Fig. 12 a–f. *Amylascus verus* ascoma (CORDC4248, holotype), g–i. mitotic spore mat of *A. verus* (MES-4218, MES-4227). a. Fresh ascoma showing excipulum with low excipular warts; b. fresh ascoma showing gleba; c. section through excipulum; d. hymenium in gleba; e. amyloid ascus with mature ascospores (Melzer's reagent); f. mitotic spore mat (MES-4218); g. conidiophore with immature mitotic spores (MES-4227); h. mature mitotic spores showing warts (MES-4227). — Scale bars: a, b, f = 1 cm; c, d = 50 µm; e, g, h, = 10 µm.

maturity, ornamented with truncate spines, light yellow-brown when mature, 19–22(–23.9) µm diam, Q = 1, av. 21.5 µm diam excluding spines, spines (1–)1.7–2.9(–3.4) µm, av. 2.5 µm high, some spine tips coalesce, without a perispore covering the spines; ascospore wall 1.5 µm thick.

Distribution & Ecology — Southern South America (Argentina). Hypogeous in montane forests of *Lophozonia obliqua*, *L. alpina*, and *Nothofagus dombeyi*, autumn (Apr.). Known from a single collection.

No other ascomata collections known.
Mitotic spore mats unknown.

Notes — This is one of only two species of *Amylascus* known to have an epithecium of paraphyses with mostly free to loosely interwoven tips such that much of the epithecium is markedly different in texture than the excipulum. The epithecium in *A. luteosporus* is more rudimentary than in *A. procerus*. *Amylascus luteosporus* also has a palisade of paraphyses, but not all of them exceed the asci and those that do exceed the asci are less organized. The ascoma in the collection may not be fully mature. While we measured only ascospores that had the hallmarks of maturity (thick, refractive walls), many ascospores were immature. When additional, mature specimens are collected, the range and average of ascospore size may be amended. *Amylascus procerus* is phylogenetically supported in a clade that includes an undescribed species (*Amylascus* sp. 1) known only from DNA sequences of *Nothofagaceae* ECM root tips, mitotic spore mats, and an immature ascoma.

Amylascus verus Healy & M.E. Sm., *sp. nov.* — MycoBank MB 847737; Fig. 12

Etymology. The Latin epithet '*verus*' means true, and refers to the true amyloid character of the asci of this species in response to Melzer's reagent, in keeping with the implication of the generic name. This is the only *Amylascus* species in South America that has amyloid asci.

Typus. Argentina, Neuquén, Lanín National Park, Ruta 48A Hua Hum, 3.5 km from the border with Chile, under *Lophozonia alpina*, *L. obliqua*, and *Nothofagus dombeyi*, 25 Apr. 2001, *E. Cázares*, *L. Domínguez*, *M.A. Castellano*, *C. Barroetaveña JT26238* (holotype CORD-C4248; isotype FLAS-F-69554).

Ascomata knobby, convoluted, up to 23 × 15 mm, sessile. Excipular surface brown with reddish tints, verrucose with low warts. Interior hollow, lined with velvety hymenium, convoluted towards the surface, yellow with castaneus brown subhymenium, no epithecium. Odor not detected. *Excipulum* 150–396 µm thick, outer excipulum with low warts up to 274 µm high, brown in water, dextrinoid in Melzer's reagent and yellowish brown in KOH, composed of isodiametric to rectangular cells up to 50 × 40 µm, outermost cells with thick walls, 3 µm thick, inner cell walls 1.5 µm. No epithecium. Gleba yellow in water and in KOH, composed of hymenium and subhymenium. Subhymenium of *textura intricata*, hyphae 7–8 µm diam. Occasional oleiferous hyphae present in the gleba. Hymenium of asci and paraphyses in an irregular palisade. *Paraphyses* variable in length with some shorter than asci and others extending beyond asci, not inflated, septate, hyaline, 165–198 × 5–7 µm. *Asci* cylindrical to broadly cylindrical, evenly but diffusely amyloid, 180–260 × 28–40 µm, including short pedicel 10–38(–63) × 7.5–15 µm diam, arising from croziers, with 5–8 ascospores arranged uniseriately to irregularly biseriately in the ascus. *Ascospores* globose, ornamented with short spines, yellow, 16–18(–20) µm diam, Q = 1, av. 18 µm diam excluding spines, spines 1–2 µm, av. 1.5 µm high, walls 1 µm thick, enveloped by a perispore.

Mitotic spore mats composed of white hyphae with yellow mitotic spores (mature) that develop in small patches on soil surface. Basal hyphae yellowish brown to reddish brown, growing together in bundles of parallel hyphae, smooth, even, septate, 3.5–5.8 µm diam. Hyphae subtending the conidiophores hyaline to light yellow, smooth, loosely interwoven, septate, 4.3–7.7 µm diam. Conidiophores smooth, hyaline, branching, but branches of uneven lengths with one branch usually much longer than the other (e.g., 17 µm long and 44 µm long), gently curved at various angles, and width variable on the same branch 6.5–9.9 µm diam. Polyblastic mitotic spores produced synchronously from denticles (≤ 1 µm long) along conidiophore branches. Mitotic spores globose to subglobose, dry, hydrophobic, warted, (4.5–)4.6–5.5(–5.7) µm diam, av. 5.1 µm diam including warts, Q = 1–1.1; warts 0.43 µm high, unevenly distributed along the mitotic spore wall.

Distribution & Ecology — Southern South America (Chile and Argentina). Hypogeous in montane *Nothofagaceae* forests, autumn (Apr.). Ascomata known from a single collection.

No other ascomata collections known.

Mitotic spore mat collections examined. Chile, Maule, Cauquenes, Reserva Nacional Los Ruiles, in forest of *Fuscospora alessandrii*, *Lophozonia glauca*, *N. dombeyi* (-35.833889S – -72.508611W), 265 m a.s.l., 14 May 2022, *B. Lemmond MES-4218* (FLAS-F-70744); ibid., *B. Lemmond MES-4227* (FLAS-F-70753).

Notes — This species is characterized by its irregular palisade of cylindrical to broadly cylindrical amyloid asci with clearly differentiated paraphyses, yellow ascospores whose ornamentation is encompassed by a perispore, and the lack of an epithecium. This is the only known species of *Amylascus* from South America in which the asci are amyloid in Melzer's reagent and is also the only South American species that lacks an epithecium. Our multi-locus phylogeny (Fig. 2) places *A. verus* in a well-supported clade with the Australian species *A. hallingii*, which also has amyloid asci. In our ITS phylogeny, *A. verus* clusters closely with undescribed species *Amylascus* sp. 9 and *Amylascus* sp. 10, both of which are known from mitotic spore mats and/or environmental samples (e.g., ECM roots, bird feces samples).

Nothoamylascus Healy & M.E. Sm. *gen. nov.* — MycoBank MB 847738

Etymology. '*Notho*' meaning in close agreement with *Amylascus*, but different. '*Notho*' also refers to the occurrence of the genus in *Nothofagaceae* forests.

Ascoma a ptychothecium, hypogeous. *Excipulum* composed of *textura angularis* or *textura globulosa*, with large isodiametric to ovoid cells, intermixed with oleiferous hyphae. *Gleba* composed of asci and paraphyses in a palisade. *Asci* cylindrical, evenly amyloid in Melzer's reagent. *Ascospores* globose, ornamented with truncated spines. Mitotic spore mats in small clusters, epigeous on soil or hypogeous, white with pinkish brown areas, spore mass pink when young but yellow at maturity, mitotic spores nearly smooth to minutely warted, globose to subglobose.

Distribution & Ecology — Known only from South America. Ectomycorrhizal with species of *Nothofagaceae*.

Type. *Nothoamylascus erubescens* Healy & M.E. Sm.

Nothoamylascus erubescens Healy & M.E. Sm., *sp. nov.* — MycoBank MB 847739; Fig. 13

Etymology. The Latin epithet '*erubescens*' means 'turning red' and refers to the color change of the mitotic spore mat that is white when young but then turns pinkish red to reddish brown at maturity.

Typus. Argentina, Río Negro, Parque Nacional Huapi, Chalhuaco Rd. south of Bariloche, 6 km north of Refugio Neumeyer, 41 14.536 71 17.226, 23 Apr. 2001, *J.M. Trappe JT26170* (holotype CORD-C4252; isotype FLAS-F-69560).

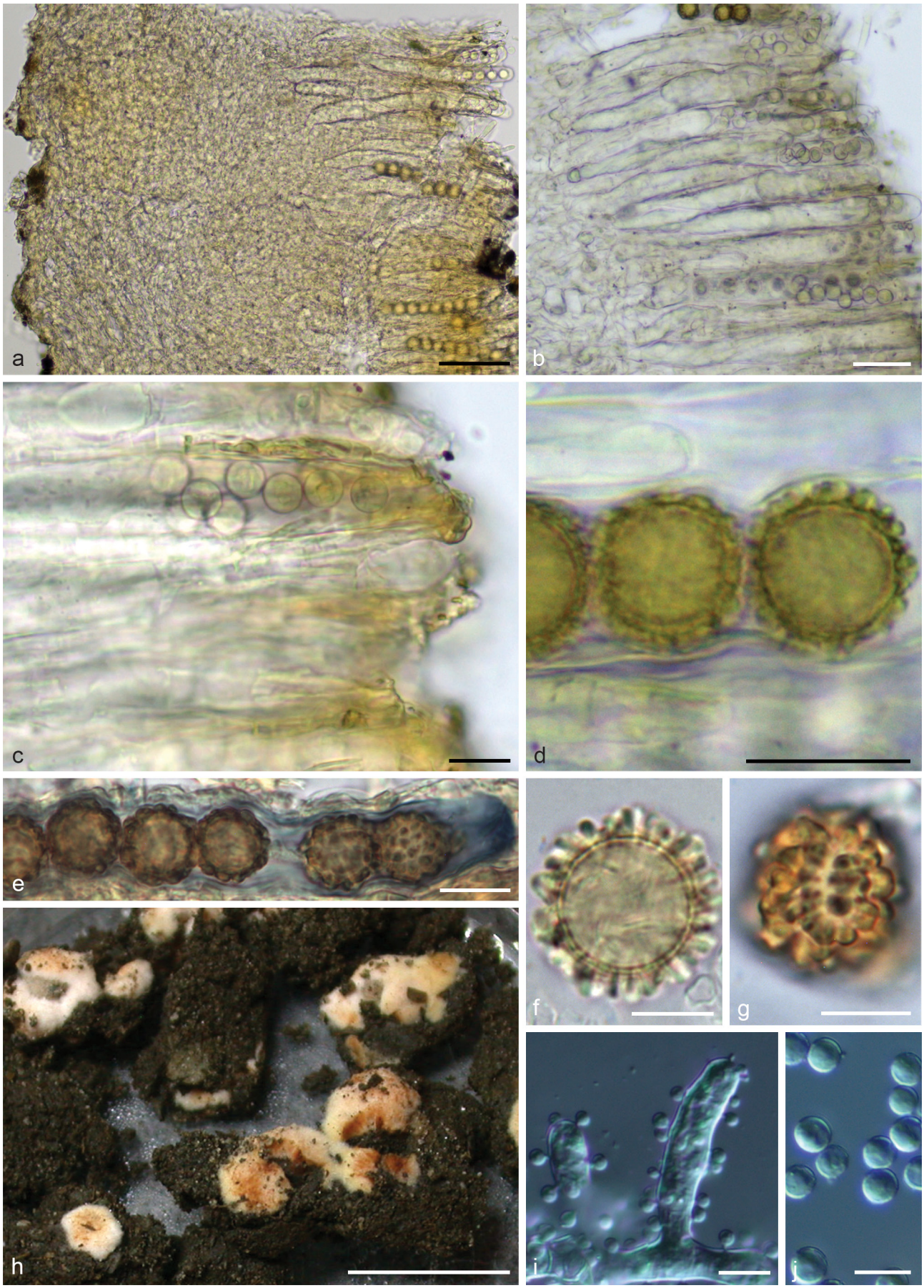

Fig. 13 a–g. *Nothoamylascus erubescens* ascoma (CORDC4252, holotype), h–j. *N. erubescens* mitotic spore mat (MES-2158). a. Cross section of ascoma; b. cross section through hymenium; c. hymenium of asci and paraphyses with yellow contents; d. mature yellow ascospores in ascus; e. amyloid ascus and ascospores (Melzer's reagent); f. ascospore (immature); g. ascospore (mature, Melzer's reagent); h. mitotic spore mat (MES-2158); i. conidiophore (MES-2158); j. mature mitotic spores (MES-2160). — Scale bars: a = 100 µm; b = 50 µm; c–e = 20 µm; f, g, i, j = 10 µm; h = 1 cm.

Ascoma infolded, convoluted, with a basal tuft of hyphae. Yellow brown, hairless except for basal attachment. Gleba grayish white when fresh then pale brown with pale orange veins when dry. Odor not recorded. *Excipulum* 269–336 µm thick, hyaline to light brown in water and KOH, brown in Melzer's reagent, composed of *textura angularis*, up to 17 × 22 µm, walls 1.5 µm thick, with occasional large, rounded cells up to 39 × 32 µm, and oleiferous hyphae, yellow, 6.6 µm diam at septum, inflated to 9.5 µm, medullary excipulum, if present, not recognizable. Gleba composed of a hymenium and subhymenium. Subhymenium of *textura intricata*, hyphal walls 1 µm thick. *Epithecium* lacking. Hymenium composed of a palisade of asci and paraphyses that are more or less equal in length to asci, hyaline to yellow (at least near tips), 8 µm diam, even to slightly inflated at tips to 12.5 µm diam, walls 1 µm thick. *Asci* cylindric, with croziers, evenly amyloid, 248–300 × 20–29 µm tapering to 8 µm diam near base, with 8 ascospores arranged uniseriately in the ascus. *Ascospores* globose, ornamented with course spines, yellow in water and KOH, brown in Melzer's reagent when mature, 15–19 µm diam, Q = 1, av. 16.7 µm diam excluding spines, spines 1–3.5 µm, av. 2.2 µm high, without a perispore covering the spines; ascospore walls 1 µm thick.

Mitotic spore mats composed of white hyphae that turns pinkish to reddish brown in some places, with white mitotic spores that become yellow at maturity. Mitotic spore mats develop in small patches on the soil surface, patches up to ~1 cm diam. Subtending hyphae smooth, hyaline to pinkish brown, branching at right angles, not conidiogenous, conidiophores rising from subtending hyphae, smooth, hyaline, branching, but branches of uneven lengths, 7–7.7 µm diam at septum, not inflated, conidiogenous tips 5.4–7.5 µm diam. Polyblastic mitotic spores produced synchronously from denticles, 0.6–0.8 µm long, 1 µm wide, along conidiophore branches. Mitotic spores globose to subglobose, dry, hydrophobic, rough, 5–6 µm diam, av. 5.1 µm diam, warts miniscule, well-separated on spore surface, hyaline under transmitted light.

Distribution & Ecology — South America (Chile and Argentina), ascomata hypogeous and mitospore mats epigeous on soil in montane forests of *Lophozonia alpina*, *L. obliqua*, *Nothofagus pumilio*, and *N. dombeyi*, autumn (Apr.).

Other ascomata collections examined. Argentina, Rio Negro, Nahuel Huapi National Park, Chalhuaco rd., south of Bariloche, 6 km north of Refugio Neumeyer, under *Nothofagus pumilio*, 23 Apr. 2001, *C. Barroetaveña JT26175* (CORD, FLAS-F-69552).

Mitotic spore mats examined. Argentina, Cerro Otto, up the mountain from Bariloche, on soil in *Nothofagaceae* forest, 17 Mar 2012, *M.E. Smith & D.H. Pfister MES-574* (DUKE); Río Negro Arroyo Goye, along a slope at trail edge on exposed soil in *N. dombeyi* forest (41 06 24.8S – 71 31 9.7W) 926 m a.s.l., 29 Oct. 2015, *A. Mujic AR15-002* (FLAS-F-63723, CORDC5253); Nahuel Huapi National Park, 1 km before Lago Hess on soil in *Nothofagus antarctica* and *N. dombeyi* forest, 18 May 2016, *N. Policelli MES-2158* (FLAS-F-64773); ibid., *R. Healy MES-2160* (FLAS-F-64774). – Chile, Osorno Puyehue National Park, foothills of Volcan Puyehue, up the road past El Caulle north of Rio Golgol, on bare soil in *Nothofagus dombeyi* forest, along bank of dry creek bed, 4 May 2016, *R. Healy MES-1651* (FLAS-F-64664).

Notes — As with many of the taxa discussed here, ascomata of *N. erubescens* are apparently quite rare. The ascomata of this species are known only from two collections, but DNA sequences could not be produced from one of the two specimens (JT26175). Unfortunately, our description of the ascoma is solely from dried, fragmented material and no photographs of the fresh collections exist. Future collections will be needed to describe all the variation present in this species, particularly the appearance of the fresh ascoma. This species can be recognized by the combination of the truffle-like form, amyloid asci, regularly formed hymenium, lack of an epithecium, and yellow ascospores that turn brown in Melzer's solution and lack a perispore.

Although ascomata of this species are rare or difficult to find, the mitotic spore mats have been collected from multiple sites in *Nothofagaceae* forests of Chile and Argentina, and a sequence of this species was also detected from an ectomycorrhizal root of *Nothofagus pumilio*. The mitotic spore mats of this species differ from those produced by *Amylascus* in the reddish brown strands of hyphae in the otherwise white mitotic spore mat. The mature mitotic spores are light yellow, like those of *Amylascus* species. We also detected morphologically similar mitotic spore mats at several sites in Chile that were resolved as a second, undescribed species of *Nothoamylascus* (Fig. 2) for which no ascomata have yet been found.

DISCUSSION

Phylogeny, morphology, ecology, distribution, and revised concept of Amylascus

Here we provide the first revision of *Amylascus* since Trappe (1971) erected the genus, based on *A. herbertianus* as the type. Trappe (1975) added a second species, *A. tasmanicus*, and for more than 40 years *Amylascus* was thought to include only two species and to be restricted to Australia. However, we show that *Amylascus* is considerably more phylogenetically diverse, morphologically varied, and geographically dispersed than previously realized. To this point, our description of five new species from South America and two new species from Australia substantially expands the morphological concept of *Amylascus*, and also demonstrates its widespread 'southern Gondwanan' distribution (Fig. 1). Interestingly, a similar biogeographic distribution pattern has been found with several other lineages of ectomycorrhizal *Pezizales*, including members of the genera *Aleurina*, *Geomorium*, *Nothojafnea*, and *Ruhlandiella* (Pfister 1984, Bonito et al. 2013, Kraisitudomsook et al. 2019, 2020).

Through phylogenetic analyses we also discovered a unique lineage that we describe here as *Nothoamylascus*. The ascomata of this group appear to be quite rare, thus far consisting of only two individual ascomata of the type species, *N. erubescens.* Despite the small amount of material available for morphological characterization, we felt it necessary to describe *Nothoamylascus*, in part because this new genus is the well-supported sister taxon of a clade that includes both the Southern Hemisphere genus *Amylascus* and the Northern Hemisphere genera *Pachyphlodes* and *Plicariella*. The discovery of this new lineage in South America also indicates that additional cryptic diversity within this group of *Pezizaceae* may also be present, but not yet discovered, in Australia and other parts of Australasia.

Our phylogenetic analysis (Fig. 2), which includes ectomycorrhizal root tip sequences from multiple species of South American *Lophozonia* and *Nothofagus*, also adds additional new evidence that *Amylascus* is ectomycorrhizal with *Nothofagaceae*, providing important context regarding the trophic ecology of these fungi and ecological constraints on their distribution. Although there are no available DNA sequences from ectomycorrhizal root tip sequences of *Amylascus* species from Australia, the described Australian species have been collected in soil and leaf litter beneath species of *Myrtaceae*, and thus likely form ectomycorrhizas with *Eucalyptus* and related host plants. Further studies looking at ectomycorrhizal roots of Australasian species of *Myrtaceae* and *Nothofagcaeae*, especially from New Zealand, are needed to bring to light the evolutionary history and host range of *Amylascus* in the southern hemisphere.

Even though ascomata of *Amylascus* are rarely found during truffle surveys, by including data on mitotic spore mats, ectomycorrhizal roots, and mycophagous bird feces we nonetheless documented high species diversity and prevalence in

native forest soil based on ITS rDNA sequences. Although mitotic spore mats have been previously documented in the closely related genera *Pachyphlodes* and *Plicariella* (Healy et al. 2013, 2015, 2018), our specimens constitute the first report of mitotic spore mats from *Amylascus* (Fig. 2). The role of these mitotic spores is unknown, however, they are thought to play a role either in sexual outcrossing (e.g., spermatia), as vegetative propagules (e.g., conidia), or both (Healy et al. 2013). Altogether, we documented an estimated 13 species of *Amylascus* and two species of *Nothoamylascus* from mitotic spore mats, including ten species that remain undescribed because ascomata for these taxa have yet to be found. We found mitotic spore mats to be particularly informative because in many cases they significantly expanded the known range of a species. For example, *Amylascus fuscosporus* and *A. verus* were each known from only a single collection of ascomata from Lanín National Park in Argentina, yet both were documented as mitotic spore mats from Chile in 2022 (*A. fuscoporus* from Cerro Cayumanque in the Ñuble region and *A. verus* from the Reserva Nacional Los Ruiles in the Maule region).

Mitotic spore mats of *Amylascus* are more variable in their sporulating habit than those found thus far in the Northern Hemisphere genera *Pachyphlodes* and *Plicariella*. Specifically, while mitotic spore mats of *Pachyphlodes* and *Plicariella* are typically found on top of the soil and leaf litter, *Amylascus* mitotic spore mats have sometimes been found on top of the soil but are more frequently detected below the surface of the soil and litter. Although we cannot explain why *Amylascus* mitotic spore mats occur belowground, we hypothesize that this growth form could be an adaptation to, or consequence of, the mycophagous animals that inhabit the South American *Nothofagaceae* forests. A recent study by Caiafa et al. (2021) found that two species of common endemic birds, *Scelorchilus rubecula* and *Pteroptochos tarnii*, regularly consume hypogeous fungi in Chilean *Nothofagaceae* forests, including *Amylascus* species. Although Caiafa et al. (2021) produced relatively short ITS1 fragments of the fungi from fecal samples, we were nonetheless able to detect three distinct species of *Amylascus* that were consumed by birds. All of these remain undescribed but have been found as mitotic spore mats and on *Nothofagaceae* ECM root tips (Fig. 2 – *Amylascus* sp. 4, sp. 10 and a species whose molecular signature is between sp. 6 and sp. 7). When *Scelorchilus rubecula* and *Pteroptochos tarnii* are foraging for fungi and other foods in the understory, they regularly disturb large patches of soil and leaf litter. We have observed individuals of *Pteroptochos tarnii* using their feet to forcefully throw litter and soil up to a meter or more, suggesting that this bird may be responsible for dispersing mitotic spores from *Amylascus* mitotic spore mats (Smith & Caiafa, pers. obs.) in addition to eating the ascomata and dispersing the ascospores in their feces. Clearly, more studies are needed to assess this hypothesis, and to determine which animals might be involved in dispersing spores of *Amylascus* species in Australia and potentially also in New Zealand, where bird mycophagy seems to be a common phenomenon (Elliot & Vernes 2019, Elliot et al. 2019).

In addition to the new observations on the ecology and distribution of *Amylascus* in South America described above, the seven new *Amylascus* species that are described here significantly expand the morphological diversity within the genus. *Amylascus* originally included only two species, *A. herbertianus* and *A. tasmanicus* (Fig. 4, 7), which shared features such as a well-developed epithecium and globose to irregular amyloid asci that are not organized in a hymenium. With the addition of the new taxa described here, we expand the diversity in a number of morphological characters, including ascus shape (ovoid to cylindrical), ascospore color (hyaline, yellow, or dark brown), ascospore ornamentation (free spines or with spines encompassed by a perispore), epithecial structure (hyphal with free ends or composed of *textura angularis* similar to the excipulum), and glebal tissue (ranging from hymenial with a palisade of asci and paraphyses to disorganized with asci randomly distributed in the gleba). The amyloid response of the ascus in Melzer's reagent is a defining feature of the epigeous *Pezizaceae* that forcibly discharge their ascospores, but this feature is absent in *Marcelleina*, and many truffle-like *Pezizaceae*, including species of *Cazia*, *Eremiomyces*, *Mattirolomyces*, *Terfezia*, and others (Hansen et al. 2001, Læssøe & Hansen 2007, Kovács et al. 2011). Intrageneric variability of an amyloid reaction is unusual within the *Pezizaceae*, although it has been reported for the truffle-like *Hydnobolites*, *Pachyphlodes* (Læssøe & Hansen 2007, as '*Pachyphloeus*'), and *Ruhlandiella* (Kraisitudomsook et al. 2019). Within *Amylascus* we also see intrageneric variability of the amyloid reaction of asci in Melzer's solution. Amyloid asci are present in known species of Australian *Amylascus* as well as the new South American species *A. verus*, but absent in the South American *A. cineraceus*, *A. dominguezia*e, *A. fuscosporus*, and *A. procerus* (Fig. 1). These four inamyloid species form a well-supported phylogenetic clade. *Nothoamylascus erubescens* retains the amyloid asci and has other characteristics of epigeous *Pezizaceae*, including a hymenium of cylindrical asci and paraphyses in a palisade.

Lineage divergence time analysis

Our dating analysis of the larger clade (which includes *Amylascus*, *Luteoamylascus*, *Nothoamylascus*, *Pachyphlodes*) suggests an origin that is consistent with the breakup of the Gondwana supercontinent during the late Cretaceous (60–100 Ma) (Fig. 3). *Nothoamylascus* started to diverge 55–89 Ma, prior to the final breakup of Gondwana and the initiation of the Antarctic glaciation near the Eocene/Oligocene boundary c. 35 Ma (DeConto & Pollard 2003, Sanmartín & Ronquist 2004). At the time, Antarctica had a warm-temperate climate and is thought to have been forested (until c. 25 Ma). This timeline is consistent with the divergence time of *Amylascus* (20–37 Ma), and a Gondwanan origin. Diversification within the *Amylascus* clade is more recent, with divergence time estimates between Australian and South American species starting around 25 Ma and up to 14 Ma, as a result of biotic isolation and drastic climatic changes generated from the onset of the Antarctic glaciation. Although no *Amylascus* species occurs on both continents, long-distance dispersal between South America and Australia cannot be excluded as a possibility to explain species distribution patterns, especially for the *A. verus*-*A. hallingii* clade for which tMRCA was estimated at 8–22 Ma. However, all estimates must be regarded with caution when considering the possibility of extinct taxa and the undersampling of Australia, and potentially New Zealand, and the underlying assumptions involved in estimating divergence times. Our estimates for divergence times for the *Helvellaceae* and for the *Morchellaceae*-*Discinaceae* clades closely follow previous studies based on the absolute rate of molecular evolution for 28S (Bonito et al. 2013, Kraisitudomsook et al. 2020), which gave a mean of 35 Ma for *Helvellaceae* and 90 Ma for the *Morchellaceae*-*Discinaceae* clade.

Phylogenetic relationships and morphological connections

Pachyphlodes is sister to *Amylascus* in our analyses, a result that is consistent with previous findings. Dissing & Pfister (1981) made the connection between *Pachyphlodes* and *Scabropezia* (= *Plicariella*) based on morphological similarity, and this was later verified via molecular phylogenetic analyses (Norman & Egger 1999, Hansen et al. 2005). *Pachyphlodes* species are mostly truffle-like fungi, but this genus also includes cup-shaped species (these are currently assigned to the genus *Plicariella* but will be transferred to *Pachyphlodes* at a later date). *Plicari-*

ella species are the only known cup-shaped, epigeous members in the *Amylascus-Pachyphlodes-Nothoamylascus-Luteoamylascus* clade. Species in the genus *Pachyphlodes* share many morphological features with species of *Amylascus*. Specifically, species of *Pachyphlodes* have large-celled *textura angularis* in the ectal excipulum, often have a hyphal tuft at the base, have globose, ornamented ascospores with a single large guttule, form ectomycorrhizas with forest trees, and produce anamorphs that are morphologically similar to those in *Amylascus* species (Healy et al. 2013, 2015). *Pachyphlodes* species also have a similar range of variation in characters as reported here for species of *Amylascus*. *Pachyphlodes* species are tomentose (e.g., *P. conglomerata*) or hairless except for the basal tuft of hyphae, have asci that are globose, clavate, or cylindrical, have asci that are amyloid (e.g., *P. melanoxantha*) or inamyloid, have spores that are arranged uniseriately, biseriately, or irregularly in the ascus, have spores that are hyaline, yellow, or brown, and have spores that possess or lack a perispore (Dissing & Pfister 1981, Hansen et al. 2005, Tedersoo et al. 2010, Healy et al. 2015, 2018).

Luteoamylascus aculeatus is the earliest diverging lineage of the *Amylascus-Pachyphlodes-Nothoamylascus-Luteoamylascus* clade. It is a monospecific truffle genus described from the Mediterranean Basin and is strongly supported as sister to the *Amylascus-Pachyphlodes-Nothoamylascus* lineage, but *L. aculeatus* is consistently resolved on a long branch (Fig. 2; Cabero et al. 2016). Cabero et al. (2016) provided molecular phylogenetic evidence for an ectomycorrhizal ecology and for mitotic spore mat production within the *Luteoamylascus* lineage, although no ECM root data or mitotic spore mat data are specifically linked to *L. aculeatus*. *Luteoamylascus aculeatus* can be distinguished from most *Pachyphlodes* and *Amylascus* species by its smooth excipular surface, but the appearance of the excipular surface of *Nothoamylascus* is currently poorly characterized and requires additional new specimens so that it can be compared accurately to that of *L. aculeatus*.

In our lineage divergence time analysis, *Sarcopeziza sicula* was inferred as sister to the *Amylascus-Pachyphlodes-Nothoamylascus-Luteoamylascus* clade, albeit with low statistical support. This relationship was also suggested in the multi-locus analyses by Agnello et al. (2018) and Van Vooren et al. (2021). *Sarcopeziza* is monospecific and known only from the Mediterranean Basin. This species produces semi-hypogeous, urn-like apothecia on soil, has amyloid asci, and its trophic ecology has not been resolved (Agnello et al. 2018). This hypothesized sister relationship requires further investigation.

CONCLUSIONS

In the first revisionary systematics of *Amylascus* in over 40 years, we used multi-locus phylogenetic inference to generate a nearly fully resolved phylogeny for Southern Hemisphere *Amylascus*. In doing so, we discovered and described a new truffle genus (*Nothoamylascus*) and species (*N. erubescens*), to accommodate a new lineage based on collections of an ascoma and multiple mitotic spore mats that share key features with *Amylascus* species. Divergence time estimates further clarify their distant relationships to Northern Hemisphere genera, including *Pachyphlodes* and *Luteoamylascus.* By integrating environmental sequence data into the phylogeny (e.g., mitotic spore mat collections, ectomycorrhizal root surveys, and bird feces samples), we were able to describe seven new *Amylascus* species, including five from South America and two from Australia, expand the current known range and ecology for most of these species, and further phylogenetically characterize 14 other *Amylascus* species and one *Nothoamylascus* species known only from environmental sequence data. We document the morphological diversity of ascomata and mitotic spore mats that are phylogenetically affiliated with *Amylascus* and morphologically similar to those in *Pachyphlodes*. Finally, we revise the morphological descriptions of the two described species (*A. herbertianus* and *A. tasmanicus*). Overall, this new morphological delimitation expands the definition of *Amylascus* to include taxa with a wide range of features, including variation in the amyloid reaction of the ascus in Melzer's reagent, ascospore color (hyaline, yellow, or dark brown), ascospore ornamentation (free spines or with spines encompassed by a perispore), epithecial structure (hyphal with free ends, or composed of *textura angularis* like the excipulum), and glebal tissue that ranges from hymenial with a palisade of asci and paraphyses to disorganized with asci randomly distributed in the gleba.

KEY TO DESCRIBED SPECIES OF AMYLASCUS AND NOTHOAMYLASCUS

1. Ascus wall rapidly turning blue in Melzer's reagent 2
1. Ascus wall not turning blue in Melzer's reagent 7
2. Ascomata found in South America with *Nothofagaceae* . 6
2. Ascomata found in Australasia with *Eucalyptus* or other ectomycorrhizal *Myrtaceae* 3
3. Ascospores yellow and (15–)17–22(–23) µm, ornamented with long spines, sometimes recurved, lacking a perispore . . . . . *A. herbertianus*
3. Ascospores with short, straight truncate spines, spine tips embedded in a perispore 4
4. Asci ovoid to oblong, mature ascospores 18–24 µm and light brown . . . . . *A. tasmanicus*
4. Asci cylindrical to clavate, mature ascospores yellow . . . 5
5. Asci clavate, up to 181 × 82 µm wide, paraphyses not in a pallisade, lacking an organized hymenium, ascospores yellow and 17.7–20.3(–21.5) µm . . . . . *A. hallingii*
5. Asci cylindrical to broadly cylindrical, up to 265 µm long, 40 µm wide, asci and paraphyses in a palisade with a loosely organized hymenium, ascospores yellow and 16.5–20.6 µm . . . . . *A. luteosporus*
6. Epithecium absent, ascospores yellow and 15–19 µm, no perispore . . . . . *N. erubescens*
6. Epithecium present or absent, ascospores yellow and 16–18(–20) µm, perispore present . . . . . *A. verus*
7. Asci and paraphyses in a palisade, paraphyses exceeding asci in length and forming a patchy epithecium that is notably different in structure from the excipulum, ascospores 19–22(–24) µm, lightly pigmented (almost hyaline) . . . . . *A. procerus*
7. Asci and paraphyses not in an easily recognizable palisade, epithecium resembling the excipulum, ascospores obviously pigmented some shade of brown or gray. 8
8. Ascospores light brown and less than 18 µm . . . . . *A. domingueziae*
8. Ascospores dark brown to gray, ≥ 18 µm 9
9. Ascospores (22–)24–27(–28) µm, av. 24.4 µm . . . . . *A. cineraceus*
9. Ascospores 18–22(–24) µm, av. 20.5 µm *A. fuscosporus*

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Supplementary material

Fig. S1 Best ML phylograms from separate RAxML analyses of 28S rDNA, *EF1α*, *rpb1* and *rpb2* sequences. Branch supports show bootstrap values ≥ 70 % on top, and posterior probability ≥ 0.95 on bottom. Phylogenetic trees rooted with *Ruhlandiella* and *Phylloscypha* (Pezizaceae) as outgroup taxa.

Table S1 Taxa included in RAxML analysis of ITS sequences. Fields include species, the country where collected, collector number, herbarium accession number, the lifestage that was sequenced (ascoma, mitotic spore mat, or ectomycorrhiza, ECM) or if the sequence was detected from bird feces, the GenBank accession number, and the published reference(s) for the GenBank accession numbers. Types (holotype or isotype) are in **bold** text.

Table S2 Sequences of 28S rDNA, *EF1α*, *rpb1* and *rpb2* downloaded from GenBank for the lineage divergence time analysis. Fields include species, the country where collected, the collector number, the herbarium or culture accession number, the source of the DNA (ascoma or mitotic spore mat), the GenBank accession number, and the published reference(s) for the GenBank accession numbers. Types (holotype or isotype) are in **bold** text.