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High diversity and widespread occurrence of mitotic spore mats in ectomycorrhizal *Pezizales*

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Abstract

Fungal mitospores may function as dispersal units and/ or spermatia and thus play a role in distribution and/or mating of species that produce them. Mitospore production in ectomycorrhizal (EcM) *Pezizales* is rarely reported, but here we document mitospore production by a high diversity of EcM *Pezizales* on three continents, in both hemispheres. We sequenced the internal transcribed spacer (ITS) and partial large subunit (LSU) nuclear rDNA from 292 spore mats (visible mitospore clumps) collected in Argentina, Chile, China, Mexico and the USA between 2009 and 2012. We collated spore mat ITS sequences with 105 fruit body and 47 EcM root sequences to generate operational taxonomic units (OTUs). Phylogenetic inferences were made through analyses of both molecular data sets. A total of 48 OTUs from spore mats represented six independent EcM *Pezizales* lineages and included truffles and cup fungi. Three clades of seven OTUs have no known meiospore stage. Mitospores failed to germinate on sterile media, or form ectomycorrhizas on *Quercus*, *Pinus* and *Populus* seedlings, consistent with a hypothesized role of spermatia. The broad geographic range, high frequency and phylogenetic diversity of spore mats produced by EcM *Pezizales* suggests that a mitospore stage is important for many species in this group in terms of mating, reproduction and/or dispersal.

Keywords: cryptic diversity, ectomycorrhizal *Pezizales*, environmental sequencing, mitospore, truffle

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Introduction

Ectomycorrhizal (EcM) fungi are important plant symbionts that improve plant nutrient status (Baxter & Dighton 2001), mediate drought effects (Warren *et al.* 2008) and enhance seedling establishment (Ashkannejhad & Horton 2006; Nara 2006). EcM fungi are diverse and are comprised of an estimated 20 000–25 000 species (Rinaldi *et al.* 2008) from 66 lineages (Tedersoo *et al.* 2010). Within the *Pezizales* (*Ascomycota*), the order that includes morels and truffles, EcM symbioses have evolved independently at least 16 times (Tedersoo *et al.* 2010). Although *Basidiomycota* often dominate EcM root communities, *Pezizales* are diverse and are prevalent EcM symbionts in many ecosystems, particularly habitats subjected to drought (Gehring *et al.* 1998; Smith *et al.* 2007b) or frequent fires (Warcup 1990; Fujimura *et al.* 2005). Some EcM *Pezizales* proliferate in response to disturbance and at forest edges (Dickie & Reich 2005; Tedersoo *et al.* 2006b). Many pezizalean EcM species show some degree of affinity for mineral soils or soils with high pH (Petersen 1985; Tedersoo *et al.*

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2006a; García-Montero *et al.* 2008; Iotti *et al.* 2010; Bonito *et al.* 2011). Other pezizalean EcM taxa such as *Tuber* spp. are also frequently detected taxa in molecular studies of undisturbed forests (Walker *et al.* 2005; Morris *et al.* 2009) and managed tree plantations (Bonito *et al.* 2011).

Reproduction and dispersal in fungi is carried out through the production of mitospores (spores produced by mitosis) and/or meiospores. Previous research suggests that EcM fungi reproduce and disperse exclusively or primarily through meiospores produced inside or on the surface of fruit bodies (Hutchison 1989). Types of fruit bodies produced by EcM fungi include above-ground mushrooms, cup fungi, jelly fungi and resupinate crusts from which meiospores are forcibly discharged to be dispersed in the wind, or below-ground fruiting structures that in most cases are truffle-like (closed), lack forcible spore discharge and disperse their meiospores passively or through animal mediation (e.g. truffles) (Tedersoo *et al.* 2010). Many saprotrophic and pathogenic relatives of EcM fungi produce mitospores (Nobles 1958; Walther *et al.* 2005), but it has been suggested that the EcM symbiosis may in some way be incompatible with mitospore production (Hutchison 1989; Walther *et al.* 2005). However, most research on sporogenesis and spore dispersal in EcM fungi has focused on species of *Basidiomycota* (Hutchison 1989); *Ascomycota* have received considerably less attention.

Even though *Ascomycota* are noted for their ability to form mitospores, many of these forms have not yet been linked to a meiosporic species (Shenoy *et al.* 2007). This disconnect may be due to spatial and temporal differences in production of these two spore types and also to the difficulty of stimulating spore production in pure culture. In addition, some fungi may have lost the ability to produce meiospores (Taylor *et al.* 1999). The trophic habits of the majority of ascomycetes known to produce mitospores are saprobic and parasitic (Kendrick & DiCosmo 1979). The few reports of mitospore formation by EcM *Pezizales* in culture include *Tarzetta catinus* (Dodge 1937; as *Peziza pustulata*), *Tricharina hiemalis*, *T. ochroleuca*, *Wilcoxina mikolae* (Yang & Korf 1985a) and *Muciturbo reticulatus* (Warcup & Talbot 1989). Only a few EcM fungi have been unequivocally linked to mitosporic stages in nature. The first was *Muciturbo*, which forms a spore mat (clump of mitospore-bearing mycelium visible to the unaided eye) on the soil surface prior to fruit body formation (Warcup & Talbot 1989). ITS sequences were used to link spore mats on soil to an unknown species in the /pachyphloeus–amylascus lineage (Norman & Egger 1999) and two species of *Tuber* (Urban *et al.* 2004). ITS sequences of asexual spore mats also matched *Fagus* and *Quercus* EcM root tip sequences (Urban *et al.* 2004; Tedersoo *et al.* 2006b; Palmer *et al.* 2008).

During preliminary surveys of *Pezizales* spore mats in 2009, we found that mitospores of *Pachyphloeus* and *Tuber* are widespread and conspicuous in hardwood and mixed forests of the Eastern USA. These findings led us to ask the following (i) what proportion of EcM *Pezizales* lineages produce spore mats? (ii) what habitats are EcM *Pezizales* spore mats produced in? (iii) what is the phylogenetic and geographic distribution of EcM *Pezizales* that produce spore mats? and (iv) can EcM *Pezizales* mitospores germinate and can the resulting hyphae form ectomycorrhizas on forest trees? We discovered that the majority of known lineages of EcM *Pezizales* commonly produce spore mats; spore mats are produced mainly on exposed soil or woodland debris, and they are distributed on four continents, and in both hemispheres. We encountered novel examples in the /fischerula, /hydnobolites, /hydnotrya, /pachyphloeus –amylascus, /terfezia–peziza depressa and /tuber–helvella lineages (*sensu* Tedersoo *et al.* 2010). Our results call for a reassessment of the life cycles of EcM *Pezizales*.

Materials and methods

Fungal material

During spring, summer and fall of 2009–2012, spore mats were encountered in a variety of habitats with EcM trees, such as forested hiking trails, washes, creek edges, parks and urban wooded areas. We opportunistically collected these spore mats across the Eastern USA during 2009–2011, in northeast Mexico and southeast China in August and September of 2010 and in Chile and Argentina in March and April of 2012. Surveyed forest types included broadleaf deciduous, oak savanna, *Nothofagus*-dominated, mixed broadleaf-*Pinaceae* and pure *Pinaceae* forests. Spore mats were photographed in the field, placed in clean plastic containers or wrapped in aluminium foil. Collecting implements were cleaned between uses to prevent cross-contamination. For all collections, we recorded the date, location, the EcM canopy plants and basic habitat information. Specimens were dried in a forced air dryer or in a closed plastic container with silica gel drying beads (Henkel *et al.* 2006). Each collection was glued to archival paper cards and stored in herbarium boxes for morphological examination, molecular study and voucher accession. Specimens are deposited in the Duke University Herbarium (DUKE), the Farlow Herbarium at Harvard University (FH), the Herbarium Jose Castillo Tovar (ITCV) Mexico, Kunming Institute of Botany (KUN) and the University of Minnesota Herbarium (MIN).

To assess whether meio- and mitospores are produced concurrently, we also collected truffles and other *Pezizales* fruit bodies in the vicinity of spore mats. These

were examined microscopically for identification, and approximately 3 mm^3 of clean tissue was sampled for DNA. EcM root tips were collected as described in Guevara *et al.* (2012) in Mexico in August 2008 and Eastern United States in July 2010. To obtain broader diversity and better phylogenetic placement of our samples, fruit body collections of EcM *Pezizales* were incorporated into this study. These included personal herbaria materials and loans from the following institutions: the Farlow Herbarium at Harvard University (FH), Oregon State University (OSC), Cornell University Herbarium (CUP), University of Bergen (BG) and Real Jardín Botánico-CSIC (MA). Voucher information is listed in Table S1, Supporting information.

Molecular protocols

DNA was extracted from spore mats, fruit bodies and EcM root tips using a modified CTAB protocol (Gardes & Bruns 1993) or an Extract-N-Amp Plant PCR kit (Sigma-Aldrich) following the manufacturer's instructions, but with 20% of the recommended volume of extraction and dilution solutions. In the Vilgalys Laboratory, miniscule pieces of spore mats were added to PCRs for direct amplification following the method described by Bonito (2009). This latter technique was effective in amplifying from small or thin spore mats, to avoid soil particles.

PCR products were run on 1.5% agarose gels containing ethidium bromide or stained with SYBR Green I (Molecular Probes). Amplicons were digested with the EXO and AP enzymes (Glenn & Schable 2005) or cleaned by standard ethanol precipitation. Amplicons were sequenced in both directions with an ABI Big Dye Terminator Sequencing Kit (v3.1) and run on an ABI 3730xl capillary sequencer (Applied Biosystems) at the Duke University sequencing facility and the University of Minnesota Biomedical Genomics Facility. Sequences were trimmed, edited and assembled in Sequencher v. 4.10.1 (Gene Codes Inc.).

Species determination and phylogenetic analysis of ITS

The ITS region of rDNA, an official barcode for fungal species identification (Schoch *et al.* 2012), has proven effective for delimiting *Pezizales* at the species level (Smith *et al.* 2007a; Bonito *et al.* 2010). We used PCR to amplify the entire ITS rDNA repeat with combinations of primers ITS1, ITS1F, ITS5 (forward) and ITS2, ITS4 or LR3 (reverse) (White *et al.* 1990; Gardes & Bruns 1993). After sequences were obtained and assembled, we performed BLAST searches on all and downloaded similar sequences from GenBank for phylogenetic comparisons. Lastly, to find closely related EcM fungal sequences, we used the Emerencia 'genus search' function to search for insufficiently identified sequences using queries for *Fischerula, Hydnobolites, Hydnocystis, Pachyphloeus, Peziza, Ruhlandiella, Scabropezia* and *Tuber* (Nilsson *et al.* 2005; Ryberg *et al.* 2009). We then trimmed all sequences to begin after the 'CATTA' motif of 18S and to end before the 'NACCTCNNATCAGGTAGGGAT' motif at the beginning of 28S. We uploaded trimmed sequences into a Sequencher file and sorted them into OTUs based on 96% sequence similarity using the 'dirty data' algorithm. Phylogenetic relationships among closely related OTUs were inferred within the four most speciose genera. The typical cut-off for species approximation using% similarity of nucleotides in the ITS region is 97% (Smith *et al.* 2007a). We used 96% because there was not a clear gap for four of the OTUs at 97% (Bonito *et al.* 2010). With the caveat that our species delimitation is broader than usual, OTUs here are handled as species. Sequences from each OTU were selected to represent unique geographic localities and isolation sources. Lineage nomenclature is preceded by a forward slash and follows Moncalvo *et al.* (2002), while *Pezizales* lineage circumscription follows Tedersoo *et al.* (2010). Four sets of ITS sequences were aligned including 41 sequences of *Hydnobolites* from the /marcelleina–peziza gerardii lineage (from 14 fruit bodies, 10 EcM roots and 17 spore mats); 94 sequences of /pachyphloeus–amylascus (from 36 fruit bodies, 25 EcM roots or environmental samples and 33 spore mats); 45 sequences of *Tuber* from the /tuber–helvella lineage (from 19 fruit bodies, 16 EcM roots and 11 spore mats) and 45 sequences of /terfezia–peziza depressa (from 12 fruit bodies, 16 EcM roots and 17 spore mats). Sequences were aligned in MAFFT v 6.822 (Katoh & Toh 2010), and alignments manually improved in Se-Al v 2.0a11 (Rambaut 2007). Ambiguously aligned regions were excluded in GBlocks using the least stringent setting (Castresana 2000; Talavera & Castresana 2007). Phylogenetic inferences from alignments were estimated under Bayesian posterior probability (BPP) and maximum-likelihood (ML) analyses. ML was estimated using RAxML 7.2.8 (Stamatakis 2006) with a GTR + G model of nucleotide substitution. Rapid bootstrapping (Stamatakis *et al.* 2008) was implemented with 1000 replicates. The best scoring ML tree and bootstrap (BS) values $\geq$70% are reported. For Bayesian analysis, a model of substitution and the priors were determined in JModelTest 0.1.1 (Posada 2008) under the Akaike Information Criterion, and posterior probabilities were estimated using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). Two million generations were run in two parallel searches on four chains, and trees sampled every 100 generations. The first 25% of samples in each set were discarded as burn-in. Stationarity was evalu-

ated based on the SD of split frequency (≤ 0.01), and mixing behaviour of the chain was checked in Tracer (Rambaut & Drummond 2007), to ensure that coverage was adequate. Posterior probability (PP) values >95% were considered significant. ML and BPP were run on XSEDE on the CIPRES web portal (Miller *et al.* 2010). Our ITS data sets included 171 newly generated sequences (Table S1) and 99 sequences downloaded from GenBank (Table S2, Supporting information).

Placement of OTUs within a phylogenetic context

After unique OTUs were determined, we examined diversity of mitospore-producing *Pezizales* within a phylogenetic context based on domains D1 and D2 of the LSU. The LSU was selected because many representative *Pezizales* sequences are available in GenBank. The LSU has also been well sampled in previous phylogenetic analyses of the *Pezizales*, providing a backbone of taxa representing known lineages within the order (Hansen & Pfister 2006; Tedersoo *et al.* 2006a; Perry *et al.* 2007). From these previous studies, we chose representative sequences from each major clade to provide a framework to place our newly generated sequences. The LSU was amplified and sequenced for representative spore mats from each OTU with combinations of primers ITS3, ITS5 or LROR (forward) and LR3, LR5 (Vilgalys & Hester 1990; White *et al.* 1990) or LR5F (reverse) (Tedersoo *et al.* 2008). Our LSU data set included 192 sequences: 66 newly generated for this study (Table S1) and 126 downloaded from GenBank (Table S2). In addition to taxa used to build the phylogenetic framework, downloaded sequences also included those from EcM root tips and nonmycorrhizal mitosporic *Pezizales*. Due to difficulty in aligning across the order, we aligned sequences in two subsets: subset one with the *Pezizaceae* and subset two with the *Pezizales* exclusive of the *Pezizaceae*. Subset one had 135 sequences from 72 fruit bodies, 23 EcM roots and 40 asexual spore mats with 816 basepairs (bp). Subset two had 76 sequences from 61 fruit bodies, 4 EcM roots and 11 asexual spore mats with 761 bp. The LSU sequences were aligned by hand in SeAl. *Orbilia vinosa* served as the outgroup in phylogenetic analyses for both subsets. Ambiguous region exclusion, selection of model of substitution and phylogenetic analyses of the LSU data set were as described for the ITS region except that for BPP the data sets were run for 20 million generations.

Culturing protocol

Intact fruit bodies of *Pachyphloeus* and *Hydnobolites* were surface-sterilized by submergence in 10% bleach for 10 min, rinsed three times in sterile water and then broken open by grasping the truffle from opposite ends and pulling the fruit body apart. Interior tissue was removed and placed on modified Melin Norkrans agar, malt extract agar (1/2 strength) and modified woody plant medium (1/2 strength). These agar media were supplemented with 10 mg/L each of the antibiotics Streptomycin and Chloramphinicol. Direct culturing and dilution plating of asexual spore mats on these same media were carried out in order to germinate the spores and grow these fungi. Direct culturing entailed sampling of spores and/or mycelia (*Hydnobolites*, *Pachyphloeus*, *Pezizaceae* 2 and *Tuber*) directly and plating with sterile technique embedded either in the media or on the surface. For dilution plating, a small clump of spores was homogenized in an eppendorf tube with 2 mL of sterile water and left to sit for 1 h. Three serial dilutions were made (10^{-3}), and 30 μL was plated and spread evenly with a sterile glass rod. Cultures were maintained in a growth chamber and examined weekly over the following 6 months.

EcM root inoculation

Quercus, *Pinus* and *Populus* species are dominant EcM hosts in Northern Hemisphere forests, and in many cases, asexual spore mats were present near these hosts. Consequently, we chose *Quercus phellos*, *Pinus taeda* and *Populus deltoides* for our inoculation experiments. One batch of inoculum was made with fresh spores harvested from spore mats the same day, and a second batch of inoculum was made with spores that had been air-dried at room temperature for 3 days. Plant roots were inoculated at Duke University following similar methods successfully used by Bonito *et al.* (2011) for inoculating seedlings with truffle spores. Briefly, a given mass (0.20–1.20 g) of spores was mixed into an appropriate volume of double autoclaved soil-less potting mixture composed of vermiculite, perlite, peat and kaolin clay (4:4:1:1). We used five OTUs from four different lineages, representing the /tuber–helvella, /pachyphloeus–amylascus, hydnotrya, /terfezia–peziza depressa lineages. We included five seedling replicates for each treatment. Spore inoculum level was calculated for a subsample of spores in a hemacytometer, with the addition of 0.1% tween 20 (to reduce spore clumping and surface tension). Spore inoculation densities ranged between 100 million and 1.0 billion spores per plant. Seedlings (oak & pine) and cuttings (poplar) were planted in 'cone-tainers' containing a soil volume of approximately 250 mL^2 (Stuewe & Sons, Inc.). Plants were maintained in the Duke greenhouses and were watered every 3 days. After 180 days of growth (18 h days/6 h nights), plants were harvested and the roots were washed clean. Root tips were then examined

under a stereoscope for EcM colonization by pezizalean fungi, characterized by a smooth, thin, brown mantle and lack of rhizomorphs (Agerer 1987–2002; Bonito *et al.* 2011). Observed EcM root tips were collected, and the ITS region of rDNA was sequenced.

Measurement of spores and spore mats

Spore mats were photographed *in situ*. To measure and quantify mitospores, 20 spores from representative spore mats from each lineage were measured in 2.5% KOH, and their size ranges and averages determined. Spore densities (spores/area) for representative OTUs of each of the major clades were quantified with a hemacytometer (Propper Manufacturing Co.), according to manufacturer instructions, by suspending 2.5 mm^2 cores into 100 mL of a 0.1% solution of Tween 20. Count averages are reported from three excised plugs per sample of three representative OTUs from the four most speciose clades (/marcelleina–peziza gerardii, /pachyphloeus–amylascus, /terfezia–peziza and /tuber–helvella). The areas of imaged spore mats were found using Image J64 (Rasband 2011).

Results

Species determination

A total of 245 spore mats, 83 sporocarps and 10 EcM root tips from North America, Europe, South America and China were sequenced for this study (Table S1). Sequences of ITS were sorted into 48 OTUs (Table 1). Independent phylogenetic analyses based on ITS and LSU placed them in six lineages. The /pachyphloeus–amylascus lineage (*Pezizaceae*) (Fig. 1) comprised 26 OTUs, including the cup fungus *Scabropezia* (1 OTU), the truffle genus *Pachyphloeus* (14 OTUs), 8 OTUs close to *Pachyphloeus* or *Scabropezia* sequences and 3 OTUs basal to *Amylascus* that are henceforth referred to as *Pezizaceae* 1-1 and 1-2, and *Pezizaceae* 3. The *Pezizaceae* 1 and *Pezizaceae* 3 OTUs were not included in the phylogenetic analyses of ITS because their sequences were too divergent to be aligned. The /marcelleina–peziza gerardii lineage (*Pezizaceae*) comprised 13 OTUs in the truffle genus *Hydnobolites*. The /tuber–helvella lineage (*Tuberaceae*) comprised 3 OTUs of the truffle genus *Tuber*. The truffle genus *Fischerula* comprised 1 OTU. The /hydnotrya lineage (*Discinaceae)* comprised 1 OTU. The /terfezia–peziza depressa lineage comprised 5 OTUs, including a *Ruhlandiella*-like species (1 OTU), and 4 OTUs of an undescribed genus for which no meiospore stage is known. The latter are henceforth referred to as *Pezizaceae* 2-1, -2, -3 and -4.

The /pachyphloeus–amylascus lineage (21 OTUs) accounted for 44% of species diversity of sequenced spore mats (Table 1). Among the /pachyphloeus–amylascus OTUs, 15 spore mat sequences matched fruit bodies, 14 matched EcM root tip sequences and 13 matched both (Fig. 1, Table 1). Four of the 21 /pachyphloeus–amylascus spore mat OTUs matched described species, while 17 represent unknown or undescribed species. The most frequently collected and widely distributed species of the /pachyphloeus–amylascus lineage was *P. thysellii*. Pink-coloured spore mats (Fig. 7c) of this species were collected in the USA and China and also detected on EcM roots or environmental samples from Canada and Europe. *Pachyphloeus citrinus* also has a broad geographic range that includes Europe, Mexico and the USA. Species in the /pachyphloeus–amylascus lineage were associated with several genera of angiosperm host plants (Table 1).

Twenty-five per cent (13) of the OTUs were in the /marcelleina–peziza gerardii lineage and highly similar to *Hydnobolites* sequences (*Pezizaceae*) (Fig. 2, Table 1). *Hydnobolites* (Fig. 7i) is a truffle genus with only two accepted species (*H. californicus* and *H. cerebriformis*) and no previous reports of mitospore production. Sequences from the two described species did not match spore mats, whereas five spore mat sequences matched fruit bodies of undescribed *Hydnobolites* species (M. E. Smith & R. A. Healy, unpublished), and two matched European orchid mycorrhizae sequences (*Epipactis*, Table 1).

Three OTUs in the /tuber–helvella lineage were allied with the genus *Tuber* (*Tuberaceae*) but could not be assigned to any described species (Fig. 3, Table 1). *Tuber* 1 was common and fruited in extensive patches, but did not match sequences from fruit bodies or EcM roots. Phylogenetic analyses placed this OTU close to *T. borchii* and *T. dryophilum*, for which spore mats were previously described (Urban *et al.* 2004). *Tuber* 2 and *Tuber* 3 matched fruit body sequences of undescribed *Tuber* species from Minnesota that are nested within the Maculatum and the Puberulum clades (Fig. 5) of Bonito *et al.* (2010). *Tuber* 2 matched German *Epipactis* orchid root tips, and *Tuber* 3 matched North American *Quercus* EcM root tip sequences. These results constitute the first report of spore mats in the Maculatum clade and double the number of species with mitosporic states previously reported in the Puberulum clade.

A single spore mat of a *Hydnotrya* sp. (/hydnotrya lineage, *Discinaceae*) and a single spore mat of *Fisherula* (/fischerula lineage, family uncertain) were discovered in Fall 2010 and 2011, respectively (Fig. 7l,m). The growth forms of both were similar to that of *Tuber* (Table S4, Supporting information). The /fischerula and /hydnotrya spore mat sequences did not match any

Table 1 Asexual spore mats, fruit bodies and ectomycorrhizal root tip matches based on ≥96% similarity in internal transcribed spacer region of nuclear ribosomal DNA

				Geographic range of sequence source[‡] and EcM hosts[§]			
Lineage/OTU	Rep. seq.	Seq Nos*.	Habitat[†]	spore mat	fruit body	EcM	host
/fischerula	JX414173	1/0/0	A	US			
/hydnotrya	JN102492	1/0/0	A	US			
/marcelleina–peziza gerardii 1	JN102392	1/0/0	P	US			
/marcelleina–peziza gerardii 2	JN102436	2/0/0	M	CN			
/marcelleina–peziza gerardii 3	JN102390	4/2/0	A, M, P	US	US		
/marcelleina–peziza gerardii 4	JN102425	1/0/0	M	US			
/marcelleina–peziza gerardii 5	JN102440	3/1/0	M	CN	CN		
/marcelleina–peziza gerardii 6	JN102384	1/3/0	A	US	US		
/marcelleina–peziza gerardii 7	JN102388	2/0/0	A	US			
/marcelleina–peziza gerardii 8	JN102372	1/0/0	A	US			
/marcelleina–peziza gerardii 9	JN102394	1/0/0	A	US			
/marcelleina–peziza gerardii 10	JN102377	4/0/0	A	US			
/marcelleina–peziza gerardii 11	JN102393	6/1/0	A, S	US	MX		
/marcelleina–peziza gerardii 12	JX414187	2/5/0	A	US	US		
/marcelleina–peziza gerardii 13	JX414188	1/0/2	A	US		G, IT	EP
Pachyphloeus citrinus	JN102363	8/9/1	A, D	MX, US	IT, MX, UK, US	G	CP, FG, TL
Pachyphloeus marroninus	JN102364	5/4/2	A, S	US	MX, US	MX	QC
Pachyphloeus thysellii	JN102370	24/7/4	All	CN, US	US	CA (env), CN, EE	Al, QC
/pachyphloeus–amylascus 5	JN102389	2/0/0	A	US			
/pachyphloeus–amylascus 6	JN102414	1/0/0	A, M	US			
/pachyphloeus–amylascus 7	JN102432	1/0/0	D	US			
/pachyphloeus–amylascus 8	JN102431	1/1/2	M	US	US	MX, US	QC
/pachyphloeus–amylascus 9	JN102430	6/3/3	M	MX, US	SP, UK	DK, EE, IT	FG
/pachyphloeus–amylascus 10	JN102368	6/0/0	M	US			
/pachyphloeus–amylascus 11	JN102439	1/0/1	A, M, S	CN		MX	QC
/pachyphloeus–amylascus 13	JN102395	3/4/2	D	US	US	US	QC
/pachyphloeus–amylascus 14	JN102435	1/0/1	A, D, S	CN			
/pachyphloeus–amylascus 15	JN102367	5/1/0	M	US	US		
/pachyphloeus–amylascus 16	JN102433	11/0/2	S	US		US	
/pachyphloeus–amylascus 17	JN102421	11/16/2	M	MX, US	MX, US	MX	QC
/pachyphloeus–amylascus 18	JN102404	6/1/0	A	US			
/pachyphloeus–amylascus 20	JN102409	11/16/0	A	MX, US	MX, US		
/pachyphloeus–amylascus 21	JN102380	5/14/2	A, D, M	US	MX, US		
/pachyphloeus–amylascus 22	JN102375	13/4/1	A, D	US	US	US	
/pachyphloeus–amylascus 23	JN102434	1/4/1	A, M	CN	EU	JP	CP
Pezizaceae 1-1	JN102379	1/0/0	A	US		US (env)	
Pezizaceae 1-2	JN102406	2/0/0	M	US		US (env)	

Table 1 *Continued*

Lineage/OTU	Rep. seq.	Seq Nos*.	Habitat†	Geographic range of sequence source‡ and EcM hosts§			
				spore mat	fruit body	EcM	host
Pezizaceae 2-1	JN102366	49/0/10	M	US		EE, G, NZ, PL, US	LX, PN, QC, SX
Pezizaceae 2-2	JN102422	33/0/5	A, D, M, S	US		G, PL, US	BT, PN, HM, QC
Pezizaceae 2-3	JN102438	5/0/0	A, D, M, S	CN			
Pezizaceae 2-4	JN102426	2/0/1	M	US		US (env)	
Pezizaceae 3	JX414201	3/0/0	A	AR			
Ruhlandiella sp. nov.	JX415205	16/1/0	A	AR, CH			
Scabropezia flavovirens	JN102402	4/3/1	A	US		EE	Al
Scabropezia sp.	JN121319	3/0/0	A	US	FR, US		
/tuber helvella 1	JN102420	22/0/0	A	US			
/tuber helvella 2	JN102385	1/2/1	A, M	US	US	G	EP
/tuber helvella 3	JN102387	4/5/3	A	US	US	G, MX, US	EP, CY, QC

*Sequence sources for OTU are listed in the order: asexual spore mat/ fruit body/ ecomycorrhizal root tip.
†Habitats are listed for asexual spore mat collections only. Abbreviations: A (angiosperm-dominated woods); D (disturbed angiosperm-wooded lot such as campus lawn and picnic ground in park); M (mixed *Pinaceae* and angiosperm); P (*Pinaceae* woods); S (oak savanna).
‡Countries: AR (Argentina), CA (Canada), CH (Chili), CN (China), DK (Denmark), EE (Estonia), FR (France), GR (Germany), IT (Italy), JP (Japan), MX (Mexico), NZ (New Zealand), PL (Poland), SP (Spain), UK (United Kingdom).
§Hosts: Al (*Alnus*), BT (*Betula*), CP (*Carpinus*), CY (*Carya*), EP (*Epipactis*), FG (*Fagus*), HM (*Helianthemum*), LX (*Larix*), PN (*Pinus*), QC (*Quercus*), SX (*Salix*), TL (*Tilia*).

fruit body or EcM root tip sequences and were not included in the phylogenetic analyses of ITS. The ITS from a single spore mat of the truffle genus *Hydnocystis* (*Pyronemataceae*), discovered in Fall of 2011, matched a fruit body from the same woods. However, *Hydnocystis* is not known to be EcM and so is not included in any further discussion of EcM *Pezizales*. This was the only non-EcM spore mat sequenced, and it was found on woody debris rather than on the soil surface.

Two clades with spore mat sequences are in the /terfezia–peziza depressa lineage. One OTU from spore mats collected in Argentina and Chile was shared with a fruit body of an undescribed *Ruhlandiella*-like species (/terfezia–peziza depressa lineage) collected previously in Chile (M. E. Smith & D. H. Pfister, unpublished). Four spore mat OTUs (*Pezizaceae* 2-1 to 2-4) were similar or identical to sequences from EcM roots but not close to any fruit body sequences. The /terfezia–peziza depressa lineage (*Pezizaceae*) includes both truffles (*Terfezia, Mycoclelandia, Tirmania, Cazia and Peziza* in part) and epigeous cup fungi (*Peziza* in part; Fig. 4). *Pezizaceae* 2-1 and 2-2 are geographically widespread as spore mats in the Eastern USA (Table 1) and have been sequenced from EcM root tips in Europe and Argentina. *Pezizaceae* 2-1 and 2-2 also have a broad host range, including woody broadleaf, and *Pinaceae* trees, as well as herbaceous species. The *Pezizaceae* 2 clade of spore mats did not share any well-supported nodes with available fruit body sequences (Fig. 4).

Phylogenetic analysis of LSU

Topologies of strongly supported nodes resulting from ML and BPP analyses were similar. Except for the /leucangium clade, there was no major disagreement among strongly supported nodes in our analyses or with previous analyses by Læssøe & Hansen (2007), Perry *et al.* (2007) or Tedersoo *et al.* (2006a). The *Pezizaceae* ML tree is shown in Fig. 5. The ML tree of *Pezizales* excluding *Pezizaceae* is shown in Fig. 6. The /leucangium lineage identified in Tedersoo *et al.* (2010) included *Fischerula*, based on strong maximum parsimony (MP) bootstrap support in a study by Hansen & Pfister (2006). A monophyletic relationship between *Fischerula* and *Leucangium* lacked strong support in our analyses (Fig. 6). Hence, we refer *Fischerula* taxa hereafter to a putatively independent /fischerula lineage.

Here, we report mitospore production by five defined EcM fungal lineages and three putative lineages that are yet to be defined. Mitospores from defined EcM lineages include /pachyphloeus–amylascus (Fig. 5); /marcelleina–peziza gerardii and /terfezia–peziza depressa (Fig. 5); /hydnotrya and /tuber–helvella (Fig. 6). Undefined lineages include /fischerula (Fig. 6), *Peziza-*

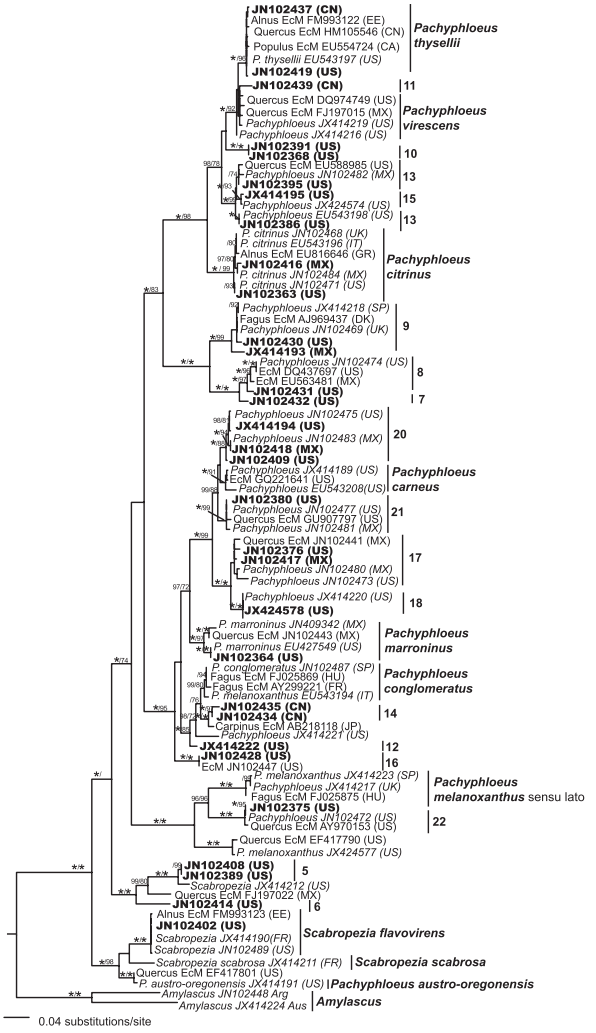

Fig. 1 Best ML (-ln 6191.356398) phylogram of 102 taxa, 530 bp of the internal transcribed spacer rDNA in the /pachyphloeus–amylascus lineage rooted with *Amylascus*. Model of evolution selected for Bayesian analysis was TVM + I + G. Numbers to the right of phylograms refer to operational taxonomic units listed in Table 1. **Following are the complete notes for Figs 1–6**: Best maximum-likelihood (ML) trees calculated with 1000 bootstrap replicates. All ML analyses were based on the GTR + G model of nucleotide substitution. Support values on branches indicated on the left side for MB posterior probablilities >95% and on the right side for ML bootstrap proportions $\geq$70%. 100% support indicated by '*'. Sequences derived from fruit bodies are italicized, spore mats are bolded and ectomycorrhizal or *Epipactis* orchid mycorrhizal root tips are preceded by 'EcM' or 'EpM', respectively. Sequences from previously reported asexual spore mats are indicated by '+'. Countries of origin, in parentheses, are abbreviated as follows: AR (Argentina), AT (Austria), AU (Australia), CA (Canada), CH (Chile), CI (Canary Islands), CN (China), DK (Denmark), DR (Dominican Republic), EE (Estonia), FR (France), GL (Greenland), GR (Germany), HU (Hungary), IL (Israel), IT (Italy), LY (Libya), JP (Japan), KW (Kuwait), MX (Mexico), NO (Norway), NZ (New Zealand), PG (Papua New Guinea), PL (Poland), PR (Puerto Rico), PT (Portugal), SAf (South Africa), SP (Spain), UK (United Kingdom), US (United States).

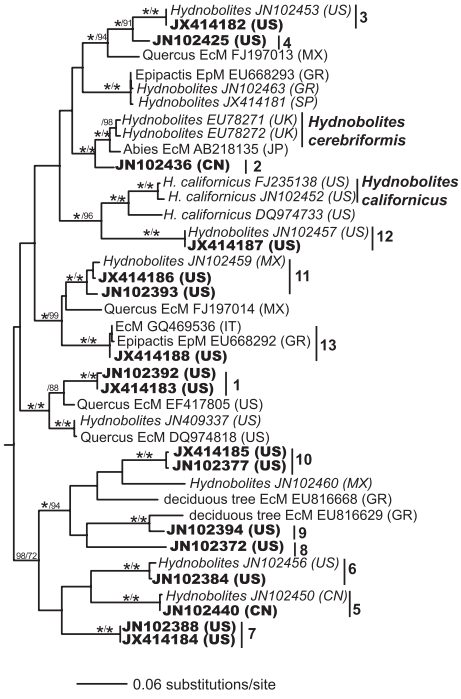

Fig. 2 Best ML (-ln -4328.002853) phylogram of 43 taxa, 577 bp of the internal transcribed spacer rDNA in the *Hydnobolites* clade of the /marcelliena–peziza gerardii lineage. Model of evolution selected for Bayesian analysis was HKY + I + G. Numbers to the right of phylograms refer to operational taxonomic units listed in Table 1. Complete notes for Figs 1–6 see Fig. 1.

ceae 1 and *Pezizaceae* 3 (Fig. 5). While *Pezizaceae* 1 occurs in a strongly supported clade with EcM root tips, there is no evidence for the trophic status of *Pezizaceae* 3. As phylogenetic analyses of the LSU place this OTU among EcM clades, we suspect an EcM habit for *Pezizaceae* 3 and include it in our analyses. Spore mats were previously unknown in the /marcelleina–peziza gerardii, /hydnotrya and /fischerula lineages. When these results are compiled with previous reports of mitospore production by EcM *Pezizales* species (indicated by '+' in Figs 5 and 6), the LSU analyses suggest that at least nine of the 16 EcM *Pezizales* lineages identified in Tedersoo *et al.* (2010), and one additional lineage preliminarily identified in this study can produce mitospores: /pachyphloeus–amylascus (Fig. 5a), /marcelleina–peziza gerardii, /terfezia–peziza depressa (Fig. 5b), /geopora, /hydnotrya, /fischerula, /sphaerosporella, /tarzetta, /tuber–helvella and /wilcoxina (Fig. 6). A breakdown of the 48 newly identified OTUs by family is 43 *Pezizaceae* (Fig. 5), 3 *Tuberaceae*, 1 *Discinaceae* and 1 *incertae sedis* (Fig. 6). An additional 30 saprotrophic, biotrophic and pathogenic species that produce mitospores are included in the phylogeny to illustrate the phylogenetic distribution of Pezizales known to produce

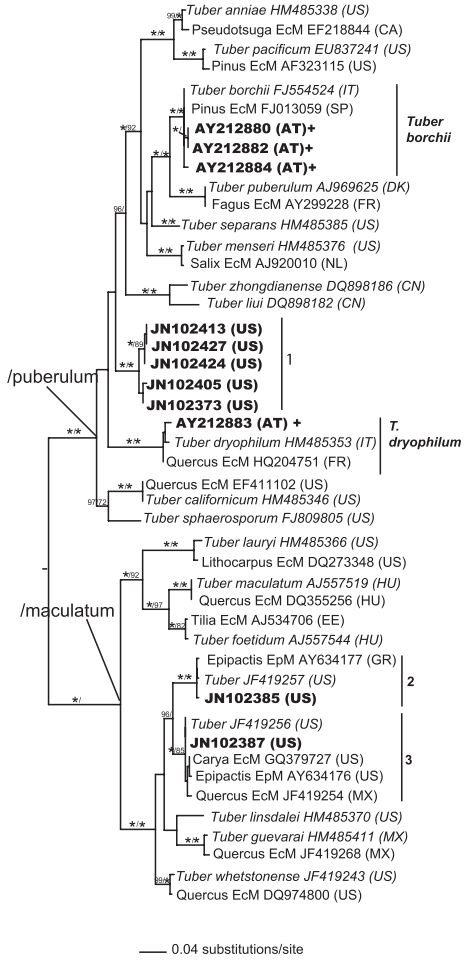

Fig. 3 Best ML (-ln 3229.833889) phylogram of 46 taxa, 454 bp of internal transcribed spacer rDNA in the *Tuber* clade in the /tuber–helvella lineage. Model of evolution selected for Bayesian analysis was TIM2 + I + G. Numbers to the right of phylograms refer to operational taxonomic units listed in Table 1. Complete notes for Figs 1–6 see Fig. 1.

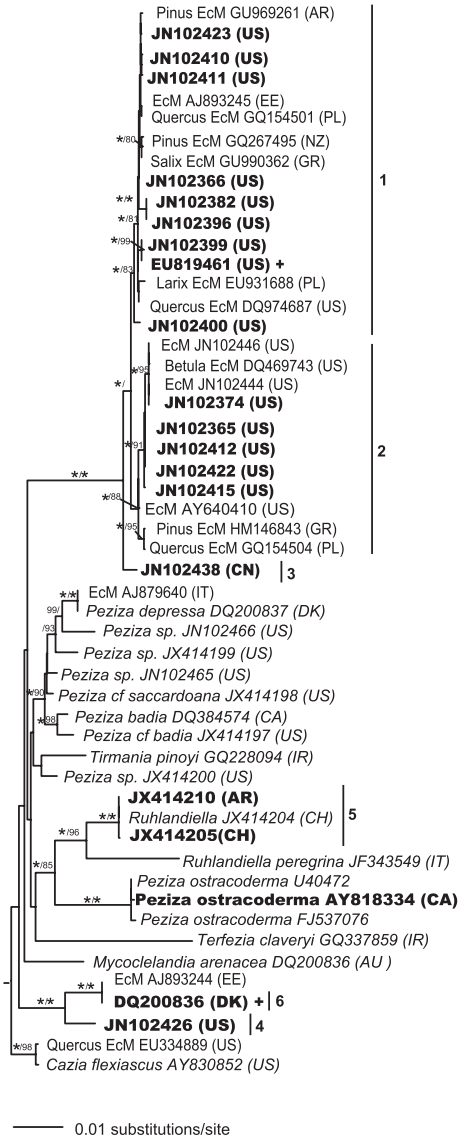

Fig. 4 Best ML (-ln 4218.648461) phylogram of 54 taxa, 523 bp of the internal transcribed spacer rDNA in the /terfezia–peziza lineage. GTR + G selected as model of evolution for Bayesian analysis. Phylogram includes sequences from *Peziza* collected in the vicinity of spore mats during this study. Complete notes for Figs 1–6 see Fig. 1.

mitospores (bolded in Figs 5 and 6). Most well-supported major clades have members that produce mitospores. Exceptions include two clades with EcM *Peziza* and *Hydnotryopsis* and one clade with saprobic *Pachyella* (and others) in *Pezizaceae* (Fig. 5b); EcM *Helvella* and *Otidea*, saprobic *Pulvinula* and *Psilopezia* and parasitic *Rhizina* in *Pezizales* excluding *Pezizaceae* (Fig. 6).

Biogeography, phenology, habitat and spore mat size

Spore mats of pezizalean EcM fungi were diverse and common over a wide geographic area in the Northern Hemisphere, including the Eastern USA (6 lineages, 40 OTUs), Mexico (1 lineage, 3 OTUs), China (3 lineages, 7 OTUs) as well as a few sites in South America, including Argentina (2 lineages, 2 OTUs) and Chile (1 lineage, 1 OTU) (Fig. S1, Supporting information). There was a lag time in production of spore mats in Minnesota (MN) compared to North Carolina (NC) by at least 1 month (Fig. S2, Supporting information). Spore mat production approximately corresponded to above-freezing temperatures and moderate precipitation. Collections during 2011 expanded the fruiting dates from April in NC to October in MN and December in NC (Table S1). Spore mats were not detected during drought conditions. At the other extreme, heavy rainfall tended to obliterate the mats, washing away the spores. In general, spore mats were collected on bare soil, rocks or woodland debris on the ground. They were most

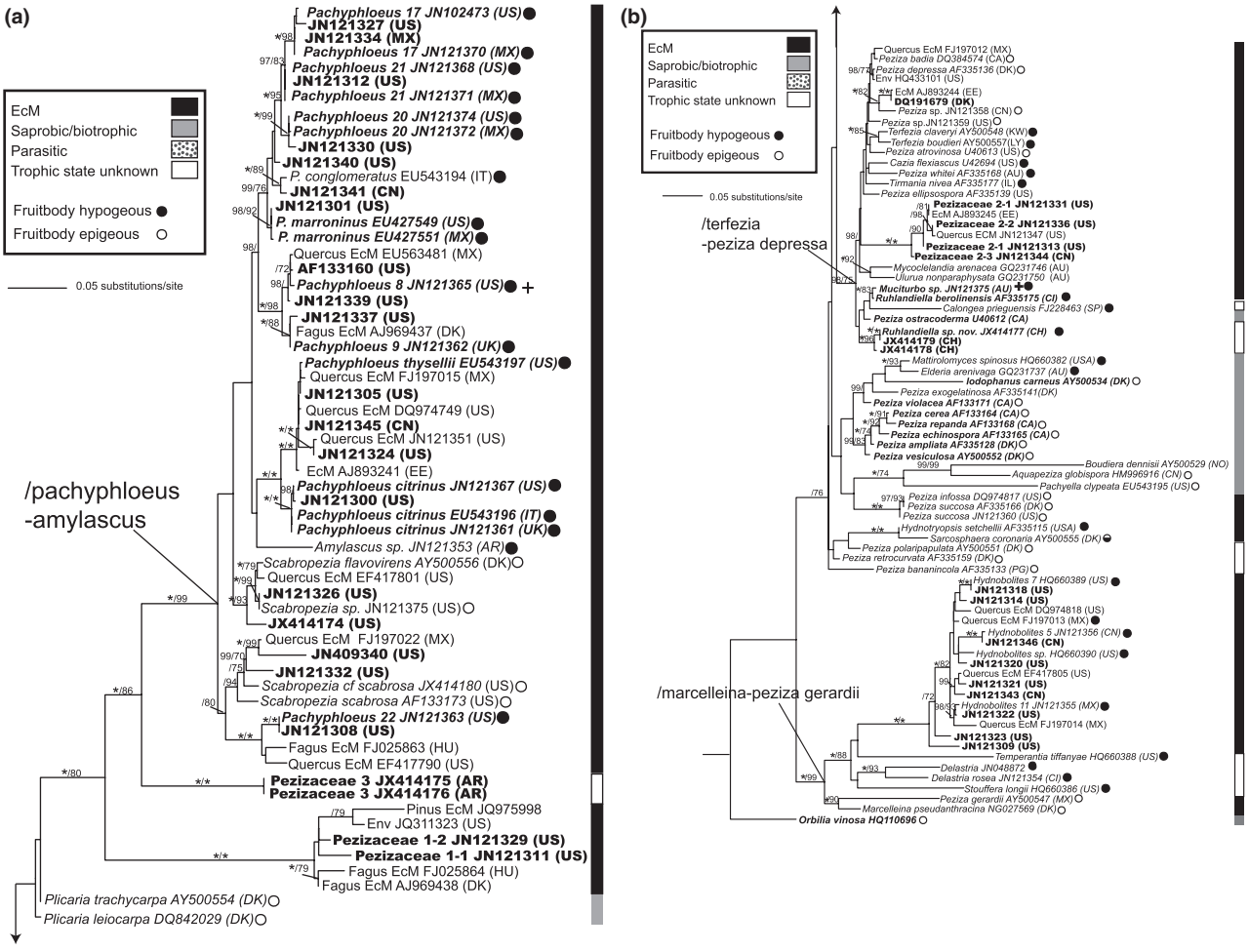

Fig. 5 The best ML phylogram from 135 taxa, 816 bp of the LSU rDNA from *Pezizaceae* (–lnL = 10873.195389). Model of evolution selected for Bayesian analysis was TIM3ef + G (Fig. 5a, *Pezizaceae* part 1; Fig. 5b, *Pezizaceae* part 2). The trophic status for each taxon, as designated by shade in the key at the top left, is displayed on the bar to the right of the phylogram. The outgroup was *Orbilia vinosa*. Complete notes for Figs 1–6 see Fig. 1.

diverse and abundant in woodlands that included EcM hardwoods, or a mixture of hardwoods and *Pinaceae*. They were not found under *Pinaceae* where heavy duff layers were present (Table 1). The most ubiquitous OTUs (*Pezizaceae* 2-1, *Pezizaceae* 2-2 and *P. thysellii)* were found on multiple continents in woodlands protected from human disturbance (although usually on bare soil due to natural disturbance), as well as human-disturbed areas (Table 1, Fig. S1). Spore mats produced between 1.5×10^3 and 11×10^3 spores/mm^2, depending on the lineage (Table S3, Supporting information). In general, *Pezizaceae* spore mats were dense with sporogenous hyphae and determinate in growth, forming cushion-like mounds on the soil (Fig. 7a,c,e,g,i,j), while /fischerula, *Discinaceae* and *Tuberaceae* spore mats were single to sparsely layered and grew indeterminately and effusely in a dendroid pattern over the surfaces of soil, leaves, rocks and twigs (Fig. 7k,m; Table S3).

Culturing of meio- and mitospores and EcM root inoculation

Attempts to culture *Pachyphloeus* and *Hydnobolites* from fruit body pieces or mitospores from spore mats were unsuccessful, producing only bacteria, nontarget fungi or no growth. Ectomycorrhizae failed to establish from mitospore inoculation with any OTU.

Contaminating fungi

Multiple genera of spore mats from MN, NC and Mexico collected during humid weather were contaminated by one of three species in a complex around *Paecilomyces penicillatus* (*Hypocreales*) (Table S4). These were not included in analyses of anamorph-producing EcM *Pezizales*. There were three nontarget EcM species sequenced in the root inoculation experiment. They included *Tuber*

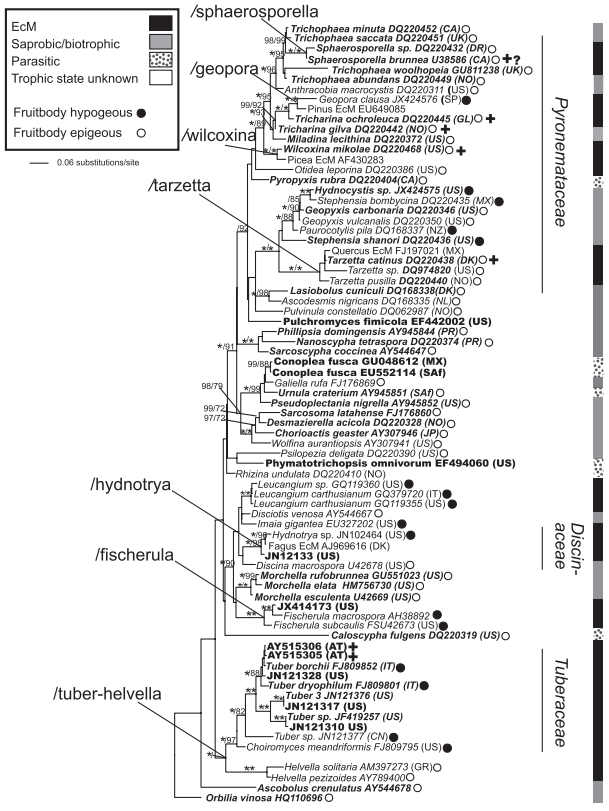

Fig. 6 The best ML phylogram from 77 taxa, 895 bp of the *Pezizales* exclusive of *Pezizaceae* (-lnL = 12207.201668). Model of evolution selected for Bayesian analysis was GTR + I + G. The outgroup was *Orbilia vinosa*. '?' indicates discrepancy in the literature regarding mitospore production. The trophic status for each taxon, as designated by shade in the key at the top left, is displayed on the bar to the right of the phylogram. Complete notes for Figs 1–6 see Fig. 1.

borchii, an unknown *Tuber* of the Maculatum clade, and *Tomentella*. *Tuber borchii* was purposely inoculated onto roots in neighbouring pots in the plant growth room (Table S5, Supporting information). The sequence of the unknown *Tuber* was unique from any collected anamorphs and GenBank submissions.

Discussion

Contrary to previous suggestions that EcM fungi generally do not produce mitospores, our data demonstrate that a majority (nine) of the 16 EcM *Pezizales* lineages defined by Tedersoo *et al.* (2010), plus one putative lineage identified here, produce mitospores. We show that the production of spore mats is geographically widespread in temperate areas, includes a high diversity of cup fungi (including a preponderance of truffles) and includes known EcM lineages for which sporocarp records are lacking. Collections from Eastern USA, Mexico, China and South America, along with previous reports from Europe, indicate that mitospore-producing EcM *Pezizales* occur with EcM angiosperms in temperate zones on at least four continents and in both hemispheres.

Our analyses suggest that mitospores are a common feature among *Pezizales* in general, regardless of lifestyle. The *Orbiliales*, which have many mitosporic species, are inferred as basal to the *Pezizales* (James *et al.* 2006; Kumar *et al.* 2012), implying that the production of mitospores in the *Pezizales* is a plesiomorphic condition.

By including sequences derived from spore mats and EcM root tips in phylogenetic analyses, we were able to improve resolution of fine-scale phylogenies in /marcelleina–peziza gerardii, /pachyphloeus–amylascus and /terfezia–peziza and to match life-cycle stages (i.e. ectomycorrhizae, fruit bodies and mitosporic forms) in taxa of /marcelleina–peziza gerardii, /pachyphloeus–amylascus, /terfezia–peziza depressa and /tuber–helvella. Spore mat data contributed to geographic distribution and habitat profiles for specific taxa and also revealed a greater diversity of cryptic truffle-like species than was previously known in *Hydnobolites* (16 undescribed species), *Fischerula* (one undescribed species), *Hydnotrya* (one undescribed species), a *Ruhlandiella*-like taxon (one undescribed species) and species in the truffle–cup fungus lineage of /pachyphloeus–amylascus (21 undescribed species). Truffles are produced belowground, so they can be difficult to find, but spore mats are readily visible on the soil surface. Unlike fruit bodies, mitospores are apparently produced over a full season, given adequate moisture, thereby increasing their chances of detection. Among pezizalean families, the large, brightly coloured *Pezizaceae* spore mats are the most obvious, which may be why they were the most commonly collected in this study (43 out of 48 OTUs). Spore mats of /tuber (*Tuberaceae*, 3 OTUs), /hydnotrya (*Discinaceae*, 1 OTU) and /fischerula (1 OTU) are less noticeable, and the latter two were only collected once. As our survey turned up such high diversity while being carried out over a relatively short time, it is possible that there are other lineages (particularly in Europe, Asia and in the Southern Hemisphere) that produce spore mats that were either not encountered during this study, were not in the geographic areas we searched or were overlooked.

Asexual spore mats allowed us to detect cryptic diversity in several well-known ECM lineages but also revealed a geographically widespread clade within the /terfezia–peziza depressa lineage that was previously known only from a single spore mat and numerous EcM root tips. Although the terfezia–peziza depressa lineage includes both truffles and cup fungi, our analyses gave no strong support for a sister lineage to the *Pezizaceae* 2 clade.

Fig. 7 (a–h) Spore mats and corresponding fruit bodies of representative OTUs of EcM *Pezizales*. (a) Spore mat of /pachyphloeus–amylascus 21 (RHAM15), bar = 0.5 cm. (b) *Pachyphloeus* fruit body of /pachyphloeus–amylascus 21 (MX32624), bar = 1 cm. (c) Spore mat of *P. thysellii* (RHAM116), bar = 0.5 cm. (d) Fruit body of *P. thysellii* (RH1180), bar = 1 cm (e) Spore mat of /pachyphloeus–amylascus 22 (RHAM126), bar = 1 cm. (f) *Pachyphloeus* fruit body of /pachyphloeus–amylascus 22 (RH735), bar = 1 cm. (g) Spore mat of /pachyphloeus–amylascus 4 (RHAM102), bar = 1 cm. (h) *Scabropezia flavovirens* (RH1209), bar = 1 cm. (i) Spore mats of *Hydnobolites* 12 (RHAM483) with fruit body of matching internal transcribed spacer sequence (RH1358), bar = 0.5 cm. (j) Spore mat of *Tuber* sp. 3 (RHAM226), bar = 1 cm. (k) Fruit body of *Tuber* sp. 3 (RH1279), bar = 1 cm. (l) Spore mat of /terfezia–peziza depressa 2-1 (RHAM371), bar = 1 cm. (m) Spore mat of *Fischerula* (RHAM489). (n) Close-up image of 8L taken through a dissecting microscope, bar = 1 mm.

The function(s) of the EcM spore mats collected during this study remains unknown. One working hypothesis is that spore mats are an ecologically adaptive mechanism for contacting and colonizing new flushes of fine roots. It is known that many groups of pezizalean fungi are adapted to disturbed, or edge habitats (Petersen 1985; Egger 1986). One possible advantage of mitospore production is the ability to reproduce quickly

following rainfall. If the soil with extramatrical mycelium were bare, the mycelium in upper soil horizons would have a greater chance of capturing incident rainwater necessary for mitospore production. High numbers of mitotic propagules could serve as a quick means for colonizing roots, an idea that is compatible with the ruderal strategy previously hypothesized for *Pachyphloeus* (Dickie & Reich 2005; Tedersoo *et al.* 2006a). Woodlands that experience litter-clearing disturbances, such as fire, may provide similar conditions favourable for EcM fungi that produce spore mats.

Morphology of the spores may give some clues to their potential function(s). The mitospores of most OTUs are in general only 1/3 to 1/5 the size of meiospores and are colourless to lightly pigmented. The walls of mitospores are between 0.3 and 0.5 μm thick, while walls of meiospores are 1–2 μm thick and overlaid with an additional 1–7 μm of ornamentation in *Fischerula, Hydnobolites, Hydnotrya*, *Pachyphloeus and Tuber*. With the exception of *Hydnobolites*, the meiospore walls in most species of the aforementioned genera have brown pigmentation. Unpigmented walls would do little to protect the mitospores from UV radiation, and thin walls on relatively small spores may argue against a role in long-term survival or overwintering. When compared to meiospores, mitospores have less mechanical protection against dessication and predation, and they also have comparatively less storage space for nutrients.

Testing of hypotheses regarding conditions that affect mitospore development or longevity should be possible for species that have been cultured from mitospores and have produced mitospores in culture such as in some EcM *Pyronemataceae*. Mitospores from *Tricharina hiemalis* and *Wilcoxina mikolae* germinated and produced fruit bodies in culture (Yang & Korf 1985a,b). Only polyspore isolates produced fertile fruit bodies of *W. mikolae* (Yang & Korf 1985a), consistent with heterothallism (obligate outcrossing). Two conidia of *Tarzetta* germinated in culture after heat shock, but only one, an unusually large mitospore, developed into normal mycelium (Dodge 1937). These reports suggest that mitospores in the *Pyronemataceae* may serve as propagules in some cases, but may be involved as spermatia in other cases. It should be noted that the mitospores of *Tricharina* and *Wilcoxina* are not formed in spore mats but are intercalary in the filaments. Therefore, they may not be comparable with the spore mats presented here. We did not find any EcM *Pyronemataceae* spore mats in our surveys.

Muciturbo reticulatus is apparently the only EcM *Pezizaceae* species reported to produce mitospores in culture, although the spores did not germinate (Warcup & Talbot 1989). Attempts to germinate mitospores of other EcM *Pezizales* have likewise been unsuccessful (Table S6, Supporting information). To understand the role of mitospores in EcM *Pezizales*, it may be useful to ascertain the role of mitospores in close relatives that are saprobic or plant-pathogenic. Mitospores of at least 13 *Pezizaceae* species have been produced in culture, mitospores of five of these germinated (Table S6), but there are no unambiguous records of ascocarps produced in cultures generated from mitospores. The requirements to axenically manipulate mitospores of most *Pezizaceae* are elusive (see Table S6 for unsuccessful attempts), and so failure to germinate them, or to produce ascocarps in culture from mycelia obtained from mitospores, may reflect our ignorance regarding their requirements for germination, growth and sexual reproduction. However, in the light of the successful germination of some species of saprobic *Pezizaceae*, the multiple independent failures to germinate EcM *Pezizales* mitospores in culture in previous studies and in our study may indicate that these spores are not propagative in nature. Likewise, in the light of the successful production of mycorrhizae from *Tuber* sporocarp tissue in the presence of fine roots, the failure to form mycorrhizae following the same techniques with mitospores suggests that their function may be for outcrossing rather than germination and growth.

A hypothesis posed by Urban *et al.* (2004) regarding *Tuber* mitospore mats is that these spores serve as spermatia, necessary for fertilization in sexual reproduction. Only recently was outcrossing among *Tuber* species verified with molecular evidence, but how this occurs is still a mystery (Riccioni *et al.* 2008). It is possible that for heterothallic species, establishment of the dikaryotic phase in truffles such as *Tuber* may be impeded by subterranean location. We propose that mitospores produced on the soil surface are subsequently carried by rainwater, arthropods or other animals to EcM hyphae in the soil, facilitating the coming together of compatible nuclei. A function of spermatia for outcrossing has been suggested for mitospores in other ascomycetes (Kohn 1993).

Either function, to provide for genetic exchange or to disperse propagules to infect new root tips, may help to explain why spore mats were rarely found in *Pinaceae* forests, and then only on bare soil. A thick duff layer may physically or chemically prevent spore mat formation or may prevent the dissemination of nuclear donors or propagules. Alternatively, absence of spore mats may be due to a preference of EcM *Pezizales* for deciduous tree hosts that is unrelated to spore mat requirements.

Morphologies of most *Pezizaceae* spore mats reported here fit previously described mitosporic forms (reviewed in Hennebert 1973). Mitosporic forms were previously classified as form genera; thus, the saprobic cup fungus *Peziza ostracoderma* has a mitosporic state that was named

Chromelosporium fulvum (Hennebert & Korf 1975). Woodland terricolous species described in Hennebert (1973) are morphologically similar to some of the mitosporic forms sequenced here. Spore mats of both the /terfezia–peziza depressa and /pachyphloeus–amylascus lineages have previously been classified under *Chromelosporium* (Palmer *et al*. 2008). *Glischroderma*, another form genus, has also been tied to *Pachyphloeus* (Norman & Egger 1999). *Glischroderma* spore mats were described as having a covering (Malençon 1964), which was not detected on *Pachyphloeus* spore mats in this study, although the long hyphal projections can sometimes cause the spore mat to appear covered when the projections are matted down.

Although the role(s) of mitospores of EcM *Pezizales* was not fully established in this study, the discovery of spore mats for *Pachyphloeus* and *Tuber* and for four additional hypogeous lineages (/hydnobolites, /hydnotrya, /fischerula and a *Ruhlandiella*-like taxon in /terfezia–peziza depressa) signals that the life cycle of these truffles is more complex than previously known. The high diversity and broad geographic distribution of EcM *Pezizales* that produce spore mats suggests that production of mitospores is more important in the life history of this ecological guild of fungi than has previously been appreciated.

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R.A.H. carried out all work (except for EcM synthesis and sequencing) that was done in the Midwest United States with field and laboratory assistance from L.K. and T.L. and wrote the article with M.E.S. and G.M.B. M.E.S. and G.M.B. carried out all the work that was done in the SE United States with field and laboratory assistance from K.S., R.A.H., and G.M.B. did the culture work on truffles and anamorphs and G.M.B. synthesized ectomycorrhizae. G.W. did the root sampling and sequencing of Mexican oak ectomycorrhizae. Diversity of mitospore-producing EcM *Pezizales* in countries outside the United States was enabled by the following: M.E.S. and D.H.P. in Chile and Argentina; M.E.S. and Z.-W.G. in China; G.G.G. in Mexico; C.H. in England. D.H.P. shared his considerable knowledge of *Pezizales* anamorphs, J.T. shared worldwide collections of truffles, R.V. advised the principle investigators and hosted DNA extraction and sequencing by R.A.H., M.E.S. and G.M.B. D.J.M. advised R.A.H. and hosted DNA extraction and sequencing.

Data accessibility

DNA sequences: GenBank accessions JN102363 - JN102492, JN409337- JN409345, JN121300 JN121377, JF419257, JX414173-JX414224, JX967996-JX968002.

Spore and spore mat measurements, root inoculation data and sequence alignments are available in Dryad under doi:10.5061/dryad.c70gv

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 New sequences of *Pezizale*s listed by lineage, sequence source (asexual spore mat, fruit body, ectomycorrhizal root tip), collector number, herbarium for voucher, geographic origin, and GenBank Accession Numbers for internal transcribed spacer and/or large subunit locus.

Table S2 Downloaded sequences used in phylogenetic analyses in this study.

Table S3 Morphological comparisons of asexual spore mats in six lineages of ectomycorrhizal *Pezizales*.

Table S4 Spore mat contaminant operational taxonomic units, based on 96% similarity of internal transcribed spacer sequenced from spore mats of diverse EcM *Pezizales* lineages.

Table S5 Fungal contaminants on roots inoculated with *Pezizales* mitospores.

Table S6 Reports on *Pezizales* that have produced mitospores under axenic conditions; and results of attempts to germinate mitospores, and to produce fruiting bodies from mito- or meiospores.

Fig. S1 Geographic distribution of operational taxonomic units of EcM pezizalean spore mats and fruit bodies collected in the Eastern USA, Northeastern Mexico, and Southeastern China.

Fig. S2 Monthly spore mat diversity as measured by number of operational taxonomic units, juxtaposed with monthly precipitation (in inches) in North Carolina and Minnesota in 2011.