Molecular and morphological diversity of pezizalean ectomycorrhiza

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Summary

• A growing body of molecular research is discovering a high diversity of pezizalean ectomycorrhiza (EcM), yet most remain unidentified at the genus or species level. This study describes EcM-forming taxa within the Pezizales.
• EcM-forming Pezizales were revealed by morphotyping and sequencing of EcM root tips from forests in Estonia and Denmark. The taxa on EcM root tips were identified using phylogenetic analyses of large-subunit rDNA sequences derived from sporocarps of 301 pezizalean species, and comparisons with internal transcribed spacer rDNA sequences.
• Thirty-three species are suggested as EcM symbionts, representing all three major clades of Pezizales, the genera Genea, Geopora, Humaria, Tarzetta, Trichophaea, Wilcoxina, Helvella, Hydnotrya, Tuber, Pachyphloeus, Peziza and Sarcosphaera, and two Pezizaceae anamorphs. EcM of Pezizales species are easily distinguished by their anatomy, particularly thick cell walls and stout hyphae.
• This study demonstrates that Pezizales species constitute a considerable proportion of the mycobionts in EcM fungal communities in mature boreal deciduous and coniferous forests, in several soil types. Fruit-body sequences and EcM descriptions will facilitate identification of pezizalean EcM in future studies.

Key words: Pezizales, molecular phylogenetic analysis, ectomycorrhiza descriptions, morphotyping, large subunit (LSU), internal transcribed spacer (ITS).


Introduction

The Pezizales are the basal order of Euascomycetes (Lutzoni et al., 2004) and comprise 1125 species (Kirk et al., 2001). Most Pezizales form cup-shaped fruit bodies (apothecia) on soil, dung or plant debris. However, several soil-inhabiting taxa have developed hypogeous fruiting in parallel with the loss of forcibly discharged ascospores. In these taxa, small mammals act as vectors for spore dispersal. Hypogeous fruiting has evolved multiple times independently from apothecial ancestors (O’Donnell et al., 1997; Percudani et al., 1999; Hansen et al., 2001, 2005). The trophic status of pezizalean species ranges from saprobic to mycorrhizal, with a few known to be parasitic (Egger & Paden, 1986). Overall, however, very little is known about the trophic status of most taxa. The majority of species are considered to be saprobic, although most hypogeous (previously in the order Tuberales, now spread across the Pezizales) and a few epigeous taxa are claimed, or have been shown, to be mycorrhizal.

The ectomycorrhizal (EcM) lifestyle has developed in several distantly related lineages of ascomycetes, including Elaphomyces within Eurotiales; Cenococcum within Luluisoascomycetes; the Rhizoscyphus (syn. Hymenoscyphus) ericae complex within Helotiales; and Tuber within Pezizales (LoBuglio et al., 1996; Percudani et al., 1999; Vrålstad et al., 2000). Maia et al. (1996) reviewed numerous reports of the EcM status of pezizalean taxa. However, these reports rely predominantly on observations of fruitbody habitats rather
than on resynthesis or identification of the EcM-forming fungi on root tips. In addition to EcM, ascomycetes also form ectendomycorrhiza. The pezizalean genera *Wilcoxina*, *Sphaerosporella*, *Balsamia* and *Geopora* have been reported to form ectendomycorrhiza with a thin or fragmented mantle, poorly developed Hartig net, and intracellular colonization on *Pinus* and *Larix* spp. (Mikola, 1965; Danielson, 1984; Palfner & Agerer, 1998; Yu et al., 2001; Fujimura et al., 2005). Similarly, *Terfezia* spp. have been shown to form ectendomycorrhiza on Cistaceae (Dexheimer et al., 1985) and possibly on some non-EcM plant genera (Bratek et al., 1996). Danielson (1982) hypothesized that ectendomycorrhizal associations on conifer roots involve several putative genera of Pezizales. Some pyrenomycous members of the Pyronemataceae display a continuum of biotrophic interactions with conifer seedlings, including both pathogenic and EcM associations (Egger & Paden, 1986).

Identification of pezizalean EcM from root tips has remained problematic at both species and genus levels. Septal pore ultrastructure, in particular the presence of simple septa and Woronin bodies, has traditionally been used to confirm ascomycete EcM (Berndt et al., 1990) and orchid mycorrhiza (Selosse et al., 2004). However, septal pore ultrastructure provides low resolution at the genus level (Kimborough, 1994). Unlike many basidiomycetes, EcM ascomycetes lack hyphal strands that can be followed from the base of a fruit body to an EcM (Agerer, 1991, 2001). Many pezizalean taxa produce inconspicuous or hypogeous sporocarps that are easily overlooked unless specifically searched for. Identification of EcM fungi has benefited from molecular tools, including restriction fragment-length polymorphism (RFLP) and sequencing of the rDNA internal transcribed spacer (ITS) region (reviewed by Horton & Bruns, 2001). But ‘universal’ primer mismatches and length polymorphism of the ITS region still hamper amplicon amplification and sequencing of some pezizalean taxa (Aviram et al., 2004; L.T., personal observation). Primarily because of identification problems, EcM anatomy of pezizalean taxa is little studied. Previously published morphological and anatomical descriptions of pezizalean EcM cover only a few genera, including *Tuber*, *Wilcoxina* and *Genesa* (Berndt et al., 1990; Ingleby et al., 1990; Ursic & Peterson, 1997; Jakucs et al., 1998). Generally, pezizalean EcM possess a thin pseudoparenchymatous mantle, infrequent emanating hyphae (EmH), no rhizomorphs and no clamp connections (Agerer, 1991, 2001). Recent sequencing studies have revealed a high diversity of pezizalean EcM (Teredosso et al., 2003; Izzo et al., 2005) and orchid mycorrhizal symbionts (Selosse et al., 2004; Jolou et al., 2005), which have remained unidentified at the genus level. The purpose of this study is to uncover the EcM-forming fungi within the Pezizales. We identify EcM Pezizales on root tips using direct sequencing of the rDNA ITS region and/or large-subunit (LSU) fragment. We demonstrate that the EcM lifestyle is present in several lineages of Pezizales, including both epigeous and hypogeous fruiting species. Most lineages or genera of Pezizales possess distinct EcM anatomy. To improve the identification of pezizalean EcM, we provide morphological and anatomical EcM descriptions.

**Materials and Methods**

**EcM sample preparation**

Root samples were taken at four sites in Estonia and Denmark in 2001–2004 in connection with studies describing the overall community structure of EcM fungi (Table 1). In Estonia, pezizalean EcM were specifically sought at five additional sites (Table 1). EcM morphotypes lacking both hyphal strands and clamp connections were considered to be possibly formed by pezizalean taxa. These morphotypes were further anatomotyped and described according to Agerer (1991), and photographed using Axiovision 3.0 software (Carl Zeiss Vision, Munich, Germany). Plan views were taken at ×20–50 magnification, and mantle structure was recorded at ×1000 magnification with Nomarski differential interference contrast. The images of the mantle were further sharpened using Adobe Photoshop 5.5 software (Adobe Systems, Inc., San Jose, CA, USA).

Longitudinal sections of EcM root tips (0.25–4 µm thick) were prepared to check for the presence of a Hartig net and intracellular colonization. EcM root tips were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by dehydration in an ethanol series (50–100% ethanol, increment, 10%). EcM were then rinsed in acetone, a mixture of acetone and araldite (1 : 1), and finally embedded in araldite (araldite CY212, 48.8%; 2-dodecyl succinic acid anhydride, 48.8%; araldite DY964, 1.4%, Serva, Wichita Falls, TX, USA). Sectioning was performed on a Reichert ultramicrotome equipped with glass knives. The sections were stained in methylene blue to visualize hyphae, and studied at ×400 magnification.

**Molecular techniques**

DNA was extracted from fruit bodies and whole EcM root tips as described by Hansen et al. (2005) and Gardes & Bruns (1996), respectively. Forward primers ITS1F (5′-cttggcctattagagagaattc-3′) and LR0R (5′-accgcctgaacttaagc-3′) were used in combination with reverse primers ITS4 (5′-tctccggtcattattgagtc-3′), LR5 (5′-accgcctgaacttaagc-3′), LR5 (5′-tctcctgaagacttag-3′), TW13 (5′-gtctcctctcctgccattc-3′), LR5 (5′-ctcttcgcctgccatcctc-3′) to amplify and sequence the ITS and/or LSU regions (Fig. 1). Additional primers Ctb6 (5′-gtctcctctcctgccattc-3′), Ctb5 (5′-ggttggtttcttttccct-3′), LR3 (5′-ccctgtgaaagaaagacg-3′) and/or LR3R (5′-gtctggaacaaagacc-3′) were used for sequencing. Contigs were assembled using Sequencher 3.0 or 4.2 (GeneCodes Corp., Ann Arbor, MI, USA). All new sequences of fruit bodies and EcM root tips were submitted to the European Molecular Biology Laboratory (EMBL) database or GenBank (NCBI) (Table 2; Table S1). The ITS sequences from fruit bodies were submitted to the UNITE database (Kõljalg et al., 2005).
Table 1 Characteristics of the study sites

<table>
<thead>
<tr>
<th>Site</th>
<th>Geocode</th>
<th>Ecosystem: age, forest site type, hosts</th>
<th>Soil type†</th>
<th>Samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Järvselja, Estonia (Jä)</td>
<td>58°17’ N; 27°19’ E</td>
<td>Old-growth forest; 100–160 yr; Oxalis type; Picea abies, Tilia cordata, Betula pendula, Populus tremula</td>
<td>Umbric podzol; haplic luvisol</td>
<td>15 × 5 × 5 cm (n = 108); 15 × 15 × 5 cm (n = 45); all EcM fungi</td>
<td>Tedersoo et al. (2003); L. Tedersoo et al. (unpublished)</td>
</tr>
<tr>
<td>Tagamõisa, Estonia (Ta)</td>
<td>58°27’ N; 22°00’ E</td>
<td>Semi-natural woodland; 40–200 yr; Hepatica type; Betula spp., Quercus robur, T. cordata, Corylus avellana</td>
<td>Mollisihumi rendzic leptosol</td>
<td>15 × 15 × 5 cm; n = 166; all EcM fungi</td>
<td>L.T. et al. (unpublished)</td>
</tr>
<tr>
<td>Lille Bøgeskov, Denmark (LB)</td>
<td>55°29’ N; 11°38’ E</td>
<td>Mature forest; 85 yr; Asperula type; Fagus sylvatica</td>
<td>Luvisol</td>
<td>2.5 cm diam. × 15 cm; n = 153; all EcM fungi</td>
<td>R.K. et al. (unpublished)</td>
</tr>
<tr>
<td>Gribskov, Denmark (Gr)</td>
<td>55°58’ N; 12°15’ E</td>
<td>Mature forest; 100 yr; Luzula type; F. sylvatica</td>
<td>Arenosol</td>
<td>2.5 cm diam. × 15 cm; n = 120; all EcM fungi</td>
<td>K. Føns et al. (unpublished)</td>
</tr>
<tr>
<td>Lelatu, Estonia (La)</td>
<td>58°35’ N; 22°34’ E</td>
<td>Semi-natural woodland; 80–250 yr; Hepatica type; Q. robur, C. avellana, Helianthemum nummularium, B. pendula</td>
<td>Mollisihumi rendzic leptosol</td>
<td>20 × 20 × 15 cm; n = 7; Pezizales only</td>
<td>This study</td>
</tr>
<tr>
<td>Kärla, Estonia (Kä)</td>
<td>58°20’ N; 22°18’ E</td>
<td>Mature forest; 100 yr; Arctostaphylos type; Pinus sylvestris, P. abies, Arctostaphylos uva-ursi</td>
<td>Haplic podzol</td>
<td>20 × 20 × 15 cm; n = 5; Pezizales only</td>
<td>This study</td>
</tr>
<tr>
<td>Holdre, Estonia (Ho)</td>
<td>57°58’ N; 25°41’ E</td>
<td>Mature forest roadside; 100 yr; Vaccinium myrtillus type; P. sylvestris, Salix sp.</td>
<td>Umbric podzol</td>
<td>20 × 20 × 15 cm; n = 3; Pezizales only</td>
<td>This study</td>
</tr>
<tr>
<td>Naissoo, Estonia (Na)</td>
<td>58°37’ N; 22°07’ E</td>
<td>Mature forest; 100–160 yr; Hepatica type; Q. robur, C. avellana, B. pendula, P. tremula, P. abies</td>
<td>Calcaric cambisol</td>
<td>15 × 15 × 5 cm; n = 1; Pezizales only</td>
<td>This study</td>
</tr>
<tr>
<td>Kehila, Estonia (Ke)</td>
<td>58°26’ N; 22°04’ E</td>
<td>Coastal sand dunes; 10–40 yr; grey dune type; P. sylvestris, Salix sp.</td>
<td>Salic fluvisol</td>
<td>15 × 15 × 5 cm; n = 2; Pezizales only</td>
<td>This study</td>
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* Abbreviation in parentheses.
† Following FAO et al. (1998).

n, Number of soil samples.
To determine the phylogenetic placement of EcM root samples within Pezizales, LSU rDNA sequences derived from sporocarps of 301 pezizalean species (represented by 384 specimens) were used in the analyses. Published sequences are primarily from B.A.P. and co-workers (unpublished data); Hansen et al. (2001, 2005); O'Donnell et al. (1997). Sequences of the LSU region were aligned by hand using the software SE-AL ver. 2.0a8 (Rambaut, 1996). The LSU rDNA contains highly divergent regions across all the Pezizales. Therefore three subset alignments were constructed, each representing one of three distinct clades identified within the Pezizales (Fig. 2; Landvik et al., 1997, K.H. and B.A.P., unpublished data). The three alignments include representative taxa from the families Pyronemataceae (clade I); Morchellaceae, Discinaceae, Helvellaceae, Tuberaceae, Rhizinaeae and Caloscyphaceae (clade II); and Pezizaceae (clade III). Sequences of the families Sarcoscyphaceae and Sarcosomataceae (clade I) and Ascobolaceae (clade III) were not included because no EcM sequence types were affiliated with these presumably strictly saprotrophic families. The LSU sequences obtained from unknown EcM root tips were initially aligned in more inclusive data sets of the most suitable clade and analysed (trees not shown). The three data sets were then pruned down to focus mainly on EcM or possible EcM lineages, still including representative taxa from most genera. The final data sets included 67 known species (from 83 specimens) and 21 unknown EcM symbionts (clade I); 35 known species (37 specimens) and 12 unknown EcM symbionts (clade II); and 60 known species (65 specimens) and 12 unknown EcM symbionts (clade III). Based on analyses of higher-level
relationships (K.H. and B.A.P., unpublished data), *Ascobolus* and *Peziza* were used as an outgroup for clade I; two species of *Peziza* and *Iodophanus* for clade II; and two species of *Ascobolus* for clade III. The LSU alignment of clade I included 905 characters, clade II 699 characters, and clade III 720 characters. Alignments are available from TreeBase (http://treebase.bio.buffalo.edu/treebase) as accession nos M2538 (clade I), M2537 (clade II) and M2539 (clade III). Sequence accession numbers are given on the phylograms (Figs 3–5).

The ITS sequences were obtained from 21 fruitbody collections and 39 EcM root tips with identical or nearly identical LSU sequences, to further confirm specific matches between these. The differences between ITS sequences of the taxa are given as pairwise percentage identity of the whole ITS region (ITS1, 5.8S rDNA and ITS2).

**Results**

Occurrence of pezizalean EcM

Pezizales species comprised 3.7–13.0% of the taxa in the four EcM fungal communities at Järvselja, Tagamõisa, Lille Bøgeskov and Gribskov, colonizing 4.5–6.1% of the root tips. At Laelatu, Kärla, Naissoo, Holdre and Kehila, where pezizalean EcM were specifically sought, several additional Pezizales species were discovered. *Humaria hemisphaerica*, *Genea hispidula*, *Genea* sp., *Wilcoxina* sp., *Geopora* sp., *Hydnora* sp. and *Peziza michelii* were found in more than one study site. Among these, *H. hemisphaerica* and *Geopora* sp. colonized root tips in both coniferous and deciduous woodlands.
Fig. 3 Phylogeny demonstrating the placement of 21 unknown ectomycorrhizas (EcM) within Pyronemataceae (clade I, Fig. 2), inferred from large-subunit rDNA sequences. One of > 15 000 most parsimonious trees. Terminal taxa represent individual specimens (from B.A.P. and co-workers, unpublished data). EMBL accession numbers are shown in parentheses. Numbers above branches represent posterior probabilities (≥95%); numbers below branches represent bootstrap support (≥70%). Five resolved fine-scale lineages including EcM samples are indicated for discussion in the text. o, Hypogeous fruiting taxa.
Molecular identification of pezizalean EcM

Parsimony and Bayesian analyses of the three LSU data sets resulted in tree topologies, and moderate to highly supported groups, that are generally in agreement with B.A.P. and co-workers (unpublished data, using LSU) for clade I; O’Donnell et al. (1997, using LSU and rDNA small subunit) for clade II; and Hansen et al. (2005, using LSU, RNA polymerase II subunit 2 (RPB2) and β-tubulin) for clade III. The trees are well resolved, but the deeper-level relationships are not supported. All EcM sequences are, however, nested within strongly supported, more-or-less inclusive lineages.

Clade I: Pyronemataceae Most (44.7%) of the pezizalean LSU EcM sequence types are nested within several lineages of Pyronemataceae (Fig. 3). Jä-O35, Kä-L795, LB-776, Na-L141 and Ta-L785 form a distinct clade with two specimens of H. hemisphaerica (BP/PP 100%). Additionally, ITS sequences of Jä-O35 and LB-776 are 99.4–100% identical to ITS sequences of H. hemisphaerica (UNITE accession UDB000988, DQ200832). LB-133 and Jä-C40 form a strongly supported group with the hypogeous fruiting G. hispidula (BP 92%, PP 100%). Supporting this identification, ITS sequences of LB-133 and Jä-C40 are 100 and 99.9% identical, respectively, to the G. hispidula specimens (AJ969622, AJ969623). Ta-L155, Ta-L168 and LB-927 form a distinct group within Genea (BP/PP 100%) that did not match any known fruitbody sequences. La-L780 and Ta-TS102 are highly supported as successive sister taxa to the Genea–Humaria lineage (BP 100%, PP 95–100%). The ITS sequence of LB-1491 is 98.6% identical to T. catinus (DQ200833).
Fig. 5 Phylogeny demonstrating the placement of 12 unknown ectomycorrhizas within Pezizaceae (clade III, Fig. 2), inferred from large-subunit rDNA sequences. One of 4268 most parsimonious trees. Terminal taxa represent individual specimens (from Hansen et al., 2001, 2005). EMBL accession numbers are shown in parentheses. Numbers above branches represent posterior probabilities ($\geq 95\%$); numbers below branches represent bootstrap support ($\geq 70\%$). ○, Hypogeous fruiting taxa.
Clade II: Discinaceae, Tuberaceae and Helvellaceae  
Clade II includes 27.7% of the LSU EcM sequence types, which are nested within lineages of *Helvella, Tuber* and *Hydnophoraya* (Fig. 4). La-L783 clusters with *Helvella aff. cupuliformis*, sharing 99.8% LSU sequence identity. Ta-L528 forms a distinct lineage with *H. crispa* (BP/PP 100%). LB-756 and Ho-TS601 are nested among *Helvella* spp., but their species-level identities are not resolved. Ta-L420 and La-L788 form a strongly supported group with *Tuber rufum var. rufum* (BP 88%, PP 100%), but with high LSU sequence variation. LB-8 groups with two specimens of *Tuber puberulum* (BP 94%, PP 100%), and the identification of LB-8 is supported by 100% ITS sequence identity to *T. puberulum* (AJ969625, AJ969626). Já-A58 is highly nested within *Tuber* spp., but forms a separate, unidentified lineage. Ta-L546 and Já-J56 form a strongly supported group with *Tuber maculatum* (BP 99%, PP 100%). LB-623 and Já-B48, which have 99.8% identical LSU sequences, form a distinct sister group to *Hydnophora cubispora* (BP 98%, PP 100%). In addition, ITS sequences of LB-623, Já-B48 and an additional EcM sample, Gr-1143, are 94.5–99.8% identical to the ITS sequence of *Hydnophora tulasnei* (AJ969621, not in Fig. 4).

Clade III: Pezizaceae  
Clade III includes 27.6% of LSU EcM sequence types that are nested within lineages of Pezizaceae: the *Pachyphloeus–Amylascus, Terfezia–Peziza depressa, Sarcosphaera–Hydnophoraya* and *P. michelii–Peziza succosa* lineages (Fig. 5). Ta-L473 and La-L806 form a distinct group with two specimens of *P. michelii* (BP/PP 100%). The identifications of Ta-L473 and La-L806 are confirmed by 100% identical ITS sequences to *P. michelii* (DQ200838, DQ200839). La-L786 and *P. succosa* form a sister taxon to *Peziza succosella* (BP/PP 100%), and together they form a sister group to *P. michelii* (BP 73%, PP 100%). The ITS sequence of La-L786 is 100% identical to ITS sequences of *P. succosella* specimens (UD8000984, DQ200840). Kä-L794 groups with three specimens of *Sarcosphaera coronaria* (PP 97%), showing the highest LSU sequence identity (98.7%) to a North American specimen (AF133172). Já-L717A forms a highly supported group with two specimens of *P. depressa* (BP 70%, PP 97%), sharing 99.7% LSU sequence identity. Supporting this grouping, the ITS sequences of Já-L717A and *P. depressa* (DQ200837) are 96.1% identical. Ta-L233 is nested within the *Terfezia–P. depressa* lineage, but its placement within this group remains unresolved. LB-472 and Ta-TS147 form a distinct lineage with an unidentified Pezizaceae anamorph (a field-collected specimen; BP/PP 100%). Confirming this, ITS sequences of LB-472, Ta-TS147 and an additional EcM sample, Gr-193 (not in Fig. 5), are 100% identical to the Pezizaceae anamorph (DQ200836). LB-1274 clusters with the anamorph *Glomoderma* sp. (BP 90%, PP 100%) among the hypogeous fruiting *Pachyphloeus* and epigeous *Scabropezia*. Ta-TS162 and Ta-L177 are closely related, and form a moderately supported group with *Pachyphloeus virescens* (BP 82%, PP 100%). LB-805 forms a sister group to the *Pachyphloeus–Amylascus* lineage (BP 97%, PP 100%), but does not match any known fruitbody sequences.

Morphology of Pezizalean EcM  
Pezizales species formed various EcM morphotypes with different colour, thickness and anatomy of the mantle and EmH (Fig. 6). Pezizalean EcM were predominantly characterized by a pseudoparenchymatous mantle, well developed Hartig net and infrequent, thick, stout, thick-walled EmH (Table 3; Item S1, Figs S1–S3). Clamps, cystidia and rhizomorphs were not observed in the described EcM. Only sequence types Kä-L800 (*Wilcoxina* sp.), Ke-L1056 (*Geopora* sp.) and Já-L1075 (*T. hybrida*) occasionally colonized cortical cells of *Pinus sylvestris* and *Picea abies*, visually causing no damage. Based on anatomical characters, EcM of the *Genea–Humaria* lineage was twice confused with nonclamped *Tomentella* spp. (Basidiomycota) that differed in having a thicker mantle and the absence of such extremely thick-walled EmH and outer mantle cells.

Discussion  
Identification and morphology of pezizalean lineages  
The phylogenetic analyses of the LSU data sets resolved the identity of most pezizalean EcM sequence types to genus or species level. The ITS region provided additional evidence for species-specific identification. All three major clades of Pezizales include EcM-forming taxa, in five families (Fig. 2). Moreover, our results indicate that hypogeous members of the Pezizales are EcM-forming and tend to occur in lineages with epigeous EcM-forming taxa or taxa with unresolved biotrophic status. Based on these results, and because evidence suggests that hypogeous or semihypogeous fruiting fungi are derived from epigeous fruiting ancestors (Trappe, 1979; Thiers, 1984; O’Donnell et al., 1997; Peter et al., 2000; Hansen et al., 2001; Peintner et al., 2001), we hypothesize that the EcM lifestyle is a precondition for the switch to hypogeous fruiting. Within the EcM lineages, selection for a hypogeous habit is probably driven and maintained, in part, by the advantageous deposition of spores into the rooting zone (Miller et al., 1994). Other likely selective factors for a hypogeous habit include resistance to desiccation in dry climatic conditions (Thiers, 1984), and protection from surface fires. Hypogeous fruiting taxa develop closed, more-or-less compact ascomata, with thick-walled hyphae and ascospores. Within the Pezizales, hypogeous ascomata evolved independently at least 10 times in six different families (Figs 3–5).

The epigeous fruiting *H. hemisphaerica* and hypogeous *Genea* spp. form a distinct lineage within Pyronemataceae. EcM types of these taxa are nearly identical in EcM plan morphology and mantle anatomy, sharing an orange-brown.
Fig. 6 Plan views of pezizalean ectomycorrhizas. (a) *Humaria hemisphaerica* Na-L141; (b) *Genea* sp. Ta-L155; (c) *Genea–Humaria* lineage La-L780; (d) sister to the *Genea–Humaria* lineage Ta-TS102; (e) *Trichophaae woolhopeia* La-L809; (f) *Trichophaae hybrida* Jä-L1075; (g) *Wilcoxina cf. rehmi* Ta-L343; (h) *Wilcoxina rehmi* Kä-L800; (i) *Geopora* sp. Kä-L799; (j) *Geopora* sp. Ke-L1056; (k) *Helvella* sp. Ho-TS601; (l) *Helvella* sp. Ta-L528; (m) *Helvella* sp. La-L783; (n) *Hydnotrya tulasnei* Jä-B48; (o,p) *Peziza micheli* La-L806; (q) *Peziza succosa* La-L786; (r) *Sarcosphaera coronaria* Kä-L794; (s) *Peziza depressa* Jä-L717A; (t) *Terfezia–P. depressa* lineage Ta-L233; (u,v) *Pezizaceae* anamorph Ta-TS147; (w) *Pachyphloeus* sp. Ta-L177; (x) *Pachyphloeus* sp. Ta-TS162. Bar, 0.3 µm. (k,u,x) by T. Suvi.
Table 3 Descriptions of pezizalean ectomycorrhizas with references to example illustrations

<table>
<thead>
<tr>
<th>Taxa and sequence types</th>
<th>Plan morphology</th>
<th>Outer mantle layer cells</th>
<th>Middle mantle layer cells</th>
<th>Inner mantle layer cells</th>
<th>Emanating hyphae</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Humaria, Genea spp.</strong></td>
<td>Orange-brown, red-brown to dark brown, surface roughly granular (Fig. 6a–c)</td>
<td>PsP: subepidermoid, blunt polygonal, extremely thick-walled (Fig. S1a,e,i)</td>
<td>PsP: polygonal to oblong oval, thick-walled (Fig. S1b,f,i)</td>
<td>PsP/PL: strongly elongated, irregular (Fig. S1c,g,k)</td>
<td>Frequent, red-brown, cylindrical, thick-walled, 4–9 µm diam. (Fig. S1d,h,l)</td>
</tr>
<tr>
<td>Na-L141, Jä-O35, Ta-L155, La-L780, La-L785, La-795</td>
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<tr>
<td><strong>Sister to the Genea–Humaria lineage Ta-TS102</strong></td>
<td>Yellowish-brown to dark brown, finely granular (Fig. 6d)</td>
<td>PsP: sharply angular rhomboid, thick-walled (Fig. S1m)</td>
<td>PsP: elongated epidermoid to irregular, thick-walled (Fig. S1n)</td>
<td>PsP: blunt polygonal to spherical, nets of short inflated hyphae (5–12 µm diam.) (Fig. S1s)</td>
<td>Frequent, dark brown, stellately branching, thick-walled, 4–6.5 µm diam. (Fig. S1t)</td>
</tr>
<tr>
<td><strong>Trichophaea woolhopeia</strong></td>
<td>Red-brown, finely granular (Fig. 6e)</td>
<td>PsP: oval to polygonal, thick-walled (Fig. S1q)</td>
<td>PsP/PL: irregular, elongated, often forming highly branched structures (Fig. S1v,w,a,d)</td>
<td>PsP/PL: oblong, irregular (Fig. S1s)</td>
<td>Infrequent, hyaline, thick-walled, 4–6 µm diam. (Fig. S1t)</td>
</tr>
<tr>
<td>La-L809</td>
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<tr>
<td><strong>Trichophaea hybrida</strong></td>
<td>Red-brown, smooth, shiny, thin (0–3 hyphal layers) on conifers (Fig. 6f–h)</td>
<td>PsP/PL: irregular, elongated, blunt polygonal to subepidermoid, often forming highly branched structures, thick-walled (Fig. S1u,y,β,γ)</td>
<td>PsP: oblong, blunt polygonal to epidermoid (Fig. S2b,c)</td>
<td>PsP/PL: irregular, elongated, often forming highly branched structures (Fig. S1v,w,a,d)</td>
<td>Infrequent, light brown, thin-walled, 5–6 µm diam. (Jä-L1075; Fig. S1x) or not observed (Ta-L343, Kä-L800, Ho-TS602)</td>
</tr>
<tr>
<td>Jä-L1075, Wilcoxina spp. Ta-L343, Kä-L800, Ho-TS602</td>
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<tr>
<td><strong>Geopora spp. Ta-L159, Kä-L799, Ke-L1056</strong></td>
<td>Orange-brown to dark brown, smooth, mantle fragmented on conifers (Fig. 6i,j)</td>
<td>PsP: spherical to subepidermoid, thick-walled (Fig. S2a,e,f)</td>
<td>PsP: oblong, blunt polygonal to epidermoid (Fig. S2j,k)</td>
<td>PsP: oblong, irregular (Ta-L159) or chains of inflated hyphae (Ke-L1056) (Fig. S2w,y)</td>
<td>Infrequent, brown, 4–6 µm diam.</td>
</tr>
<tr>
<td><strong>Helvella spp. Ho-TS601, Ta-L528, La-L783</strong></td>
<td>Orange-brown to red-brown, whitish tip when young, smooth (Fig. 6k–m)</td>
<td>PsP: large spherical to subepidermoid (Ho-TS601; Fig. S2i); subepidermoid to epidermoid (Ta-L528, La-L783; Fig. S2m,p), thick-walled</td>
<td>PsP: oblong, blunt polygonal (Ho-TS601; Fig. S2j,k); epidermoid, thick-walled (Ta-L528, La-L783; Fig. S2n,q)</td>
<td>PsP: oblong, irregular (Ta-L528; Fig. S2o) or subepidermoid (La-L783; Fig. S2r)</td>
<td>Not observed</td>
</tr>
<tr>
<td><strong>Hydnotrya tulasnei</strong></td>
<td>Light yellow to beige, smooth (Fig. 6n)</td>
<td>PsP: spherical to subepidermoid, thick-walled, with abundant intercellular space (Fig. S2s)</td>
<td>PsP: large, oblong, deeply branched, epidermoid (Fig. S2t)</td>
<td>PsP: large, oblong, deeply branched, epidermoid (Fig. S2u)</td>
<td>Not observed</td>
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<tr>
<td>Jä-B48</td>
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<tr>
<td><strong>Peziza michelii</strong></td>
<td>Yellow-green to olive green, smooth to finely granular (Fig. 6o,p)</td>
<td>PsP: spherical, with abundant extracellular material in tip (Fig. S2v); triangular or rectangular, densely packed (Fig. S2w)</td>
<td>PsP: triangular or rectangular, densely packed (Fig. S2x)</td>
<td>PsP: rectangular to polygonal, thin-walled (Fig. S3c)</td>
<td>Not observed</td>
</tr>
<tr>
<td>Ta-L473, La-L806</td>
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<tr>
<td><strong>Peziza succosa</strong></td>
<td>Whitish, cottony (Fig. 6q)</td>
<td>PsP: spherical to rectangular, blunt-angled, thick-walled (Fig. S3a)</td>
<td>PsP: rectangular, blunt-angled (Fig. S3b)</td>
<td>PsP: rectangular to polygonal, thin-walled (Fig. S3c)</td>
<td>Infrequent, hyaline, thin-walled, 2–3 µm diam.</td>
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<tr>
<td>La-L786</td>
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<tr>
<td><strong>Sarcosphaera coronaria</strong></td>
<td>Red-brown, smooth, shiny (Fig. 6r)</td>
<td>PsP: loosely arranged, spherical to subepidermoid in a gelatinous matrix (Fig. S3d)</td>
<td>PsP: triangular or rectangular, densely packed (Fig. S2x)</td>
<td>PsP: rectangular to polygonal, thin-walled (Fig. S3c)</td>
<td>Not observed</td>
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<td>Kä-L794</td>
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Table 3 continued

<table>
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<tr>
<th>Taxa and sequence types</th>
<th>Plan morphology</th>
<th>Outer mantle layer cells</th>
<th>Middle mantle layer cells</th>
<th>Inner mantle layer cells</th>
<th>Emanating hyphae</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Peziza depressa</em> Jä-L717A</td>
<td>Whitish, cottony (Fig. 6s)</td>
<td>PsP: spherical to slightly angular with a net of stout bulbous cells (Fig. S3g)</td>
<td>PsP: spherical to slightly angular (Fig. S3 h)</td>
<td>Pl: short, stout hyphae, 5–8 µm diam. (Fig. S3i)</td>
<td>Frequent, inflated, stellately ramifying, thin-walled, 2.5–4 (6) µm diam. (Fig. S3j) Not observed</td>
</tr>
<tr>
<td>The Terfezia-Peziza depressa lineage Ta-L233</td>
<td>Yellowish to beige, thin, irregular (Fig. 6t)</td>
<td>PsP: large, spherical to subangular, thin-walled (Fig. S3k)</td>
<td>PsP: oblong, subepidermoid to subangular, thin-walled (Fig. S3l)</td>
<td>PsP: oblong, subepidermoid to subangular, thin-walled (Fig. S3m)</td>
<td></td>
</tr>
<tr>
<td>Pezizaceae anamorph Ta-TS147</td>
<td>Lilac-grey to violet brown, glittering (Fig. 6u,v)</td>
<td>PsP: spherical to oblong, with a superficial net of inflated hyphae (Fig. S3n)</td>
<td>PsP: spherical to oblong (Fig. S3o)</td>
<td>Pl: regularly distributed, short, inflated, 5–8 µm diam. (Fig. S3p)</td>
<td></td>
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<tr>
<td><em>Pachyphloeus</em> sp. Ta-L177</td>
<td>Yellowish to orange-yellow, smooth (Fig. 6w)</td>
<td>PsP: large, sharply rhomboid, thick-walled (Fig. S3s)</td>
<td>PsP: sharply polygonal (Fig. S3t)</td>
<td>Pl: short, inflated hyphae, 4–6 µm diam. (Fig. S3u) Infrequent, in dense tufts, irregular, 2.5–5 µm diam. (Fig. S3v) Frequent, short, inflated, 2.5–4.5 (7) µm diam. (Fig. S3z)</td>
<td></td>
</tr>
<tr>
<td><em>Pachyphloeus</em> sp. Ta-TS162</td>
<td>Whitish to beige, smooth to slightly cottony (Fig. 6t)</td>
<td>Spherical, thick-walled (Fig. S3w)</td>
<td>PsP: rectangular (Fig. S3x)</td>
<td>PsP/PL: oblong, rectangular to oval (Fig. S3y)</td>
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Each EcM sequence type is described in more detail in Item S1. PsP, pseudoparenchymatous; Pl, plectanchymatous.
to dark red-brown rough surface, an outer mantle layer of oval to rectangular, extremely thick-walled cells, and thick, straight EmH (Fig. 6a–c, Fig. 5a–l), as described for G. hispidula (Brand, 1991) and Genea verrucosa (Jakucs et al., 1998).

The genus Trichophaea is not monophyletic, based on LSU rDNA sequences (Fig. 3; B.A.P. and co-workers, unpublished data). Trichophaea woolhopeae is closely related to Sphaeroserpella brunnea, Trichophaea abundans and Anthracobia spp. Sphaerospella brunnea is able to form EcM or ectendomycorrhiza on a wide range of hosts in vitro and in vivo (Danielson, 1984; Parlade et al., 2004). Additionally, Anthracobia tristis and Anthracobia maurilabra are reported to form a thin, discontinuous mantle on roots of Pinus spp. (Egger & Paden, 1986). However, Anthracobia macrocystis, Anthracobia melaloma, T. abundans, Trichophaea minuta and Trichophaea contradicta display no biotrophic properties in aseptic synthesis trials (Danielson, 1984; Egger & Paden, 1986; Warcup, 1990). Trichophaea hemisphaerioidea and T. hybridra are phylogenetically quite distant from T. woolhopeae. Trichophaea hemisphaerioidea has been shown to colonize cortical cells of short and long roots, indicating weak biotrophism (Egger & Paden, 1986). However, Anthracobia macrocystis, Anthracobia melaloma, T. abundans, Trichophaea minuta and Trichophaea contradicta display no biotrophic properties in aseptic synthesis trials (Danielson, 1984; Egger & Paden, 1986; Warcup, 1990). Trichophaea hemisphaerioidea and T. hybridra are phylogenetically quite distant from T. woolhopeae. Trichophaea hemisphaerioidea has been shown to colonize cortical cells of short and long roots, indicating weak biotrophism (Egger & Paden, 1986). However, Anthracobia macrocystis, Anthracobia melaloma, T. abundans, Trichophaea minuta and Trichophaea contradicta display no biotrophic properties in aseptic synthesis trials (Danielson, 1984; Egger & Paden, 1986; Warcup, 1990). Trichophaea hemisphaerioidea and T. hybridra are phylogenetically quite distant from T. woolhopeae. Trichophaea hemisphaerioidea has been shown to colonize cortical cells of short and long roots, indicating weak biotrophism (Egger & Paden, 1986). However, Anthracobia macrocystis, Anthracobia melaloma, T. abundans, Trichophaea minuta and Trichophaea contradicta display no biotrophic properties in aseptic synthesis trials (Danielson, 1984; Egger & Paden, 1986; Warcup, 1990). Trichophaea hemisphaerioidea and T. hybridra are phylogenetically quite distant from T. woolhopeae. Trichophaea hemisphaerioidea has been shown to colonize cortical cells of short and long roots, indicating weak biotrophism (Egger & Paden, 1986). However, Anthracobia macrocystis, Anthracobia melaloma, T. abundans, Trichophaea minuta and Trichophaea contradicta display no biotrophic properties in aseptic synthesis trials (Danielson, 1984; Egger & Paden, 1986; Warcup, 1990). Trichophaea hemisphaerioidea and T. hybridra are phylogenetically quite distant from T. woolhopeae. Trichophaea hemisphaerioidea has been shown to colonize cortical cells of short and long roots, indicating weak biotrophism (Egger & Paden, 1986). However, Anthracobia macrocystis, Anthracobia melaloma, T. abundans, Trichophaea minuta and Trichophaea contradicta display no biotrophic properties in aseptic synthesis trials (Danielson, 1984; Egger & Paden, 1986; Warcup, 1990). Trichophaea hemisphaerioidea and T. hybridra are phylogenetically quite distant from T. woolhopeae. Trichophaea hemisphaerioidea has been

The EcM mantle anatomy of these taxa is hardly distinguishable, has not previously been identified as an EcM-forming species.

Our molecular data suggest that Tarzetta catinus may be able to form a biotrophic relationship with Fagus sylvatica. Tarzetta spp. form a highly supported sister group to Geopora and two hypogeous fruiting taxa. Geopora carbonaria forms a biotrophic relationship with spruce roots in nature (Vrålstad et al., 1998). Egger & Paden (1986) synthesized the association with a thin mantle, abundant intracellular colonization and disruption of pine root epidermal cells, suggesting a moderately parasitic relationship. Pulsinula spp., which constitute a sister lineage to the Tarzetta–Geoprysi lineage, form EcM with a thin plecetenchymatous mantle and a Hartig net (Warcup, 1990; Amicucci et al., 2001). Unfortunately, the root tips colonized by T. catinus were frozen for molecular analyses, preventing us from studying the anatomy of its association.

Helvella aestivalis and two unidentified Helvella spp. were previously shown to form EcM with Dryas sp. and Salix sp. (Weidemann, 1998). Using cloned ITS sequences, Murat et al. (2005) identified Helvella and/or Tuber spp. from EcM root tips morphotyped as Tuber or unidentified, and hinted that these genera might form a biotic association. We found the mantle structure of Helvella and Tuber spp. to be similar, which probably reflects the shared ancestry of Helvellaceae and Tuberaceae (Fig. 4; Landvik et al., 1997; O’Donnell et al., 1997). In contrast to Tuber spp., however, the EcM of Helvella spp. possess no superficial hyphal net.

The EcM, hypogeous fruiting Hydnorina spp. form a sister lineage to epigeous Gyromitra spp. Gyromitra infusa has been shown to be nonEcM in aseptic synthesis trials (Egger & Paden, 1986). However, reliable information is lacking on the trophic status of other members of the Discinaceae, which may include additional EcM taxa. The EcM of H. tulasnei are easily recognizable by large, extremely thick-walled, subepidermoid to oval cells in the outer and middle mantle layers (Fig. S2s,t).

This is the first report of EcM formation in the P. michelii–P. succosa and Sarcophaea–Hydnotryopsis lineages. EcM of P. michelii are easily distinguishable by a green to yellow colour and black-staining corners of polygonal cells in the middle and inner mantle layers (Fig. S2v–x). Sarcophaea coronaria displays a unique EcM anatomy among Pezizales with sparse hyaline hyphae in a gelatinous matrix (Fig. S3d–f).

This is the first report of an epigeous EcM forming fungus in the Terfezia–P. depresa lineage (Fig. 5). The hypogeous or semihypogeous fruiting taxa, Peziza whitei and Rublandiella (as Muciturbo), form EcM with Eucalyptus or Melaleuca (Warcup, 1990). The hypogeous fruiting genera Terfezia and Tirmania form mycorrhizal symbiosis with Helianthemum sp. (Drexheimer et al., 1985). Pachyphloeus spp. Ta-L177 and Ta-TS162 resemble EcM anatomotypes ‘ITE.1’ and ‘ITE.4’ on seedlings (Ingleby et al., 1990) with respect to inflated inner mantle layer cells and EmH (Fig. S3u–z). A closely related sequence type, ‘pezizalean II’, predominantly colonizes root tips distant from trees, suggesting its poor competitive ability (Dickie & Reich, 2005). In agreement with these results, EcM sequence types Ta-L177 and Ta-TS162 inhabit only the most open sites of a wooded meadow (L.T. and co-workers, unpublished data). These findings suggest that Pachyphloeus spp. display a ruderal strategy.

The ecological role of EcM Pezizales

Previous studies have shown that Pezizales species are often the dominant members of EcM fungal communities in early successional ecosystems and following disturbance. In particular, Wilcoxina spp. and Tuber spp. dominate the roots of seedlings in forest nurseries (Mikola, 1965; Ursic et al., 1998) and clear cuts (Mah et al., 2001). Several pezizalean species colonize seedlings established after wildfire (Warcup, 1990, 1991; Baar et al., 1999; Grogan et al., 2000) and at bonfire sites (L.T., personal observation). A variety of hypogeous fruiting fungi,
including Tuber (Pezizales), dominate the resistant propagule communities in mature forests, emerging after complete drying and mixing of soils (Baar et al., 1999; Taylor & Bruns, 1999). Hypogeous fruiting taxa produce thick-walled spores that are predominantly dispersed by rodents with a restricted home range (Maser et al., 1978). This suggests that the spores of these fungal species probably spread over a short distance and are able to persist over time. Thick-walled chlamydospores and ascospores may also be advantageous in burnt ground because of their ability to survive surface fires. Moreover, raised temperature has been shown to stimulate germination and growth of some pyrophilous taxa (El Abyad & Webster, 1968a), which probably gives them a competitive advantage (El Abyad & Webster, 1968b). Taylor & Bruns (1999), studying a resistant propagule community of EcM fungi in a soil-depth gradient, found that Pezizales species preferentially inhabit mineral soils. This was later supported in a study of an old-growth forest EcM community (Tedersoo et al., 2003). Corroborating some recent studies (Valentine et al., 2004; Izzo et al., 2005; Murat et al., 2005), we demonstrate that pezizalean mycobionts constitute a considerable proportion of EcM fungal communities in mature boreal deciduous and coniferous forests in several soil types. This suggests that, while some Pezizales species occur or fruit in early successional ecosystems and following disturbance, others are important as EcM symbionts in mature forests. The pezizalean species discovered as EcM symbionts in this study are known to fruit in mature boreal forests on the undisturbed forest floor or, in some cases more often, in locally disturbed patches in the forests (with a high pH and low organic matter) such as the edge of forest roads, sloping sides of lakes, ditches or road cuts, wheel tracks, etc. (Petersen, 1985; Dissing, 2000). We suggest that these species are present as EcM symbionts in the undisturbed mature forests, with the potential to act as pioneers, and rapidly fruit and/or establish symbiotic relationships with new seedlings recolonizing the site, if a disturbance occurs. Analogously, Vrålstad et al. (1998) suggested that the post-fire pezizalean species G. carbonaria is a widely distributed root-associated fungus in boreal spruce forests, judging from its ubiquitous occurrence on boreal spruce fire sites. These authors hypothesized that the post-fire mass production of G. carbonaria sporocarps might be an escape response of the fungus to a dying host.

The ability of some Pezizales species to produce conidia and chlamydospores in a vegetative state (anamorphs) (Paden, 1972; Yang & Korf, 1985; Urban et al., 2004) may complement their survival and dispersal mechanisms. In this investigation, two of the species we identified as EcM symbionts (Glischorodera and a Pezizaceae sp.) are, thus far, known only from anamorphic structures. These results hint that such taxa may be able to persist for extended periods in EcM communities in the absence of a sexual state.

Agerer (2001) described pezizalean taxa forming EcM types with scarce or no EmH. In agreement with this, only four of the 10 pezizalean EcM sequence types found on root tips [the hypogeous fruiting LB-133 (G. hispidula); LB-805 (sister to the Pachyphloeus–Amylascus lineage); the anamorphic LB-1274 (Glischorodera); and LB-472 (Pezizaceae anamorph)] were detected in molecular analyses of EmH in sand-filled mesh bags in Lille Bøgeskov (R.K., unpublished data). Indeed, these sequence types are closely related to taxa forming a moderate amount of EmH (this study). We can speculate that infrequent EmH and a thin mantle of most pezizalean taxa especially benefit tree seedlings with little carbon cost.

In conclusion, EcM fungal communities comprise numerous Pezizales species in different ecosystems. The results of this study suggest that Pezizales include more EcM-forming species than previously thought, and there is the potential that many more EcM taxa remain to be documented. EcM symbionts have probably evolved repeatedly in pezizalean lineages. However, the ancestral trophic condition of Pezizales and pezizalean lineages remains undetermined, and further knowledge of the phylogeny and trophic status of extant pezizalean species is needed before such a hypothesis can be tested. In general, pezizalean EcM can be recognized by a rectangular to epidermoid, thick-walled, pseudoparenchymatous mantle and thick, clampless EmH. Mantle anatomy is highly variable among different lineages of the Pezizales.

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References


Supplementary material

The following supplementary material is available for this article online:

Table S1. List of pezizalean ectomycorrhizal sequence types

Item S1. Descriptions of pezizalean ectomycorrhizas

Fig. S1. Anatomy of pezizalean ectomycorrhizas. (a–d) *Hydnotrya tulasnei* sp. Ta-L155: (e) outer layer; (f) middle layer; (g) inner layer. (h) emanating hyphae (EmH). (i–l) by T. Suvi. Bar = 7 µm.

Fig. S2. Anatomy of pezizalean ectomycorrhizas. (a–d) *Geopora* sp. KA-L799: (e) outer layer; (f) middle layer; (g) inner layer. (h) emanating hyphae (EmH). (i–l) by T. Suvi. Bar = 7 µm.

Fig. S3. Anatomy of pezizalean ectomycorrhizas. (a–c) *Peziza succisa* sp. Ta-L786: (d–f) *Sarcosphaera coronaria* KA-L794: (g) outer layer; (h) middle layer; (i) inner layer. (j) emanating hyphae (EmH). (k–m) The *Terfezia–P. depressa* lineage Ta-L233: (n) outer layer; (o) middle layer; (p) inner layer. (q) *Pachyphyloeus* sp. Ta-L177: (r) outer layer; (s) middle layer; (t) inner layer. (u) inner layer. (v) *EmH*. (w–z) *Pachyphyloeus* sp. Ta-LS162: (w) outer layer; (x) middle layer; (y) inner layer. (z) *EmH*. Bar = 7 µm.

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