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SHORT NOTE

Characterization of beech ectomycorrhizae formed by species of the *Pachyphloeus–Amylascus* lineage

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Abstract The hypogeous genus *Pachyphloeus* forms a common phylogenetic lineage with the epigeous *Scabropezia* and the hypogeous *Amylascus*, within the Pezizaceae (Ascomycota). Though the ectomycorrhiza- (EM) forming ability of this group was proposed previously, no detailed description has been published up to now, except for the characterization of EM related to *P. virecens*. During our several-year-long survey on the EM community of a beech forest reserve in Hungary, we found ten EM specimens belonging to the *Pachyphloeus–Amylascus* lineage. All of them share common morphological and anatomical characters. The densely ramifying whitish-yellow to light-brown mycorrhizal systems are pyramidal with short, stout ends. The EM surface is densely wooly with white or brown, curly hyphae. All mantle layers are pseudoparenchymatous angular, covered by a thick-walled hyphal network. Frequent emanating hyphae are densely septate without clamps. The EM can be sorted into three different morphotypes (Mt) according to their color, specific morphometric traits (cell-wall thickness, diameter of emanating hyphae, septal distance), and certain anatomical characters (structure of the surface net). Molecular identification was accomplished by the phylogenetic analysis of the ITS and LSU regions of the nrDNA, what proved that the sequences clustered into three clades corresponding to the three Mt. With the aid of fruitbody-derived sequences, obtained from GenBank, one of the Mt can be identified as *Pachyphloeus melanoxanthus* and another one as *Pachyphloeus citrinus*. The third Mt, together with another unidentified EM sequence of the GenBank, forms a distinct branch, which is a sister group to the *Pachyphloeus–Scabropezia–Amylascus* lineage. In addition to presenting the first detailed anatomical and molecular comparison of the EM related to *P. melanoxanthus* and *P. citrinus*, we call the attention to the need for further microscopical investigations amended by molecular taxonomical analyses.

Keywords Pezizaceae · Ectomycorrhiza · LSU · ITS · Molecular analysis · Anatomy

Introduction

In the past few years, detailed studies have been carried out on the ectomycorrhizae (EM) of Ascomycetes (Fujimura et al. 2005; Tedersoo et al. 2006a; Smith et al. 2006). Though these works have broaden our view on ascomycetous EM species, further detailed morphological and anatomical descriptions are required to support the selection and accurate identification of ascomycetous EM in future investigations.

Pachyphloeus is a worldwide distributed hypogeous genus of the Pezizaceae (Pezizales). The genus was previously placed into the Terfeziaceae (Eriksson and Hawksworth 1993); however, the first molecular results proved that this family is nested within the Pezizaceae (Percudani et al. 1999). Even in the early 1980s, Dissing and Pfister (1981) raised the assumption of a closer relationship with epigeous taxa based on the anatomical resemblance

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between the ascocarps of *Pachyphloeus* and *Scabropezia*. Later, molecular studies confirmed this assumption by giving evidences for the close relationship between the hypogeous *Pachyphloeus*, *Amylascus* and the epigeous *Scabropezia*, and even the mitosporic genus *Glischroderma* (Norman and Egger 1999; Hansen et al. 2001, 2005; Hansen and Pfister 2007; Læssøe and Hansen 2007).

The EM-forming ability of the *Pachyphloeus–Scabropezia* lineage was proposed by Hansen et al. (2001) and also by Agerer (2006). Accordingly, different species of the genus *Pachyphloeus* (Fogel and States 2002; Frank et al. 2006) were considered as putatively ectomycorrhizal. However, only a few detailed anatomical descriptions of the EM of these mycobionts were published up to now. The extensive work of Tedersoo et al. (2006a) on pezizalean EM provided the first unambiguous observations of *Pachyphloeus* and *Glischroderma* EM. These EM were collected in a beech forest and a wooded meadow, inhabited by different deciduous trees (Tedersoo et al. 2006a, b). *Pachyphloeus* sporocarps were also detected in communities dominated by oak (Cázares et al. 1992; Fogel and States 2002; Frank et al. 2006), pine (Fogel and States 2002), or Douglas-fir (Colgan and Trappe 2004).

During our several-year-long survey on the EM community of an undisturbed beech forest reserve in Hungary, we regularly found EM formed by mycobionts that belong to the *Pachyphloeus–Amylascus* lineage based on their anatomy and the analysis of their ITS nrDNA sequences. The aim of the present study was (1) to give detailed morphological–anatomical characterization of these EM, (2) to identify the mycobionts to the species level by molecular taxonomical methods, and (3) to compare the EM anatomy of the different species.

Materials and methods

Sample collection and preparation

Altogether, 30 soil samples were collected between 2002 and 2007 from different parts of the "Őserdő" forest reserve. The area, covered by an undisturbed beech forest, lies at 830–850 m amsl, on the district of the Bükk National Park in Hungary. For the detailed description of the site, see Kovács and Jakucs (2006). Soil cores of 25×25×25 cm size were taken randomly in two or three replications at each sampling occasion in different seasons of the year.

Soil cubes were stored at 4°C for not more than a week before the examination. The sample preparation was accomplished according to Agerer (1991). The morphology and anatomy of the EM were described using dissecting and Nomarski-DIC microscopy. Numerical data were measured on two to three different EM tips derived from the same branching systems that were analyzed by molecular methods. Voucher specimens were deposited in the Hungarian Natural History Museum, Budapest (BP99792-BP99801).

Molecular and phylogenetic analyses

DNA isolation, PCR-based amplification, and sequencing of the ITS region of the nrDNA were carried out as described previously (Jakucs et al. 2005, modified as indicated in Erős-Honti et al. 2008). We also amplified and sequenced the partial 28S nrDNA (LSU) with the primer pair LROR–LR5 (Rehner and Samuels 1994; Vilgalys and Hester 1990). Sequences were deposited in the GenBank database under the accession numbers FJ025867–FJ025875 for ITS and FJ025857–FJ025866 for LSU sequences (Table 1).

Electrophoregrams were analyzed with the programs Pregap4 and Gap4 (Staden et al. 2000). Similar sequences were selected from GenBank using BLAST homology search (Altschul et al. 1990). Multiple alignments of the obtained sequences were carried out using ClustalX (Thompson et al. 1997) and manually edited with Proseq 2.91 (Filatov 2002). Phylogenies were inferred with neighbor-joining (NJ) and maximum parsimony (MP) analyses using PAUP* 4.0 software (Swofford 2003). Besides, Bayesian and maximum likelihood (ML) analyses were also carried out with the programs MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and PhyML (Guindon and Gascuel 2003), respectively. For the MP analysis, the starting tree was obtained via random stepwise addition; gaps were treated as a fifth character, the branch-swapping algorithm was achieved by TBR, without the "steepest descent" function; "MULTrees" function was in effect, and topological constraints were not enforced. Branches were collapsed if maximum branch length was zero. Heuristic searches consisted of 10,000 replications. In NJ, ML, and Bayesian analyses, the general time-reversible substitution model (Tavaré 1986) was used. For the ML method, equilibrium base frequencies were optimized, and four different substitution categories were used; both the proportion of invariable sites and the gamma distribution parameter were estimated. In the Bayesian analysis, a proposed gamma-shaped rate variation and the proportion of invariable sites were taken into consideration. The analysis ran with the following priors: equal nucleotide frequencies, uniform prior shape parameter value and uniform proportion of invariable sites, non-constrained topology prior, and unconstrained branch length prior. The MCMC simulation ran for 1,000,000 generations and was sampled in every 100th step with a burn in at 2,500 sampled trees.

Robustness of the clades inferred by NJ, MP, and ML analyses were estimated by bootstrap (Felsenstein 1985)

Table 1 Comparison of the differentiating characters and morphometric data of the specimens belonging to the investigated morphotypes

Morphotype/ species	Herbarium number	GenBank accession number		Color of mantle cells	Cell wall thickness of outer mantle (µm)	Cell wall thickness of surface net (µm)	Cell wall thickness of emanating hyphae (µm)	Diameter of surface net hyphae (µm)	Distance of septa of surface net hyphae (µm)
		ITS	LSU						
Mt 1.	BP 99792	FJ025874	FJ025863	Mostly membranaceously and plasmatically light brown (or colorless)	(0.5)0.6–1(1.8)	(0.2)0.4–0.6(0.8)	(0.3)0.4–0.6(0.8)	(4)5–8(12)	(4)10–15(30)
P. melanoxanthus	BP 99793	FJ025868	FJ025857						
Tul. & C. Tul.	BP 99794	FJ025875	FJ025865						
Mt 2.	BP 99795	FJ025870	FJ025859	Mostly colorless (or membranaceously light brown)	(0.6)1–1.5(2)	(0.5)0.8–1.2(2)	0.6–0.8(1.2)	(4)6–8(10)	(8)15–20(32)
P. citrinus	BP 99796	FJ025871	FJ025860						
Berk. & Broome	BP 99797	FJ025872	FJ025861						
	BP 99798	FJ025873	FJ025862						
	BP 99799	FJ025867	FJ025866						
	BP 99800	FJ025869	FJ025858						
Mt 3.	BP 99801	–	FJ025864	Colorless	0.6–0.8 (1)	0.6–0.8 (1.2)	0.6–0.8	(7) 12–14 (15)	(8) 12–16 (20)

using 10,000, 1,000, and 1,000 replicates, respectively. Phylogenetic trees were edited and visualized with the program TreeView (Page 1996) and the Tree Explorer of the MEGA 3.1 software (Kumar et al. 2004).

The mean differences between the sequence groups (clades) were calculated with the PAUP* 4.0 software (Swofford 2003).

Results

We have found ten EM samples with a morphology resembling those described by Tedersoo et al. (2006a) as *Pachyphloeus* spp. These EM were present in seven of the 30 soil samples and were collected at five different sampling occasions. According to our abundance estimation (following the modified method of Gardes and Bruns 1996), these morphotypes constitute less than 5% of the examined ectomycorrhizal root tips, and thus, they are regarded as minor components of the community (Jakucs et al., unpublished data). Based on the observed microscopical-anatomical features, we sorted the samples into three different morphotypes (Mt 1, Mt 2, and Mt 3; Table 1.) Concerning the anatomical characters, these types were almost identical, yet the majority of their morphometric data were different. Below, we present the detailed morphological–anatomical description of Mt 1 and discuss the certain differences between the three morphotypes.

Characterization of the ectomycorrhiza of Mt 1 (reference EM specimen: BP 99792)

Ectomycorrhizae are whitish-yellow or ochre to light brown. Mycorrhizal system is monopodial–pyramidal, densely ramifying, with short, stout ends. Surface is densely wooly with white or brown, curly hyphae. Mantle is pseudoparenchymatous, regularly angular, slightly gelatinized, and covered by a hyphal network. Inner mantle layers are also angular. Emanating hyphae lack clamps, and they are densely septate, colorless, or brown, uneven in thickness, and slightly constricted at septa.

Morphological characters (Fig. 1 j) Ectomycorrhizal systems are abundant, monopodial, and pyramidal. Main axes are 4–5 mm long and 0.4 mm in diam., straight or slightly bent. *Unramified ends* are 1–2 mm long and 0.2–0.3 mm in diam., cylindric, or clavate. The color of the mycorrhizae is whitish-yellow to ochre or brown and darker brown at older parts. The surface is densely wooly with curly, colorless, or brown emanating hyphae. *Rhizomorphs* are lacking.

Anatomical characters of mantle in plan views Each layers of the mantle are pseudoparenchymatous angular. The

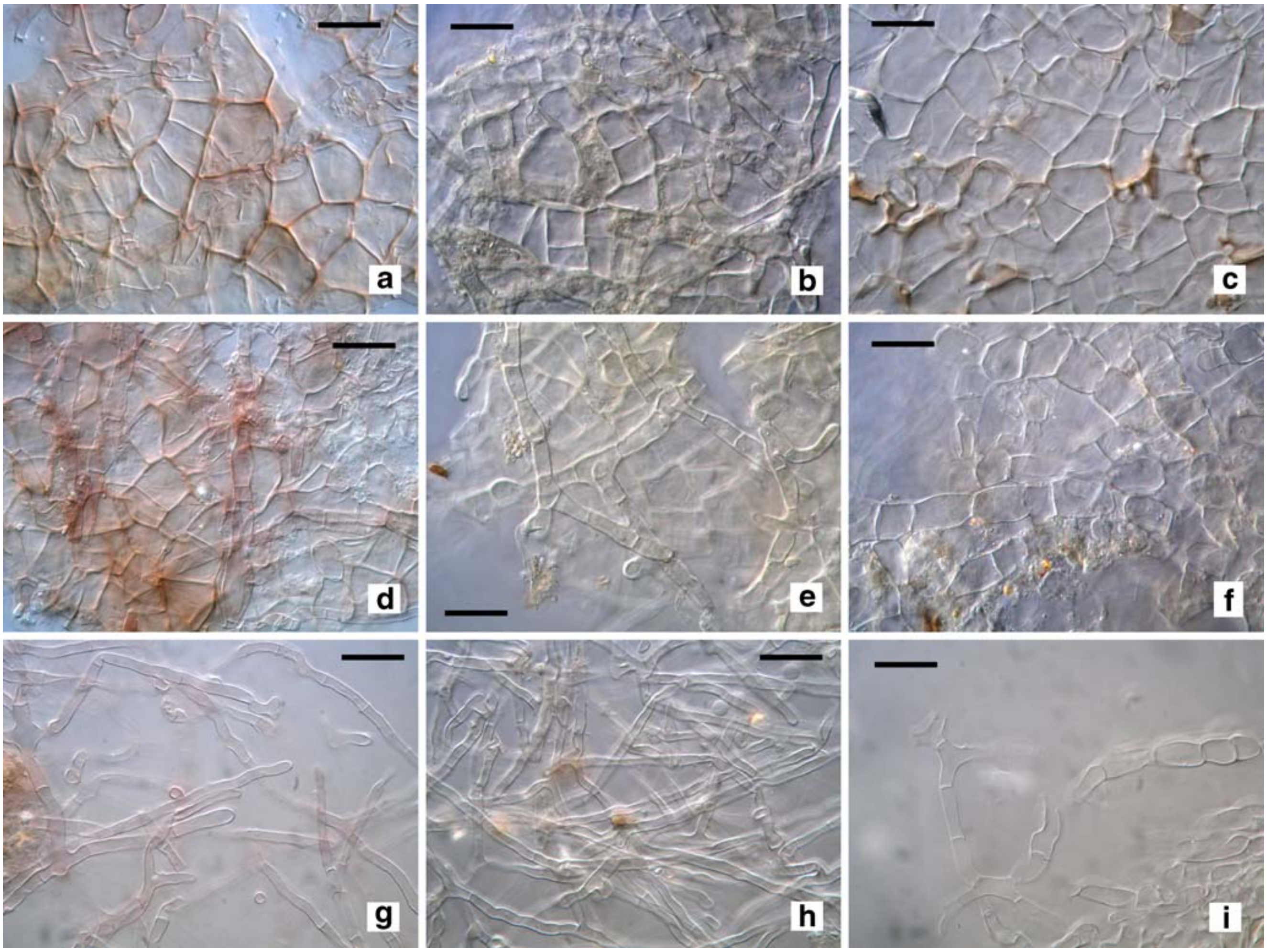


Fig. 1 Morphology and anatomy of the ectomycorrhizae of Mt 1, Mt 2, and Mt 3. Pseudoparenchymatous angular outer mantle layer of different cell wall thickness and different color, in case of Mt 1 (*a*), Mt 2 (*b*), and Mt 3 (*c*); surface net composed of thin- (*d*—Mt 1) and thick-walled (*e*—Mt 2) hyphae or loosely connected globular cells (*f*—Mt 3); emanating hyphae of Mt 1 (*g*), Mt 2 (*h*), and Mt 3 (*i*); microscopical drawings of the habit (*j*), the pseudoparenchymatous-angular structure of the outer (*k*, *n*) and inner (*m*) mantle layers, the surface hyphal net (*k*), the cystidium-like short hyphae (*l*), and the emanating hyphae (*o*) of Mt. 1. *Arrows* indicate the hyphae constricted at septa. *a–i* Nomarski-DIC, *bars* = 20 µm

mantle is covered by a dense network of surface hyphae. *Surface net* (Fig. 1 d, k) consists of densely septate hyphae, uneven in thickness, often with slightly swollen intersepta, and constricted at septa. The cell wall thickness of the surface cells is (0.2)0.4–0.6(0.8) µm. *Outer mantle layer* (Fig. 1 a, k, n) is slightly gelatinized, with large, angular cells, at some places organized in regular rays, and covered by the network of surface hyphae (mantle type L; Agerer 1991). Cells of the mantle are membranaceously and plasmatically light brown or colorless. The surface of the cells is finely granulated, and some cells contain lipid droplets (Fig. 1 n). The cell walls are (0.5)0.6–1(1.8) µm thick. There are nine to 11 cells in a square of 20 × 20 µm. *Middle mantle layers* are also pseudoparenchymatous angular but with thinner cell walls (about 0.5–0.6 µm), mostly colorless cells, and smooth cell surface. *Inner mantle layers* (Fig. 1 m) are pseudoparenchymatous angular, and cell walls are 0.5–0.8 µm thick, colorless, or membranaceously yellow; surface of cells are granulated. *Very tip* is also pseudoparenchymatous, and the cells are angular, like in other parts of the mantle.

Anatomical characters of emanating elements Hyphae lack clamps, they are frequently septate and uneven in thickness, and the apical ends are slightly clavate. *Emanating hyphae* (Fig 1 g, o) are colorless or light brown, densely ramifying. Ramification is near 90° or Y-shaped, and distance of septa is 25–45 µm. Anastomoses with septa are present, surface of hyphae are smooth, and some hyphae contain lipid droplets (Fig. 1 o). Two types of emanating hyphae, with transitional forms, can be observed: a soft, thin-walled, cylindrical, light-brown type, which often collapse and a

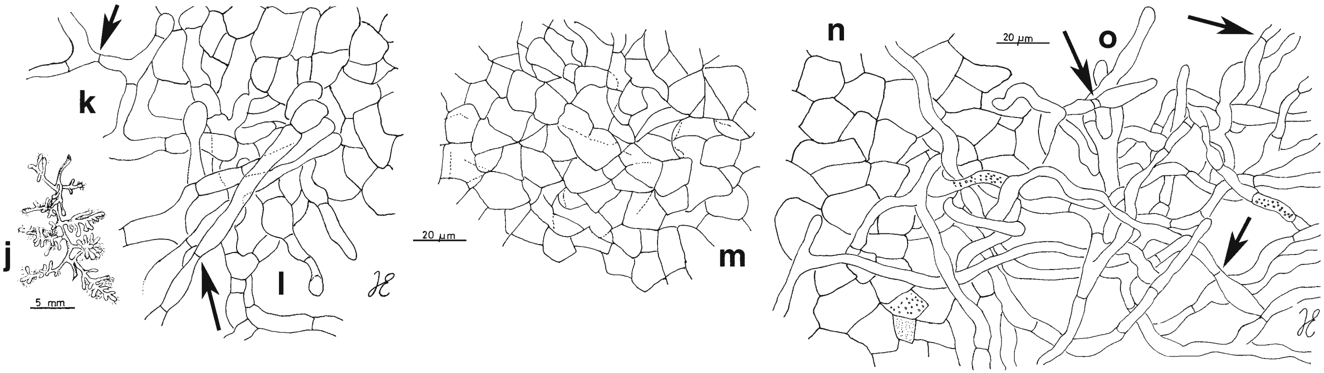


Fig. 1 (continued)

more rigid, thick-walled, colorless type with swollen parts and constricted at septa (Fig. 1 o). Thinner hyphae are 3–5 µm, and thicker hyphae are 5–8 µm in diam. The cell walls of hyphae are (0.3)0.4–0.6(0.8) µm thick. The walls of the rigid hyphae are sometimes considerably thickened at the tips (1–1.2 µm). *Cystidia* are not observed, but short, cystidium-like emanating hyphae are present (Fig. 1 l). *Rhizomorphs* are lacking.

Differentiating between the three EM morphotypes

All of the three morphotypes (Mt) are characterized by pseudoparenchymatous-angular mantle covered by a hyphal network on the surface and densely septate emanating hyphae, slightly constricted at septa (as described above in detail for Mt 1 (BP 99792)). However, there are some clear differences between the three Mt in pigmentation, in the shape of the surface network cells and cell wall thickness ranges (compared in Table 1). The EM of Mt 1 is generally browner and darker than the other two morphotypes. Mt 2 is yellow to light brown and Mt 3 is whitish-yellow. The surface network of Mt 1 and Mt 2 consists of densely septate, stout hyphae (Fig. 1 d, e), yet the surface net of Mt 3 is composed of loosely connected, globular cells (Fig. 1 f). Cell wall thickness is different in the three morphotypes (as indicated in Table 1) concerning the mantle cells (Fig. 1 a–c), the surface hyphal network (Fig. 1 d–f), and also the emanating hyphae (Fig. 1 g–i). The cell walls of the mantle and the surface network are more gelatinized and considerably thicker in case of Mt 2 than in the remaining morphotypes, and a characteristic wall thickening at the tips of the emanating hyphae can also be observed (Fig. 1 e).

Phylogenetic inference

Preliminary phylogenetic analyses were carried out, including different pezizalean sequences that we supposed to be related to our samples according to BLAST results. All our EM samples grouped into a well-supported clade formed by the sequences of the *Pachyphloeus–Scabropezia–Glishroderma–Amylascus* lineage; thus, the phylogenetic tree presented here (Fig. 2) includes only these taxa.

We sequenced the ITS region of nine and the LSU region of ten EM mycobionts (Table 1). The ITS-based trees (Fig. 2b), calculated by the different inferring methods, were principally identical concerning their topologies, and only the bootstrap support values differed slightly. In all trees, sequences obtained from Mt 1 and Mt 2 clustered in two different, well-supported groups (bootstrap values higher than 85%, posterior probability values higher than 0.90), according to the morphotypes. Both morphotypes formed common clades with GenBank-derived sequences published as *Pachyphloeus*. Mt 1 clustered together with a sequence obtained from a sporocarp that was associated with *Quercus garryana* according to the GenBank description (published by Frank; accession number: AY920528), while Mt 2 grouped together with two sequences obtained also from a *Pachyphloeus* species (*Pachyphloeus marroninus* Healy, Bonito & G. Guevara, voucher specimens Garcia3757 and RH299 (holotype); accession numbers: EU427551, EU427549, Healy et al. 2009), being under publication. The estimated mean difference between Mt 1 sequences and the sequence obtained by Frank was 14.3%; that between the group of Mt 2 and the sequences of Healy et al. (2009) was 7.8%.

In all the LSU-based trees, constructed by the different inferring methods, the sequences of the EM samples clustered into three distinct groups corresponding to the three morphotypes (Fig. 2a). The sequences of Mt 1 grouped together with a sequence derived from *Pachyphloeus melanoxanthus* (Tul. & C. Tul. ex Berk.) Tul. & C. Tul. sporocarp (DQ191674), in a common group with two *Scabropezia* sequences (however, this common clade has low bootstrap and posterior probability values). The EM samples of Mt 2 formed a common, well-supported clade

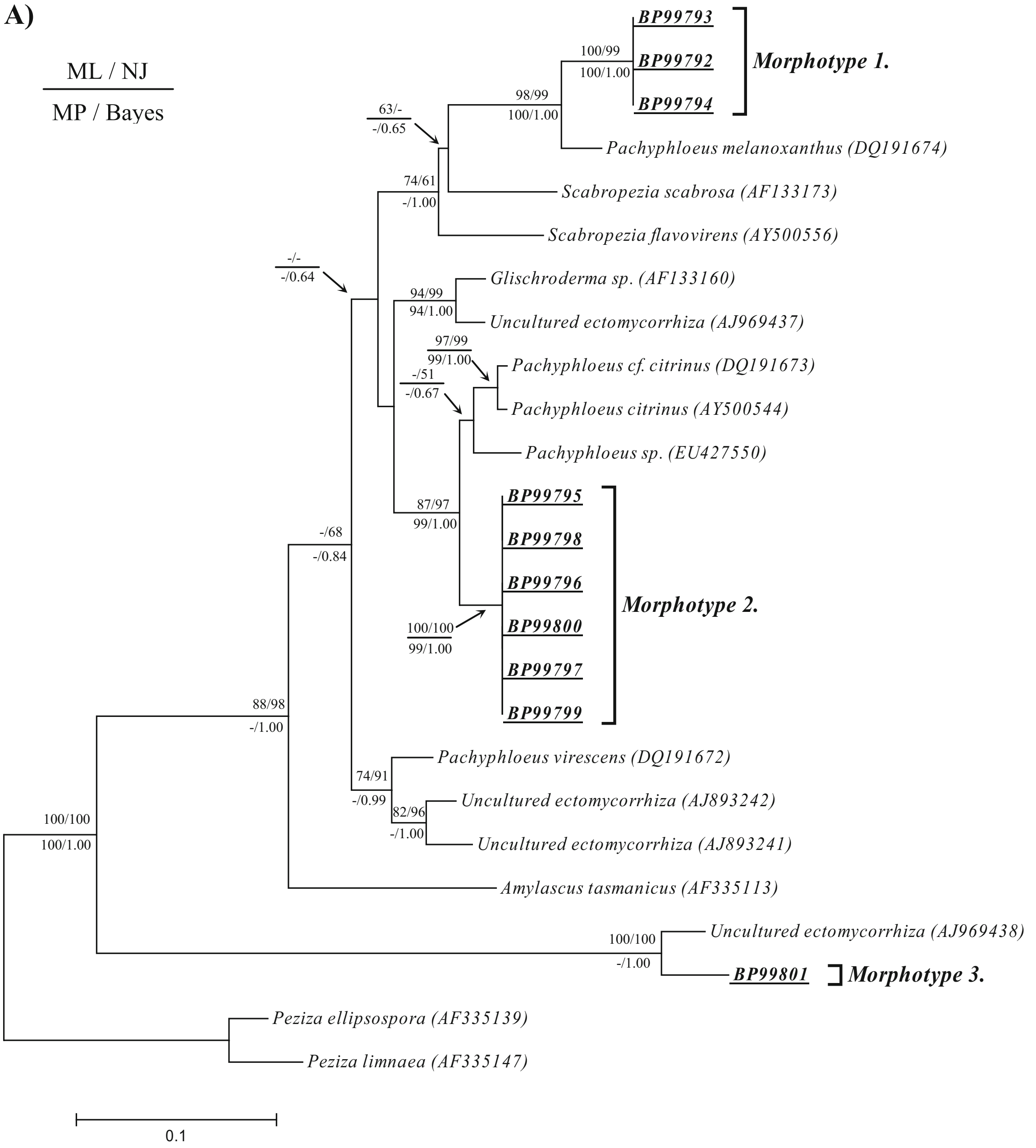
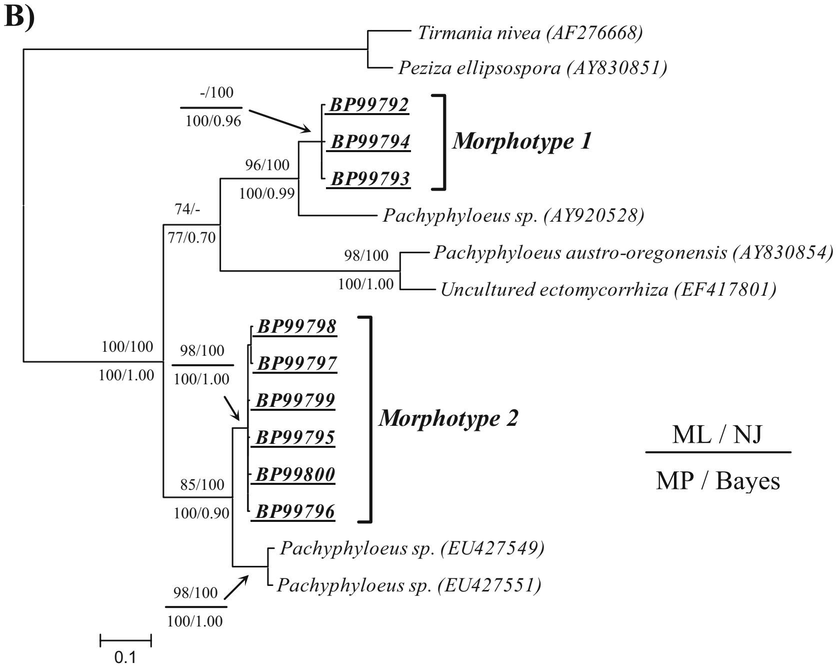


Fig. 2 Phylogenetic trees demonstrating the relationships of the large subunit rDNA (LSU; **A**) and the ITS sequences (**B**) derived from the EM specimens (*bold*, *underlined*) and those obtained from public databases (accession numbers are indicated in the *parentheses*). Sequences of the same morphotypes are joined by *vertical lines*. The presented tree of the LSU sequences was constructed by maximum likelihood analysis, while Bayesian method was applied in case of the ITS-based tree. *Numbers above the branches* (or *the horizontal lines*) represent the bootstrap values (>50%) of the ML (*left*) and NJ (*right*) analyses; numbers *below the branches* (*horizontal lines*) stands for the bootstrap values (>50%) of the MP analysis (*left*) and the posterior probability values (>0.6) of the Bayesian analysis (*right*). The outgroups of the LSU analyses were *Peziza ellipsospora* and *Peziza limnaea* and *P. ellipsospora* and *Tirmania nivea* in the analyses of the ITS. Scale stands for ten changes per 100 characters

with two published sequences of *Pachyphloeus citrinus* Berk. & Broome and a sporocarp-derived sequence identified also as *Pachyphloeus marroninus* (Healy et al. 2009, EU427550). In all but the MP phylogenetic tree, the sequence of EM specimen BP 99801 (Mt 3) paired with a GenBank-derived sequence obtained from an unidentified EM by Kjøller (host: *Fagus sylvatica*, location: Lille Bøgeskov, accession no. AJ969438; as published in Tedersoo et al. 2006a). In the MP-based tree, Mt 3 turns up as a sister lineage to all the remaining sequences,

Fig. 2 (continued)



having AJ969438 nested within. The average difference between the sequence group of Mt 1 and *P. melanoxanthus* was 2.6%, between the sequences of Mt 2 and *P. citrinus* it was 2.2%, while this value was 3.1% between the sequence of Mt 3 and the uncultured ectomycorrhiza AJ969438.

Discussion

During our studies on the EM community of the beech forest reserve of the Bükk mountains in Hungary, we regularly found EM morphotypes proven to be closely related to the genus *Pachyphloeus*, according to our molecular results. These EM seem to be constant members of the mycorrhizosphere in these forests. This is in accordance with previous observations of the presence of this genus mainly in deciduous forests, especially from mixed or oak-dominated woodlands (Cázares et al. 1992; Fogel and States 2002; Frank et al. 2006; Tedersoo et al. 2006a, b). Similar to Tedersoo et al. (2006b), we also found these EM in low abundance, yet—in contrast to them—in the closed, older parts of the forests. Thus, our results do not support the suggestion by Tedersoo et al. (2006a) of *Pachyphloeus* having ruderal life strategy.

Molecular analyses of the ITS and LSU regions of our samples proved three, well-supported clades (Fig. 2). Two of them formed monophyletic groups with species of the genus *Pachyphloeus*, while the third was nested in a sister group to the *Pachyphloeus–Amylascus* lineage. The three clades can be related to the specific morphological–anatomical differences of Mt 1, Mt 2, and Mt 3. On the base of phylogenetical distances, bootstrap, and posterior probability values, Mt 1 and Mt 2 can be regarded as identified to the species level, as *P. melanoxanthus* and *P. citrinus*, respectively. Both LSU and ITS sequences of Mt 2 grouped together with *Pachyphloeus* sequences of Healy et al. (2009, EU427549-51) having derived from sporocarp samples. Mt 3 cannot be linked to any sequence identified to the species level though it forms a sister group to the *Pachyphloeus–Amylascus* lineage, together with an EM-derived sequence of Kjøller (isolated also from beech, accession no. AJ969438; Tedersoo et al. 2006a).

The three morphotypes share common anatomy but the color is darker in case of Mt. 1 (*P. melanoxanthus*) than the remaining two (Fig. 1). Besides, quantitative differences of cell wall thickness, distance of septa, and diameter of emanating hyphae were observed between the three morphotypes.

Tedersoo et al. (2006a) characterized two *Pachyphloeus* EM, forming a common clade with *Pachyphloeus virescens* Gilkey on the phylogenic trees, in addition to the EM of *Glischroderma* and an unidentified mycobiont of the same lineage. The EM of *P. virescens* are similar to our morphotypes concerning the pseudoparenchymatous angular structure of the outer and middle mantle layers, but are different in the plectenchymatous inner layer, that we have never observed in our samples. Moreover, in contrast to the characterization of Tedersoo et al. (2006a), the EM described in the present study have a hyphal net covering the mantle and two different forms of emanating hyphae. Tedersoo et al. (2006a) observed greater anatomical difference between their two EM than what we observed between our morphotypes, though their EM samples were more closely related according to the molecular results than ours. At the present state of knowledge, this discrepancy between the observation of Tedersoo et al. (2006a) and our results cannot be satisfactorily explained because of the low number of EM investigated from the *P. virescens* clade.

The anatomical similarity of the EM structures of different species within a phylogenetic lineage is not a unique phenomenon among ascomycetes. Highly similar mycorrhizal anatomy was observed also among white truffle lineages, despite their high interspecific distances proven by ITS phylogenies (Kovács and Jakucs 2006). Even species of different genera, like the closely related epigeous *Humaria* and the hypogeous *Genea* (Pyronemataceae), may share common EM characters (Erős-Honti et al. 2008), so EM anatomy seems to be conservative in these taxonomic groups. However, in other phylogenetic lineages, e.g., within the basidiomycetous genera *Tomentella* (Jakucs and Erős-Honti 2008) or *Lactarius* (Eberhardt 2000), the anatomy of the EM formed by closely related species may differ considerably. The variable intraspecific similarities of EM morphology in different taxonomic groups may be explained by their different evolution rate, highly influenced by genetic plasticity and environmental changes.

In addition to the morphological–anatomical description, image documentation, and comparison of three new EM morphotypes within the *Pachyphloeus–Amylascus* lineage, our results call the attention to the need of more detailed microscopical investigations, parallel to molecular taxonomical analyses. These complex studies should be extended to the EM of more hypogeous ascomycetous species in order to support mycorrhizal identification and to resolve evolutionary and taxonomic problems within these ectomycorrhizal groups.

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