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ORIGINAL PAPER

Morphological and molecular characterization of *Humaria* and *Genea* ectomycorrhizae from Hungarian deciduous forests

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Abstract The ectomycorrhizae (EM) of *Humaria* and *Genea*, two closely related genera of the Pyronemataceae (Ascomycetes), were regularly found in different deciduous forests of Hungary. In the present paper, the morphology and anatomy of these EM are described in detail, including morphometric analyses. Identification of the EM was carried out by molecular taxonomic analyses of the nrDNA ITS sequences obtained from mycorrhizae, herbarium ascomata, and public databases. The anatomy of the EM, examined during this work, was almost identical. They possessed angular outer and epidermoid inner mantle layers and warted, thick-walled emanating hyphae. Ten of our EM sequences grouped into the clade of *Humaria hemisphaerica* sequences and one into the genus *Genea*. Both molecular taxonomic analysis and morphometry differentiated three sub-groups within the clade of *Humaria*, and these methods also clearly separated the EM of *Genea* from those of *Humaria*. We may suppose that the previous morphological–anatomical descriptions, lacking molecular taxonomic identification, do not concern the denominated taxa. As a consequence, we stress the importance of revaluating the literature data, based on morphotyping of *Humaria* and *Genea* EM, to prevent misidentification in future studies. The presented work demonstrates that combining molecular and morphological analysis is essential for the unambiguous identification of the EM formed by problematic taxa.

Keywords Ectomycorrhiza · *Genea* · *Humaria* · Anatomy · Morphometry · Phylogenetic analysis

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Introduction

The number of described ascomycetous ectomycorrhizae (EM) is much lower than that of basidiomycetous EM. Until the end of the 20th century, only truffles received considerable attention because of their gastronomic and economic importance. For instance, Maia et al. (1996) reviewed less than a hundred EM of non-truffle ascomycetes. Although more information has been gathered on ascomycetous EM from large-scale screening surveys using molecular taxonomic methods (Tedersoo et al. 2006a), the EM of few taxa have been characterized also by morphological methods (Fujimura et al. 2005; Smith et al. 2006; Tedersoo et al. 2006a). As previous EM descriptions based on mere morphological identification often caused confusions even in recent studies, more investigations combining morphological and molecular approach are required.

Humaria and *Genea* are two closely related genera of the Pyronemataceae (Ascomycetes) that frequently form EM (Tedersoo et al. 2006a; Perry et al. 2007). The presence of the genus *Humaria* was demonstrated in coniferous forests (Boxman et al. 1998; Brandrud and Timmermann 1998; Fay and Mitchell 1999; Izzo et al. 2005; Rudawska et al. 2006) and in broad-leaved woodlands (Fay and Mitchell 1999; Sesli 1998; Salerni et al. 2001; Richard et al. 2004; Tedersoo et al. 2006a; Mosca et al. 2007). Similarly, *Genea* species were also recorded in both deciduous (Lee et al. 1997; Ingleby et al. 1998; Rumberger et al. 2004; Valentine et al. 2004; Mosca et al. 2007) and mixed forests (Izzo et al. 2005; Tedersoo et al. 2006a, b; Smith et al. 2006).

From these two genera, detailed morphological–anatomical descriptions are available only for the EM of *Humaria hemisphaerica* (Wigg.: Fr.) Fuckel (Ingleby et al. 1990), *Genea hispidula* Berk. & Br. (Brand 1991), and *Genea verrucosa* Vitt. (Jakucs et al. 1998). In their work on

pezizalean EM, Tedersoo et al. (2006a) reported the anatomical similarity of *Genea* and *Humaria* EM. The mycorrhizae of these genera have some features in common with those of the E-strain fungi, a morphological group defined by Laiho and Mikola (1964) for particular mycorrhizal mutualists that form both ecto- and ectendomycorrhizas on different hosts (Wilcox 1983; Yu et al. 2001). The robust, septate, thick-walled, often warted, yellow or brown emanating hyphae, characteristic to these fungi, are regularly found in the rhizosphere of different soils (Yu et al. 2001; Kovács and Szigetvári 2002; Jakucs 2002).

Humaria species are also considered E-strain fungi, along with genera such as *Wilcoxina*, *Tricharina*, *Trichophaea*, and *Sphaerosporella* (Danielson 1982, 1984; Egger 1996; Yu et al. 2001). Based on molecular phylogenetic results, Tedersoo et al. (2006a) showed the close phylogenetic relationship of the hypogeous *Genea*, the epigeous *Humaria* and other E-strain fungi, and this result, was later confirmed by Perry et al. (2007).

During the investigation of the EM communities of different deciduous forests in Hungary, we regularly found EM anatomically similar to the previously described *Humaria* and *Genea* EM. The aim of our work was to characterize these EM morphotypes by morphological–anatomical methods, including morphometry, identify them by molecular taxonomic analyses of the nrDNA ITS region, and compare them in detail to the previously published descriptions.

Materials and methods

Sampling sites

Mycorrhizal samples were collected from three different sites in Hungary. One site, sampled between 1998 and 2001, was an oak woodland, located near Püspökladány (for the site description, see Jakucs et al. 2005). The second site, described in Kovács and Jakucs (2006), was a beech stand of the "Őserdő" forest reserve within the territory of the Bükk National Park. This site was sampled from 2000 to 2006. The third site, sampled in 2006, occurred in a beech forest in the hills of the Őrség National Park, near Csörötnek, at 250–300 m amsl, on clay soil. The annual rainfall of the area is 700–950 mm. In this site, *Fagus sylvatica* L. forms a mixed stand with *Carpinus betulus* L., *Pinus sylvestris* L., and *Quercus petraea* (Matt.) Liebl.

Sampling

Sampling was carried out according to Agerer (1991). The 20×20×20-cm soil cubes were taken from homogenous portions of each stand. At each occasion, we collected randomly two or three samples from each site, and sampling was repeated twice or three times a year. Altogether, 61 soil samples were collected.

The soil cubes were stored at 4°C for not more than 1 week. EM roots were washed under tap water, then the different morphotypes were separated in water under a dissecting microscope. The relative abundance of the morphotypes within the soil samples was estimated visually, based on the method of Gardes and Bruns (1996), simplified by Jakucs (2002). The abundance (expressed as a percentage) was given as the proportion of the EM morphotype to the total number of the root tips. EM tips were fixed in FEA for further light microscopy (Agerer 1991) and also in cetyl trimethyl ammonium bromide (CTAB) buffer for the DNA analysis. Voucher specimens were deposited in the collection of the Hungarian Natural Museum, Budapest (collection numbers: BP 97489-495 and BP 98698-701).

Herbarium numbers, collection data, and GenBank accession numbers are compiled in Table 1.

Characterization of EM morphology and anatomy

When describing the morphology and anatomy of the EM, we followed the methods of Agerer (1991). First, morphotypes were separated, and their morphology was characterized under dissecting microscope. Then, EM mantle structure and emanating elements were examined by Nomarski-DIC microscopy (Nikon Eclipse 80i). For semithin longitudinal and cross sections, EM were embedded in Historesin, according to the instructions of the manufacturer (LEICA HISTORESIN Embedding Kit-7022 31731). The 10-μm-thick sections were examined under phase contrast microscope (PhC).

Study design and statistical analysis in morphometry

For morphometric analysis, anatomical measuring was carried out with the software Image-Pro® Plus, version 5.1 (Media Cybernetics, Inc.). From each *Humaria* and *Genea* EM sample, two to three tips were randomly chosen, and the following anatomical parameters were measured: the cell wall thickness in the outer mantle layer, the longest diameter ("length") of the angular cells in the outer mantle layer, the shortest diameter ("width") of the angular cells in the outer mantle layer, the proportion of the length to the width of the cells in the outer mantle layer ("isodiametricity"), the diameter of emanating hyphae on the proximal parts, the diameter of emanating hyphae on the distal parts, the cell wall thickness of emanating hyphae on the proximal parts, the cell wall thickness of emanating hyphae on the distal parts, the diameter of warts on the emanating hyphae, and the distance of the septa of emanating hyphae. For comparison, the same parameters were re-measured on

Table 1 Collection data of EM samples

Herbarial number	Site	Date	Host	Relative abundance	GenBank accession number
BP 97489	Püspökladány	1998.04.08	*Quercus cerris*	D	EU024873
BP 97490	Püspökladány	2000.04.24	*Q. robur*	B	EU024874
BP 97491	Püspökladány	2001.06.10	*Q. robur*	B	EU024875
BP 97492	Bükk-Őserdő	2002.11.01	*F. sylvatica*	B	EU024876
BP 97493	Bükk-Őserdő	2003.04.18	*F. sylvatica*	A	EU024877
BP 97494	Bükk-Őserdő	2002.10.21	*F. sylvatica*	A	EU024878
BP 97495	Bükk-Őserdő	2005.10.23	*F. sylvatica*	D	EU024879
BP 98698	Őrség-Csörötnek	2006.09.16	*F. sylvatica*	A	EU024880
BP 98699	Őrség-Csörötnek	2006.09.16	*F. sylvatica*	A	EU024881
BP 98700	Őrség-Csörötnek	2006.09.16	*F. sylvatica*	A	EU024882
BP 98701	Bükk-Őserdő	2006.10.23	*F. sylvatica*	A	EU024883

Abundance categories: *A* minor component (<10%), *B* minority codominant (10–50%), *C* majority codominant (50–90%), *D* dominant component (>90%)

the original EM voucher specimen, described as *G. verrucosa* (herbarium number: BP 92140) by Jakucs et al. (1998). The anatomical features of emanating hyphae were measured only if sufficient number of hyphae were found for the statistical analysis.

At first, we analyzed our datasets with ANOVA, which showed significant differences among the datasets of the samples in each character. Subsequently, the datasets were compared to each other with Welch's modified *t* test (or *d* test; Welch 1947).

Herbarium samples

Herbarium ascocarp samples were obtained from the Hungarian National Museum, Budapest. The herbarium data and GenBank accession numbers are summarized in Table 2.

Molecular analysis

DNA extraction

DNA was isolated from EM tips collected from the same ramifying systems that had been anatomically characterized. The isolation was accomplished by the CTAB method as described previously (Jakucs et al. 2005), with slight modifications for herbarium ascocarp samples. These were put in CTAB buffer for at least 24 h before the extraction procedure and then incubated for 90 min at 50°C.

PCR and sequencing

The ITS regions of the EM and ascocarp samples were amplified with the fungal-specific primers ITS1f and ITS4 (White et al. 1990; Gardes and Bruns 1993). The compo-

Table 2 Herbarium data used for the identification of EM samples

Herbarium number	Source	Site	Date	Leg/Det	Taxon	Accession number
BP 53878	Ascocarp	Uzsapuszta	1973.10.04	S. Tóth	*H. hemisphaerica*	EU024884
BP 46092	Ascocarp	Kabhegy (Bakony Mts.)	1968.09.11	S. Tóth	*H. hemisphaerica*	EU024885
BP 45991	Ascocarp	Miklóspálhegy	1968.09.27	S. Tóth	*H. hemisphaerica*	EU024886
BP 77249	Ascocarp	Cuha-hegy (Zirc)	1981.09.26	S. Tóth	*H. hemisphaerica*	EU024887
–[a]	Ascocarp	Őrség-Szalafő	2004.06.27	I. Siller	*H. hemisphaerica*	EU024888
–[a]	Ascocarp	Kisterenye (Heves, Borsod Hills)	2004.06.12	B. Dima, M. Németh	*H. hemisphaerica*	EU024889
BP 92140	Ectomycorrhizal root tip	Miskolc-Görömböly	1996.09.27	E. Jakucs, Z. Bratek	*Genea verrucosa* + *Quercus* sp.	–

[a] Personal collection
Leg/Det Legit et determinavit

sition of the reaction mixture and the program used for the PCR followed those of Jakucs et al. (2005).

Cycle sequencing was accomplished with ABI PRISM 3.1 BigDye Terminator Kit (Applied Biosystems), according to the instructions of the manufacturer. Electrophoreses were performed on an ABI PRISM 3100 Genetic Analyzer at the service laboratory of the Biology Research Center (Szeged, Hungary). Sequences were assembled from electrophoregrams and analyzed using the programs Pregap4 and Gap4 (Staden et al. 2000), then they were deposited in the GenBank (accession numbers EU024873-83 for EM and EU024884-89 for ascocarps).

Phylogenetic analyses

Similar ITS sequences were selected from GenBank using BLAST homology search (Altschul et al. 1990) and also from the UNITE sequence database (Köljalg et al. 2005) with galaxieBLAST algorithm (Nilsson et al. 2004). Multiple alignments were done with the program ClustalX (Thompson et al. 1997) and manually edited with ProSeq 2.91 (Filatov 2002). Phylogenies were inferred by neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods with PAUP* 4.0 software (Swofford 2003), and also by Bayesian analysis using the program MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003).

The optimal nucleotide substitution model was chosen by the Akaike information criterion (Akaike 1974) using Modeltest 3.7 (Posada and Crandall 1998). The ML analysis was carried out with these parameters, using heuristic search (TBR algorithm), in ten replicates. In the NJ analysis, general time-reversible (GTR) evolution model (Tavaré 1986) was applied. When looking for the most parsimonious trees (MP analysis), gaps were treated as a "fifth base". The starting tree for branch swapping algorithm was obtained via random stepwise addition. The topology was optimized by the TBR method, without the STEEPEST function, MULTREES function in effect, and topological constrains not enforced. Branches were collapsed if the maximum branch length was zero. The phylogenies inferred by the NJ and the MP analyses were tested by bootstrap (Felsenstein 1985) using 10,000 and 100 replicates, respectively.

Bayesian analysis was carried out with the GTR evolution model of nucleotide substitution, a proposed gamma-shaped rate variation and taking the proportion of invariable sites into consideration. The priors were as follows: 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.

The phylogenetic trees were visualized and edited with TreeView software (Page 1996) and the Tree Explorer of the MEGA3.1 software (Kumar et al. 2004).

Results

During our 9-year-long study, we found EM resembling those of *Humaria* and *Genea* in 11 soil samples out of 61. The EM morphotype formed by these genera was usually a minor or minority codominant component. In two samples (one from oak, the other from beech forest), they were the dominant EM types (Table 1).

Common morphological–anatomical features of the EM

The EM summarized in Table 1 had common morphological and anatomical features. Ramifying systems were pyramidal with variable lengths, (1) 2–7 (12) mm. Individual tips were 0.1–0.5 (1)-mm wide and (0.3) 0.7–2 (3)-mm long, chestnut brown or dark brown. Mycorrhizal surface was smooth or loosely woolly. Outer mantle layer (Fig. 1a,b) was pseudoparenchymatous with angular cells, at some parts transitional to epidermoid. The thickness of the yellow cell walls varied even within a single sample between (0.3) 0.6 and 1.8 (3.5) μm, but towards the surface of the mantle, it became even thicker (8–12 μm; Fig. 1a). Anastomoses of hyphal cells were observed. Three to four (sometimes six to eight) cells were in a square of 20 μm×20 μm. In a single sample (BP 97492), some cells contained cytoplasmatic granules. Middle and inner mantle layers (Fig. 1d) were pseudoparenchymatous epidermoid with thin-walled (0.5–1 μm) cells. In some cases, the mantle type was transitional to plectenhymatous; the elongated cells of the inner mantle layer were arranged in rows.

The emanating hyphae (Fig. 2) derived from thick-walled, bulbous basal cells, resembling those of the outer mantle layer (Fig. 1c, Fig. 2a). Hyphae were wavy, hyaline, and smooth when young but yellowish brown and warted at the older parts. Proximal parts of the hyphae were not ornamented. Their diameter was (4) 5–7 (10) μm. Distance between septa was 70–80 μm at younger and (25) 30–60 (120) μm at older, ornamented parts. The hyphal walls were (2) 2.5–3 (3.5) μm thick close to the mantle and (0.8) 1–2 (2.5) μm at the distal parts. At some places, cell wall intrusions were found. Rhizomorphs and cystidia were lacking.

The thickness of the fungal mantle was (23) 25–30 (40) μm. The Hartig net was paraepidermal, mainly surrounding the cells of the outermost layer of root cortex. In some samples, it penetrated the cortex to the depth of two cell rows. Although general intracellular colonization was not observed, at some places, the cortical cells of sample BP 97493 were colonized by septate hyphae (Fig. 1e,f).

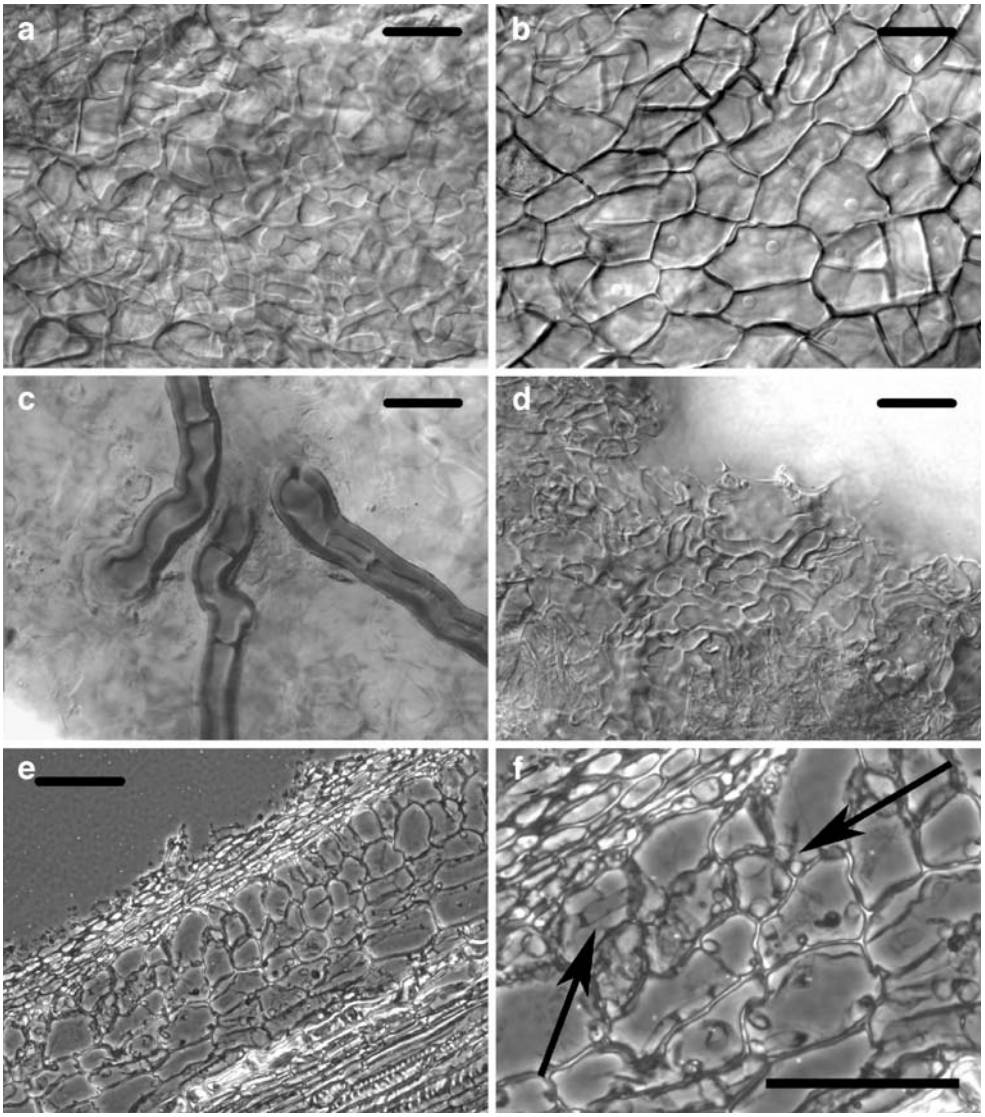

Fig. 1 The anatomy of representative EM samples of *Humaria*. **a** The outermost part of the mantle layer, **b** the thickened cell walls of the outer mantle layer, **c** the origin of emanating hyphae, **d** the inner mantle layer, **e**, **f** longitudinal section of the sample BP 97493 showing the mantle structure, the Hartig net, and intracellular penetration within the cortical cells. *Arrows* show septate colonizing hyphae within the cells. (**a**–**d** Nomarski-DIC, **e**, **f** PhC; *bars* represent 10 μm on **a**–**d** and 50 μm on **e**, **f**)

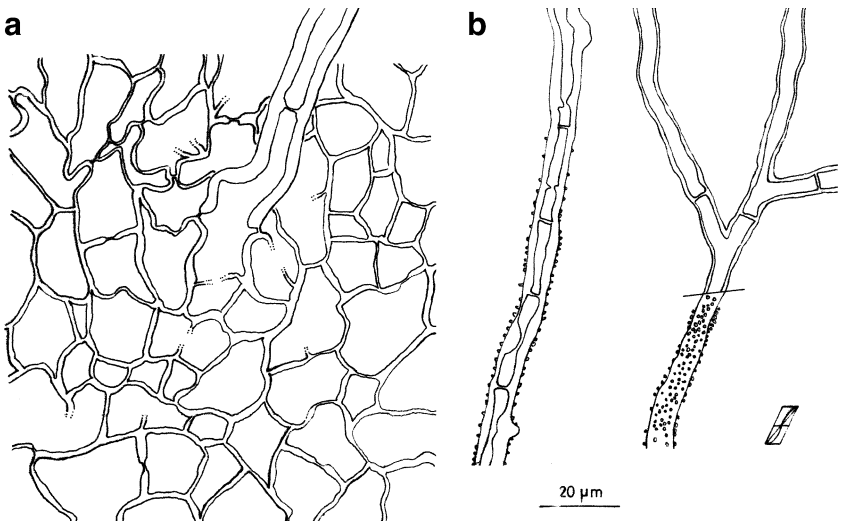

Fig. 2 **a** The outer mantle layer and the origin of emanating hyphae, **b** the structure of emanating hyphae of the representative *Humaria* sample BP 97492

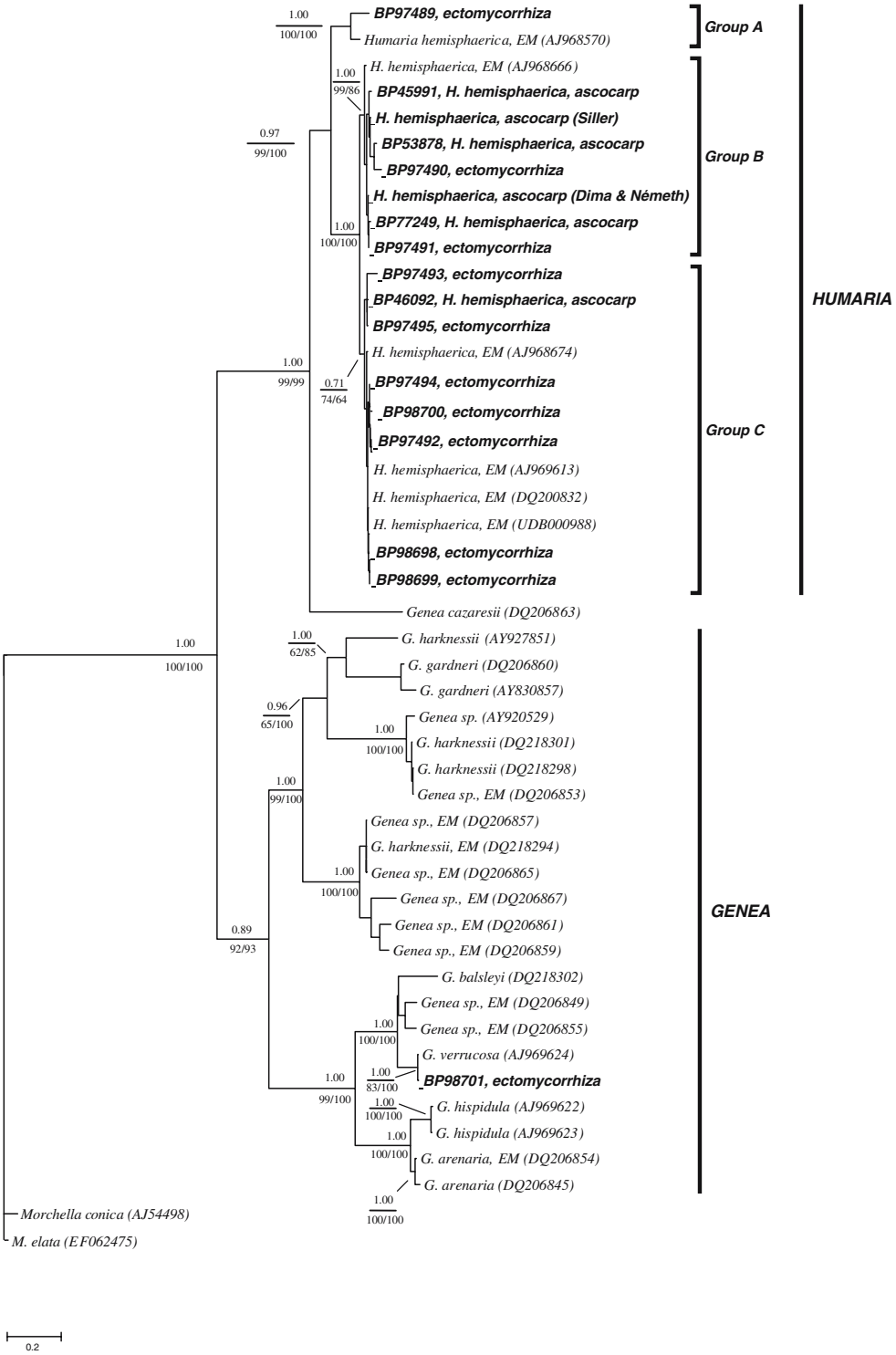

Fig. 3 The Bayesian phylogenetic tree of the rDNA ITS sequences. Sequences of 11 EM and six herbarial ascocarp samples were obtained in the present study (indicated in *bold* letters). In case of two ascocarp samples, which do not derive from the herbarium of the Hungarian National Museum, the collectors are indicated in the brackets. For the sequences from previous studies GenBank accession numbers are shown in the *brackets*. Statistical support values are shown only for the major clades because of the very short branches. The posterior probability values are *above the branches* (or *horizontal lines*). Below them are the bootstrap values of NJ (*before the slash*), and MP (*after the slash*) analyses. (Only the values higher than 50% are *indicated*.) *Groups A*, *B*, and *C* indicate major clades formed within the genus *Humaria*. (*Bar* represents 20 changes/100 characters)

Phylogenetic inference

In the preliminary phylogenetic analyses including different genera of the Pyronemataceae, all EM samples grouped into the clade formed by the species of *Humaria* and *Genea*, thus the phylogenetic tree presented here (Fig. 3) includes only these two genera.

The phylogenetic trees constructed by the different methods were similar concerning the topology, branch lengths, and statistical support values. By the MP analysis, two tree islands were found; one of them with 12, the other with one single tree. However, the topology of these 13 trees did not differ considerably.

Table 3 Statistical comparison of the anatomical characteristics of the EM samples with the application of Welch's *t* test

	Group A	Group B	Group C	ΣHumaria	BP 92140	BP 98701
Group A	–	1, 2, 3, 4, 6, 8, 10	1, 2, 3, 4, 6, 8, 9	–	1, 2, 3, 6, 8, 9, 10	1, 2, 3
Group B	–	–	1, 2, 3, 5, 8, 9, 10	–	1, 2, 3, 6, 7, 9, 10	1, 2, 3, 4
Group C	–	–	–	–	1, 2, 3, 6, 7, 8, 9	1, 2, 3, 4
ΣHumaria	–	–	–	–	1, 2, 3, 5, 6, 7, 9	1, 2, 3, 4
BP 92140	–	–	–	–	–	1, 2, 3, 4

Each cell of the table means the statistical comparison of the datasets of 2 samples or 2 groups of the samples, indicated in the heading of the respective row and column. *Numbers* within the cells refer to those measured parameters that were significantly different between the compared samples, proven by Welch's *t* test at the significance (*p*) value of 0.05. The 10 characters represented by the numbers: *1* cell wall thickness in the outer mantle layer, *2* length (maximal diameter) of the angular cells in the outer mantle layer, *3* the width (minimal diameter) of the angular cells in the outer mantle layer, *4* the isodiametricity of the angular cells in the outer mantle layer (calculated as the proportion of the length to the width), *5* the proximal diameter of emanating hyphae, *6* the distal diameter of emanating hyphae, *7* the cell wall thickness of emanating hyphae at the proximal parts, *8* the cell wall thickness of emanating hyphae at the distal parts, *9* the diameter of warts of the emanating hyphae, *10* the distance of the septa of emanating hyphae. *ΣHumaria* stands for the compiled dataset of all the samples identified as *Humaria*, and "group A", "group B" and "group C" refers to the compiled datasets of the sample groups in the phylogenetic tree of Fig. 3. The characteristics of the emanating hyphae were not measured on the sample BP 98701 because of the low number of hyphae.

One EM sample formed a common group with the sequences of *Genea* ascocarps and EM obtained from molecular databases. The closest sequence derived from an ascoma was identified as *G. verrucosa* (Fig. 3). However, the others formed a well-supported group with *H. hemisphaerica* ascocarp sequences obtained during this study and those deriving from databases. Within this *Humaria* clade, the sequences clustered in three groups (groups A, B, and C; Fig. 3). The statistical support for groups A and B was above 85% in each tested analysis and somewhat lower for group C (74% in NJ and 64% in MP analysis).

Morphometric analysis

The anatomical characteristics of eight EM samples were measured and analyzed. As no emanating hyphae sufficient for the measurements were found in the sample BP 98701, their features were not included in the statistical analyses. Descriptive statistics are summarized in the Appendix.

The majority of the characters were significantly different in each comparison. Groups B and C of the *Humaria* clade were statistically different in seven of the measured characters, while all but one measured character differentiated group B from group A and group C from group A. The compiled dataset, composed of the summarized data of all our *Humaria* EM samples, was similar to that of the voucher specimen BP 92140 (identified previously as *G. verrucosa* by Jakucs et al. 1998) concerning three characters (the isodiametricity of the angular cells in the outermost mantle layer, the cell wall thickness on the distal parts of emanating hyphae, and the distance of the septa of emanating hyphae). On the other hand, all parameters of the *Humaria* EM proved to be significantly different from those measured on BP 98701, identified as *Genea* in the present study by molecular taxonomic methods. The specimen, identified as *Genea* in this work, also differed significantly from the previously described *Genea* EM (BP 92140) in each character. The results of the statistical comparisons are summarized in Table 3.

Discussion

In all three sampling sites, we regularly found the EM of *Genea* and *Humaria*, and they shared common morphological and anatomical features. This morphotype was present in high abundance in xeric *Quercus* forests, similar to the results of Smith et al. (2006). However, we also detected these EM in the more humid beech forests.

In the molecular phylogenetic analyses, our samples grouped into two distinct genera: *Genea* and *Humaria* (Fig. 3). Within the clade of *H. hemisphaerica*, three groups were formed with high statistical support, but we cannot assume any infraspecific grouping because of the limited data set.

Some general features of the examined EM, i.e., the light-yellow or yellowish-brown pigmentation and the warted, thick-walled emanating hyphae, are similar to those formed by the E-strain fungi (Danielson 1982, 1984; Egger and Paden 1986; Scales and Peterson 1991a, b) or species previously identified as *Humaria* (Ingleby et al. 1990) and *Genea* (Brand 1991; Jakucs et al. 1998). However, in contrast to *Wilcoxina* (Scales and Peterson 1991a, b; Tedersoo et al. 2006a) and other E-strain fungi (Danielson 1982), the fungal mantle of our samples was always continuous and multilayered at the mature parts of the EM. In addition, the angular structure of the outer layer of the fungal sheath is also a differing character between the EM we found and those determined as *Humaria* by Ingleby et al. (1990) and other E-strains, because these fungi form plectenchymatous or subepidermoid EM mantles (Danielson 1984; Ingleby et al. 1990; Scales and Peterson 1991a; Fujimura et al. 2005).

The mantle of the EM samples, identified in the present work as *Genea* and *Humaria* EM by molecular taxonomic methods, is composed of thick-walled angular cells, similar to the descriptions by Tedersoo et al. (2006a, b). These EM are almost identical with that of *Genea* (Brand 1991; Jakucs et al. 1998) and do not resemble to the previously described *Humaria* EM (Ingleby et al. 1990).

The similarity in EM anatomy could have evolved as an apomorphy of these closely related genera. Agerer (2006) proposed that the pseudoparenchymatous mantle and hypogeous fruitbody are apomorphic and evolved parallel in the Pyronemataceae. If both characters are apomorphic, as *Genea* and *Humaria* are sister groups, we might suppose that the pseudoparenchymatic mantle structure evolved first in the common ancestor of these taxa and the hypogeous sporocarp appeared later in the *Genea* lineage. The similarity of EM characteristics of hypogeous and epigeous sister genera is not unique. For instance, the EM of the hypogeous *Arcangeliella borziana* (Egli et al. 2001) is almost identical with those of certain *Lactarius* species, its epigeous relatives (Brand and Agerer 1986; Brand 1991).

The statistical comparisons of the measured data of the specimens indicated significant difference between the datasets in almost each case (Table 3). Significant differences in the majority of the measured characteristics were found even between the three clades of *H. hemisphaerica*. The EM sample BP 92140, identified previously as *G. verrucosa* (Jakucs et al. 1998), was similar to the summarized dataset of all *Humaria* samples of this study in three characters, so its original identity is questionable. However, because of the wide range of the parameters within the genus *Humaria* itself, we cannot identify this previously described *Genea* EM sample unambiguously as a *Humaria* EM. Nevertheless, all the measured parameters of the EM sample BP 98701, identified as *Genea* in the present study, were proven to be statistically different from those of the compiled *Humaria* dataset. Similarly, BP 98701 is also different in all parameters from the previously described BP 92140. We can regard this result as evidence for the morphometric difference between *Genea* and *Humaria* EM. Consequently, although *Humaria* and *Genea* EM can be clearly distinguished from E-strains based on their mantle structure, EM of *Genea* and *Humaria* cannot be unambiguously separated by mere morphotyping, as also noted by Tedersoo et al. (2006a).

Based on these findings, it is likely that previous studies contain ambiguous results about the EM of *Humaria* and *Genea*. The EM of *H. hemisphaerica* described by Ingleby et al. (1990) seems to be formed by another mycobiont. As this EM has incomplete plectenchymatous mantle, it probably was an ectomycorrhiza of *Wilcoxina* sp. (Scales and Peterson 1991b). This likelihood is also supported by the observations of Rudawska et al. (2006). They found E-strain EM morphotypes similar to the descriptions by Ingleby; however, they did not find any match between the sequences of EM and those obtained from *Humaria* sporocarps collected within the same region.

In addition, it appears that the identification of the mycobiont of previously described *Genea* EM (Brand 1991; Jakucs et al. 1998) is ambiguous, so we cannot regard it univocally either as *Humaria* or *Genea*. Brand (1991) identified his EM samples by tracing the hyphal connections between the EM and the sporocarp of *G. hispidula*, and Jakucs et al. (1998) designated their EM as *G. verrucosa*, because they found it under the ascocarp of this species, and the EM was morphologically similar to Brand's description. Because these methods do not provide absolute EM fungus identification per se, the precise determination of these previously described morphotypes remains questionable and unresolved.

The taxonomic ambiguity of the descriptions of *Genea* and *Humaria* EM should be borne in mind when evaluating former studies on EM communities applying them (Boxman et al. 1998; Brandrud and Timmermann 1998; Ingleby et al. 1998; Fay and Mitchell 1999; Richard et al. 2004; Rumberger et al. 2004). Presence of these genera should be based either on the observation of sporocarps (Sesli 1998; Salerni et al. 2001; Izzo et al. 2005) or on molecular identification of the EM mycobionts (Smith et al. 2006; Tedersoo et al. 2006a, b; Mosca et al. 2007). Moreover, surveys combining anatomical and molecular identification of certain samples may also be misled by ambiguous descriptions. In these cases, certain EM were regarded as *Genea*, and no further molecular analyses were considered necessary. For example, Mosca et al. (2007) identified *Genea* EM by morphology in a survey, but only *Humaria* sequences were detected with molecular analyses.

Our presented work demonstrates that combining molecular and morphological analysis is essential for the unambiguous identification of the EM formed by problematic taxa. Although, the importance of combined approach in EM identification is stressed (Horton and Bruns 2001), several studies apply only either molecular or morphological methods.

In addition to providing a detailed morphological–anatomical and molecular taxonomical characterization of *Humaria* and *Genea* EM from Hungary, our results emphasize the need to revaluate the specifications of previous morphological EM descriptions that are not supported by molecular identification. Otherwise, the potentially inaccurate descriptions may mislead large-scale ecological studies in the future.

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Appendix

Table 4 The measured morpho-anatomical data of the EM samples examined

	BP 97489	BP 97490	BP 97491	BP 97492	BP 97493	BP 97494	BP 97495	ΣHumaria	BP 92140	BP 98701
The cell wall thickness in the outer mantle layer (μm)	0.71±0.18 (106)	0.94±0.29 (86)	2.08±0.64 (77)	1.04±0.32 (91)	1.11±0.34 (56)	0.99±0.28 (90)	0.82±0.26 (119)	1.05±0.53 (625)	1.80±0.40 (80)	0.65±0.16 (124)
Length of the angular cells in the outer mantle layer (μm)	13.41±3.82 (38)	17.48±3.98 (49)	27.38 +/−7.05 (50)	20.12±9.48 (51)	15.13±3.59 (40)	15.34±3.51 (50)	12.82±4.42 (57)	17.50±7.34 (335)	26.69±5.36 (59)	10.71±2.11 (77)
Width of the angular cells in the outer mantle layer (μm)	8.41±1.79 (39)	11.99±2.62 (49)	19.49±4.33 (48)	15.67±8.25 (55)	10.08±2.06 (41)	10.59±2.95 (49)	8.40±2.67 (56)	12.21±5.69 (337)	18.08±3.93 (58)	6.73±1.71 (77)
Isodiametricity of the angular cells in the outer mantle layer	1.61±0.43 (38)	1.49±0.36 (49)	1.37±0.24 (50)	1.44±0.30 (58)	1.46±0.31 (39)	1.42±0.25 (47)	1.53±0.41 (56)	1.47±0.34 (337)	1.48±0.29 (58)	1.65±0.42 (77)
The diameter of emanating hyphae on the proximal parts (μm)	Not measured	16.66±5.35 (8)	Not measured	15.46±1.65 (9)	12.13±1.25 (9)	11.38±0.86 (7)	11.99±1.69 (7)	13.63±3.34 (40)	15.54±1.02 (6)	Not measured
The diameter of emanating hyphae on the distal parts (μm)	5.70±0.99 (20)	7.20±1.08 (25)	6.34±0.97 (40)	8.60±1.05 (18)	5.90±1.48 (37)	6.66±1.86 (17)	5.27±0.63 (27)	6.39±1.49 (184)	4.67±0.46 (24)	Not measured
The cell wall thickness of emanating hyphae on the proximal parts (μm)	Not measured	2.77±0.34 (18)	Not measured	2.81±0.66 (22)	2.79±0.48 (21)	2.83±0.77 (16)	2.81±1.07 (14)	2.80±0.66 (91)	3.58±1.64 (17)	Not measured
The cell wall thickness of emanating hyphae on the distal parts (μm)	0.81±0.21 (18)	1.30±0.38 (41)	1.29±0.29 (63)	1.79±0.53 (27)	1.76±0.60 (43)	1.48±0.51 (26)	1.29±0.42 (50)	1.40±0.50 (268)	1.34±0.25 (46)	Not measured
The diameter of warts on the emanating hyphae (μm)	0.71±0.18 (38)	0.67±0.17 (54)	0.86±0.31 (45)	0.61±0.13 (32)	0.59±0.11 (43)	0.60±0.11 (63)	0.53±0.10 (74)	0.64±0.19 (349)	0.54±0.10 (76)	Not measured
The distance of the septa of emanating hyphae (μm)	30.11±12.11 (21)	53.83±16.76 (15)	67.16±17.5 (19)	47.12±19.63 (11)	23.89±12.48 (17)	25.24±6.25 (16)	36.82±12.98 (17)	40.26±20.76 (116)	44.29±18.69 (7)	Not measured

Mean, standard deviation, and the number (in the brackets) of the measured data are indicated. In case of the angular cells of the outer mantle layer, the maximal and minimal diameter of the cells are referred to as the length and width of the cells, respectively. Isodiametricity was calculated as the proportion of the length to the width. BP 97489-95 samples represent *Humaria* EM; BP 92140 is the voucher EM specimen, described previously as *G. verrucosa* (Jakucs et al. 1998); BP 98701 is the EM sample identified as *Genea* by molecular methods in the present study. The statistics in the column titled Σ*Humaria* were calculated on the basis of the compiled dataset of samples BP 97489-95. (In certain cases, the features of emanating hyphae could not have been measured because of the low number of them.)

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