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SHORT NOTE

Morphological and molecular comparison of white truffle ectomycorrhizae

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Abstract In the present study, white truffle ectomycorrhizae (EM) collected in deciduous forests (*Populus*, *Quercus*, and *Fagus*) from Hungary were characterized by morphological–anatomical and molecular methods. Our investigations suggest that the EM of white truffles (e.g., *Tuber rapaeodorum*, *Tuber puberulum*, *Tuber rufum*) are common and abundant members of the forest communities in the area. The ITS sequences of 14 EM specimens and 46 additional fruitbody sequences from the GenBank were clustered into four main groups in phylogenetic analyses. In the ITS-1 region, a characteristic indel pattern was found, which supports the clades. Although our analyses indicate definite genetic distance between the groups of the phylogenetic tree, these clades do not correspond to the traditional taxons identified by fruitbody characteristics. Comparison of the ectomycorrhizae shows that neither is mycorrhizal anatomy a good tool to separate the groups, because the characters (like the epidermoid or angular mantle structure, cell wall thickness, the sape and size of cystidia) are too variable and overlap between the clades. The interspecific similarity, observed both in ectomycorrhizal and fruitbody characters, strengthen the *sensu lato* morpho-species concept of this group. Our study, which combines comprehensive molecular and anatomical approach to characterize and identify ectomycorrhizae of white truffles from natural samples, stress out the need of the taxonomical revision of this group.

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Introduction

Truffles are ectomycorrhizal hypogeous ascomycetes belonging to the genus *Tuber*. There are commercially valuable species in both main groups of truffles like the black truffle *Tuber aestivum* Vitt. and *Tuber brumale* Vitt. or the white truffle *Tuber magnatum* Pico. Some species of white truffles with less gastronomic value have received high research interest, like *Tuber borchii* Vitt., which became a model organism of physiological, biochemical, and molecular biological studies.

The taxonomy of the *Tuber* species is rather problematic. Traditional description and identification of the taxa is based on ambiguous characters of the ascocarps and variable and overlapping microscopic features. These values are often continuous between the species leading to different *sensu lato* and *sensu stricto* interpretations or synonyms (Gross 1987). This is why the species group *Tuber puberulum* within white truffles has been used (Montecchi and Lazzari 1992) containing the species *T. borchii*, *Tuber dryophilum* Tul. et Tul., *Tuber foetidum* Vitt., *Tuber maculatum* Vitt., *T. puberulum* Berk. et Br. and *Tuber rapaeodorum* Tul. et Tul.. Although molecular taxonomical analyses of white truffles were carried out (Urbanelli et al. 1998; Roux et al. 1999; Mello et al. 2000; Urban et al. 2004; Halász et al. 2005) and specific molecular markers were also developed for identification of certain species (Longato and Bonfante 1997; Bertini et al. 1998; Amicucci et al. 1998, 2000), no comprehensive, multilocus molecular systematic analysis complemented with morphological characters of white truffles has been carried out up to now.

In addition to the biochemical and molecular biological results, morphological descriptions of white truffle ectomycorrhizae (EM) were also published. Using detailed microscopic characterization, the EM of *T. borchii* (Rauscher et al. 1996), *Tuber albidum* Pico (synonym of *T. borchii*; Zambonelli et al. 1993), *T. puberulum* (Blaschke 1987, 1988), *Tuber rufum* Pico (Rauscher et al. 1995), and *T. maculatum* (Zambonelli et al. 1999) were described.

The concept on the distribution of white truffles in Europe is mainly based on collections from southern and western Europe (especially Italy, France, and Spain; Urbanelli et al. 1998; Montecchi and Sarasini 2000; Moreno-Arroyo et al. 2005), but very few data are available from central and eastern Europe. Although *T. borchii*, *T. rapaeodorum*, *T. maculatum*, and *T. dryophilum* were recorded from the Carpathian Basin (Hollós 1911) and specimens with Hungarian origin have been used in a recent work (Halász et al. 2005), our knowledge on the distribution and abundance of white truffles in this region is insufficient. Mycorrhizae of white truffles were frequently found in poplar forest of the Great Hungarian Plain (Jakucs 2002).

The main aim of the work presented here was to characterize white truffle EM collected from different deciduous hosts in Hungary. Besides the distribution data of *Tuber* species in the ectomycorrhizal fungal communities in the region, we wanted to test the taxonomic level at which the morphological characteristics of EM can be used to identify white truffles.

Materials and methods

Soil samples were collected from four sampling sites in Hungary. Two of them (Tompa and Kelebia) are about 10 km far from each other in the southern part of the Hungarian Plain at 120–130 m altitude. Both sites are in *Populus alba* L. stands on sandy soils (Jakucs 2002). The third sampling plot (Püspökladány) is in the northeast part of the Hungarian Plain at 85–90 m altitude. The differently aged *Quercus robur* L. stands are on solonetz soil. The fourth site is in the mountain region of north Hungary, in the "Őserdő" (virgin forest) reserve of the Bükk National Park. The area is an autochthonous mountain beech forest elevated at 830–850 m on brown forest soil, based on limestone.

Between 1998 and 2005, soil samples were taken two to three times a year randomly from each sampling plot. The sampling and the washing of the EM were carried out as described earlier (Jakucs et al. 2005). Voucher specimens were deposited in the Hungarian Natural History Museum Budapest (BP98684–BP98697, GenBank Accession numbers are DQ355255–DQ355268, respectively) and in the collection of E. Jakucs.

Fig. 1 **a** The neighbor-joining tree of 60 *Tuber* sequences (14 presented first in this study). The bootstrap values shown above the branches are percentages of 1,000 replicates; the scores below 70% are not shown. *Bar*=10 changes on 100 characters. The accession numbers of the 46 sequences obtained from GenBank are shown in *parentheses*. The letters with *gray background* (*A–D*) correspond to the deletions presented in Fig. 1b. Hosts: *P.a.*, *Populus alba*; *Q.r.*, *Quercus robur*; *F. s.*, *Fagus silvatica*. Collecting sites: *T* Tompa, *K* Kelebia, *P* Püspökladány, Ő Bükk, (see in text). **b** The approximate position of the characteristic deletions in the ITS-1 region. The *numbers* indicating the character position of the bordering nucleotides. The *bolded* number is the 44th nucleotide of the ITS-1 region of BP98687

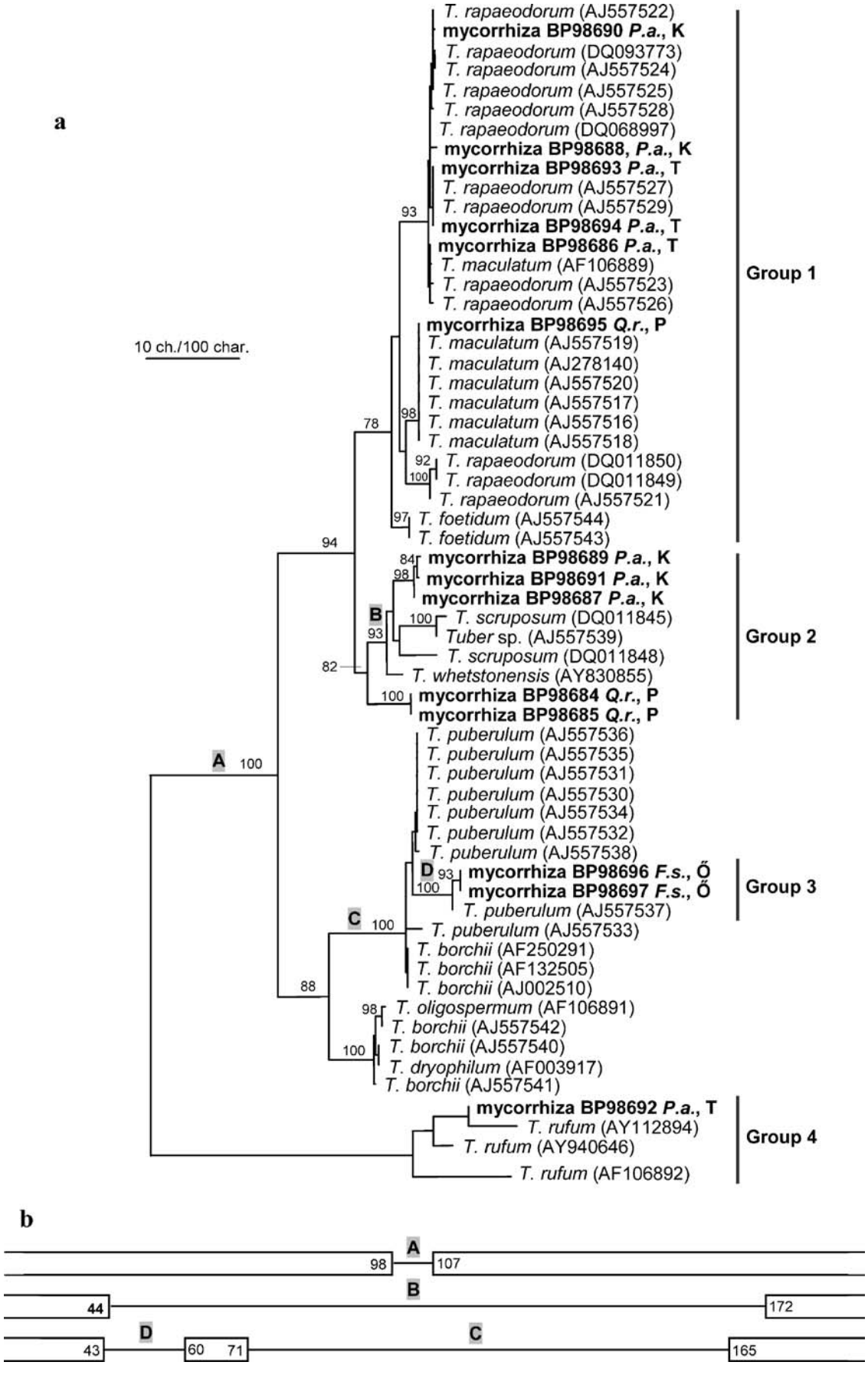
DNA isolation and the amplification and the sequencing of the ITS region was carried out as described previously (Jakucs et al. 2005).

The sequences were compiled from electropherograms using Pregap4 and Gap4 (Staden et al. 2000). Multiple alignments of the obtained data and homologous ITS sequences from GenBank were made using ClustalX (Thompson et al. 1997) and Multalin (Corpet 1988). The alignments were checked and edited with ProSeq 2.9 (Filatov 2002). For inferring phylogenies, the program PAUP* 4.0b10 (Swofford 2003) was used. The best-fit nucleotide substitution model was found with the program Modeltest 3.06 (Posada and Crandall 1998) considering the selection of Akaike Information Criterion. The best-fit model was used in maximum likelihood (ML) analysis using heuristic search and for calculating distances for neighbor-joining (NJ) analysis. The gaps of the alignment were used as missing characters. The inferred phylogenies were statistically tested by bootstrap using 100 (ML) and 1,000 (NJ) replicates.

Ectomycorrhizae were characterized and described using the microscopical–morphological methods of Agerer (1991) and documented by drawings and digital photos.

Results

In the phylogenetic analyses of the ITS sequences, the 14 EM samples together with data from the GenBank formed four main groups (Fig. 1a). Significant difference of the lengths of the ITS regions was detected between certain groups of the studied taxa. These differences resulted in a specific insertion–deletion pattern in the ITS-1 region of the aligned sequences. From the studied taxa, group 4, containing *T. rufum* has the longest ITS-1 region. Although the position and the separation of the indels are extremely sensitive to the set parameters of the alignment algorithm, the main indels could be connected to mono-


a
10 ch./100 char.
T. rapaeodorum (AJ557522)
mycorrhiza BP98690 P.a., K
T. rapaeodorum (DQ093773)
T. rapaeodorum (AJ557524)
T. rapaeodorum (AJ557525)
T. rapaeodorum (AJ557528)
T. rapaeodorum (DQ068997)
mycorrhiza BP98688, P.a., K
mycorrhiza BP98693 P.a., T
T. rapaeodorum (AJ557527)
T. rapaeodorum (AJ557529)
mycorrhiza BP98694 P.a., T
mycorrhiza BP98686 P.a., T
T. maculatum (AF106889)
T. rapaeodorum (AJ557523)
T. rapaeodorum (AJ557526)
mycorrhiza BP98695 Q.r., P
T. maculatum (AJ557519)
T. maculatum (AJ278140)
T. maculatum (AJ557520)
T. maculatum (AJ557517)
T. maculatum (AJ557516)
T. maculatum (AJ557518)
T. rapaeodorum (DQ011850)
T. rapaeodorum (DQ011849)
T. rapaeodorum (AJ557521)
T. foetidum (AJ557544)
T. foetidum (AJ557543)
mycorrhiza BP98689 P.a., K
mycorrhiza BP98691 P.a., K
mycorrhiza BP98687 P.a., K
T. scruposum (DQ011845)
Tuber sp. (AJ557539)
T. scruposum (DQ011848)
T. whetstonensis (AY830855)
mycorrhiza BP98684 Q.r., P
mycorrhiza BP98685 Q.r., P
T. puberulum (AJ557536)
T. puberulum (AJ557535)
T. puberulum (AJ557531)
T. puberulum (AJ557530)
T. puberulum (AJ557534)
T. puberulum (AJ557532)
T. puberulum (AJ557538)
mycorrhiza BP98696 F.s., Ő
mycorrhiza BP98697 F.s., Ő
T. puberulum (AJ557537)
T. puberulum (AJ557533)
T. borchii (AF250291)
T. borchii (AF132505)
T. borchii (AJ002510)
T. oligospermum (AF106891)
T. borchii (AJ557542)
T. borchii (AJ557540)
T. dryophilum (AF003917)
T. borchii (AJ557541)
mycorrhiza BP98692 P.a., T
T. rufum (AY112894)
T. rufum (AY940646)
T. rufum (AF106892)
Group 1
Group 2
Group 3
Group 4
A
B
C
D
b
98
107
44
172
43
60
71
165

phyletic groups and can be handled as their shared characters (Fig. 1b). The ITS-1 region of all other studied truffles contained an 8-bp long deletion compared to *T. rufum* (deletion "A" Fig. 1).

Due to the above-mentioned indels, almost the whole ITS-1 region was excluded from the analyses. For inferring phylogenies, a 409-character long alignment was used. The model TrN + G + I was selected as best-fit model. The assumed nucleotide frequencies were A=0.3099, C=0.2081, G=0.1966, T=0.2854; the substitution types were rAC, rAT, rCG, rGT=1.000; rAG=2.436; rCT=4.397; the gamma shape parameter was 2.2627, while the assumed proportion of invariable sites was 0.396.

The ITS region of BP98692 ectomycorrhiza showed significant similarity to *T. rufum* sequences. As *T. rufum* does not belong to the *T. puberulum* group of the white truffles, this species was selected as outgroup (group 4). The ML and the distance-based NJ analysis resulted to a similar tree topology. The only difference between the two trees was the position of the BP98684–BP98685 clade. In the ML analyses, it branched from the base of group 1 but with a very low bootstrap support, while in the NJ analysis it grouped into group 2 with strong support value (Fig 1a). The latter position was also supported by anatomical results (see below).

Excluding the outgroup (group 4), the analyzed white truffle sequences formed two main branches of the tree with strong support. Both main branches contain further groups (Fig. 1a).

Within the first branch *T. rapaeodorum*, *T. maculatum* and *T. foetidum* segregate into group 1 with six EM samples, and the first two species form mixed clades (Fig. 1a). Group 2 is branching into two main clades, one with two (BP98684, BP98684), the other with three EM specimens (BP98687, BP98689 and BP98691), and with *T. whetstonensis*, *T. scruposum*, and an unidentified *Tuber* sequence (Fig. 1a). The OTUs in this second clade have a short ITS-1 region with the 130-bp long "B" deletion (Fig. 1).

The other main branch of the tree was formed by *T. puberulum*, *T. borchii*, *Tuber oligospermum*, and *T. dryophilum*, containing two ectomycorrhizae (BP98696, BP98697; Fig. 1a). This clade separated into two well-supported groups. One of these is strengthened by the presence of the 94-bp long "C" deletion (Fig 1). *T. puberulum* and *T. borchii* do not separate clearly within this group. Moreover, one *T. puberulum* sequence and two ectomycorrhizae form a well-supported clade nested in group 3 with a 16-bp long "D" deletion (Fig. 1).

The other clade including *T. dryophilum* and *T. oligospermum* contains sequences with no extra deletion except "A." There are also some *T. borchii* sequences in this clade (Fig. 1a).

Morphology and anatomy of the EM

All EM showed similar characteristics, except clade 4, including BP 98692. The latter could be identified as the EM of *T. rufum*. All other EM included in groups 1–3 are yellow-ochre to brown when young and dark brown at maturity. Two EM samples, BP98694 and BP 98688 as representatives of group 1, have been chosen for more detailed microscopical characterization.

Morphology Ectomycorrhizal system abundant, densely ramifying, monopodial to pyramidal. Main axes 3–4 mm long, straight or slightly bent. Unramified ends up to 0.7 mm long, 0.2–0.3 mm in diameter, cylindrical or slightly tapering, tips lighter than other parts. Surface densely spiny with cystidia. Rhizomorphs lacking. Cystidia frequent, apical to lateral, colorless or yellow.

Anatomy Mantle pseudoparenchymatous-epidermoid, at some places covered by a hyphal network, which cystidia originate from. Surface net (Fig. 2b) formed by anastomosing, irregularly constricted hyphae, 4–6 μm in diameter, average distance between septa 10–15 μm. Cell walls of hyphal network 0.2 μm thick, smooth, colorless to pale ochre. Outer mantle layer (Fig. 2c): cells epidermoid, sinous, membranaceously yellow, cells smooth or at some places with amorphous, yellow layer on surface. Cell walls 0.5–2 μm thick, uneven in thickness, number of cells in a square of 20×20 μm 4–9. Middle mantle layer (Fig. 2i) pseudoparenchymatous epidermoid, at some parts cells somewhat smaller than in outer layer, cell walls 0.2–1 μm thick, membranaceously yellow, surface of cells smooth. Inner mantle layer (Fig. 2d) epidermoid, cell walls 0.2–0.5 μm thick, colorless to yellow, surface of cells smooth. Very tip: cells epidermoid, like on other parts of the mantle, but cells smaller, cell walls thin (0.2 μm), number of cells in a 20×20-μm square 10–12.

Emanating hyphae Pale yellow or colorless, growing out from the surface hyphal net of the mantle, cells 1–4 μm in diameter, cell walls 0.2–0.5 μm thick, surface smooth, distal tips simple or slightly swollen. Hyphae straight or slightly wavy, even or slightly constricted at septa, distance between septa 20–200 μm, cell walls of hyphae mostly 0.2 μm thick, clamps lacking. Ramifications not frequent, Y-shaped, not connected to septa, anastomoses with a short or long bridge, septate. Cystidia (Fig. 2a,e) frequent, needle-shaped, straight or slightly bent, foot cells with broader basis and blunt tips, 70–120 μm long, often with one or two septa. Diameter at the base 3–4 μm, in middle parts 1–2 μm, at tip 1 μm. Surface smooth to rough but not warty, colorless to light yellow. Cell walls 0.5–1 μm thick.

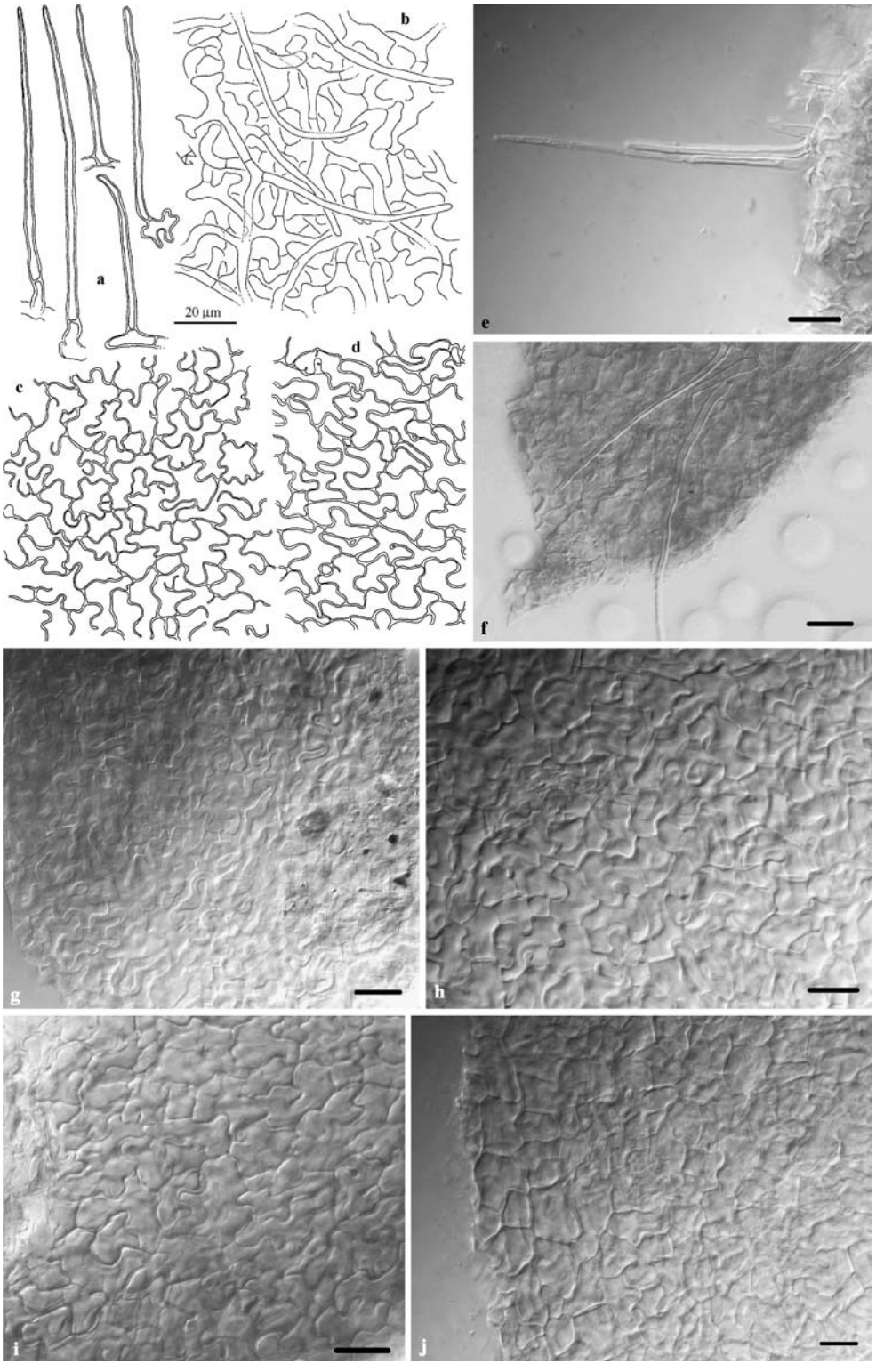


Fig. 2 Anatomy of the mycorrhizae. **a** Cystidia, **b** hyphal net on the surface of the mantle, **c** outer layer of mantle, **d** inner layer of the mantle of *T. rapaeodorum* (BP 98694). Cystidia in **e** Group 1 (BP 98688), **f** Group 2 (BP 98687). Outer mantle layer in **g** Group 3 (BP 98696), **h** Group 2 (BP 98687). Middle layer of mantle in **i** Group 1 (BP 98688) and **j** Group 2 (BP 98687). *Bar* for **e**–**j**: 10 µm

Although all samples were highly similar to those described above, minor anatomical alterations between the representatives of group 1, group 2 (represented by BP 98687), and group 3 (represented by BP 98696) could be detected. However, these features vary in a wide range even within the same specimen, and overlap between the groups.

The cystidia show the clearest difference between the groups. Cystidia of both group 1 and group 3 (Fig. 2e) are straight, similar in shape and size, but those of group 3

are more rigid, with somewhat thicker cell walls than in group 1. Cystidia in group 2 (Fig. 2f) are slightly differing from the former ones. Although similar in shape, these are shorter and more flexible, and their cell walls are thinner.

Differences in mantle structure between the groups can be also observed. While all mantle layers in group 1 are epidermoid, in the outer and middle layers in group 3 elongated, oval or even angular cells appear (Fig. 2g). The inner layers are similar in all groups (Fig. 2d). The middle layer of the mantle is distinctly angular in group 2 (Fig. 2j) and cell walls of the outer layer are thinner than in others (Fig. 2h). In contrary, cell walls are thicker and mantle cells bigger in group 3 (Fig. 2d).

Discussion

The presence of *Tuber* species in a certain area can be tested either by collecting the hypogeous ascocarps or by checking the EM of the appropriate hosts. Although the *Tuber* EM can be relatively easily determined at genus level, the differences between the white truffle EM are ambiguous.

The phylogenetic analysis of the ITS sequences reveals the taxonomical questions, the term "white truffle" does not cover a monophyletic group of *Tuber* species (Roux et al. 1999). Although the problems of the morpho-species have been raised earlier (Gross 1987; Montecchi and Lazzari 1992) and trying to solve the ambiguity of the morphological data was attempted (Halász et al. 2005), our results also indicate that the situation is still problematic. With the exception of the *T. rufum* group, the analyzed sequences form two definite branches, which prove that white truffles can be sorted into two main taxonomic groups (Mello et al. 2000). One of them contains *T. rapaeodorum*, *T. maculatum*, *T. foetidum* and the other contains *T. puberulum*, *T. borchii*, *T. oligospermum*, and *T. dryophilum*. However within these groups, some species (*T. rapaeodorum*, *T. maculatum*, and *T. borchii*) do not separate and are hardly distinguishable. These results query the reliability of identification of the species based on traditional characteristics and can support the *sensu lato* species concept in the case of the white truffle taxa (Montecchi and Lazzari 1992).

However, the molecular analysis showed definite genetical distances between the OTUs, which must reflect real taxonomical differences. The position of the characteristic indels in the ITS-1 region corresponds to the results of the phylogenetic analyses; however, this region was excluded from the analyses. Besides the importance of these features in the delimitation of the certain groups, these indels could be suitable to design specific PCR primers. Specific primers could help the fast and accurate molecular separation of morphologically similar or almost undistinguishable groups. Such systems were already developed for some white truffle species (Amicucci et al. 1998, 2000; Mello et al. 1999). Although the developed PCR systems were able to separate some species, based on ITS sequences, we suppose that, e.g., the primer designed to amplify *T. maculatum* (Amicucci et al. 1998) would amplify also *T. rapaeodorum*.

Nine of the studied EM clearly clustered together with definite species and, keeping the above-mentioned taxonomical problems in mind, they can be regarded as identified ones.

The mycorrhiza BP98692 was very likely formed by *T. rufum* and its anatomical characteristics, e.g., infrequent cystidia and a robust hyphal net on mantle surface, corresponding to the previous descriptions of *T. rufum* (Rauscher et al. 1995; Rauscher and Chevalier 1996), also supports this determination.

Five mycorrhizal specimens grouped with *T. rapaeodorum* sequences so these ectomycorrhizae might belong to this species. All these mycorrhizae were collected from poplar on the Great Hungarian Plain, but any geographic specificity can be excluded, as this group contains *T. rapaeodorum* sequences also from different areas of Hungary (Halász et al. 2005) and other countries as Lithuania (Menkis et al. 2005, 2006) and Italy (GenBank Accession number AF106889). One oak mycorrhiza was grouped with *T. maculatum*, but based on the heterogeneous origin of these ascocarp samples, we may exclude any geographic and host specificity.

In the case of the five ectomycorrhizae (two from *Quercus*, three from *Populus*) in group 2, ambiguous clustering with sequences of identified species was obtained. However, these specimens seem to be slightly different in cystidia and mantle structure.

Two mycorrhizae formed a well-supported clade within group 3 together with a *T. puberulum* sequence. These mycorrhizae were collected from beech in a mountain region of the northeast area, while the fruitbody originated from the southern part of Hungary (Halász et al. 2005). The clade is inserted between other *T. puberulum* sequences, but the genetic distance and the presence of a characteristic indel separate this clade from its surrounding.

The ectomycorrhizae of white truffles show characteristic common features. Although the molecular analyses clearly separates several clusters of the studied sequences, these genetic differences do not correspond to any morphological difference. The ectomycorrhizae generally have an epidermoid outer mantle layer with a hyphal network on the surface and needle-shaped cystidia, as it has also been detected in the previously published *T. puberulum* (Blaschke 1987, 1988), *T. borchii* (Rauscher et al. 1996), and *T. maculatum* (Zambonelli et al. 1999) ectomycorrhizae. The anatomy and size of the cystidia, cell wall

thickness of mantle cells, and a transition form between the epidermoid and angular type of the outer, and more expressed, of the middle mantle layers seem to be the only characteristics suitable to detect differences between groups 1, 2, and 3. Nevertheless, these variable features overlap, as it was observed also when comparing younger and older parts of the same mycorrhizal specimen. Similarly variable mantle patterns were observed by Giomaro et al. (2000) in the ectomycorrhizae of *T. borchii*, who explained this phenomenon as intraspecific variability and the different age of the strains.

Although the relatively easy recognition of white truffle ectomycorrhizae makes it possible to study their occurrence in plant communities, the anatomical comparison shows that the mycorrhizal characteristics are not good tools for identification of the species within this group. The interspecific similarity, observed both concerning ectomycorrhizal and fruitbody characters, strengthens the *sensu lato* morpho-species concept of this group. The classical morphological characters, which have been used for delimitation of the taxa, are too variable to separate the species unambiguously (Gross 1987; Montecchi and Lazzari 1992; Halász et al. 2005). On the contrary, molecular analyses show clear genetical distances between the clusters of OTUs, but these do not correspond to species identified traditionally. This is clearly demonstrated by the mixture of taxon names within the well-supported phylogenetic clusters of database sequences. Our study, which combines comprehensive molecular and anatomical approach to characterize and identify ectomycorrhizae of white truffles from natural samples, also stresses out the need of the taxonomical revision of this group.

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