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Morphological-anatomical characterization and molecular identification of *Tomentella stuposa* ectomycorrhizae and related anatomotypes

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Abstract Species in the genus *Tomentella* (Thelephoraceae) belong to the most frequent and widespread ectomycorrhizal (EM) fungi found in temperate and boreal forests. Although several unidentified tomentelloid morphotypes have been presented as common members of EM communities in coniferous and broad-leaved forests, few tomentelloid EM have been identified and described in detail. In this study, ten tomentelloid EM isolates collected from *Populus alba*, *Quercus cerris* and *Picea abies* stands in Hungary and Germany are characterized and documented by morphological-anatomical methods using light microscopy. The investigated ectomycorrhizae belong to the same brown-black tomentelloid morphotype but form two different anatomotype groups (At I and At II). Molecular taxonomical identification was accomplished using phylogenetic analysis (neighbor joining method) of 49 *Tomentella* nrDNA-ITS nucleotide sequences including the 10 new and 39 GenBank sequences. The EM isolates clustered into two adjoining clades identical with the two anatomotypes. At II clustered with *Tomentella stuposa* while At I could not be identified to species. Based on the morphological similarity and the low genetic difference it must be a closely related taxon. A comparison of the recently known tomentelloid EM to *T. stuposa* is presented. Ecological questions involving abundance and host relationships are discussed.

Keywords *Tomentella stuposa* · Ectomycorrhiza · ITS sequences

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Introduction

Ectomycorrhizae (EM) play a key role in nutrient cycling and energy flow of temperate and boreal forest ecosystems (Smith and Read 1997). The best known mycobionts of EM communities belong to the basidiomycota producing different types of conspicuous fruitbodies. Unfortunately, the frequency and ecological significance of fungi forming inconspicuous, resupinate sporocarps are highly underestimated as EM symbiotic partners of trees (Gardes and Bruns 1996). Until recently, only a few genera of resupinate fungi with few species have been proved to be ectomycorrhizal, i.e. *Tylospora* (Eberhardt et al. 1999), *Piloderma* (Larsen et al. 1997), *Amphinema* (Fassi and de Vecchi 1962), and *Byssocorticium* (Brand 1991). Recently, it has become generally accepted that species of the resupinate genus *Tomentella* (Thelephoraceae) belong to the most frequent and widespread EM fungi in coniferous and broad-leaved forests in Europe and North America (Gardes and Bruns 1996; Dahlberg et al. 1997; Köljalg et al. 2000). As their mostly thin basidiocarps appear often on the underside of twigs, leaves, and stones, they are easily overlooked (Köljalg 1996). *Tomentella* species have been demonstrated to form EM associations with gymnosperms (Danielson and Pruden 1989; Visser 1995; Gardes and Bruns 1996; Bradbury et al. 1998; Horton and Bruns 1998; Kranabetter and Wylie 1998; Taylor and Bruns 1999), angiosperms (Brand 1991; Raidl and Müller 1996; Jakucs 2002c) and even with orchids (Taylor and Bruns 1997).

Currently, the EM *T. albomarginata* (Bourdot and Galzin) Larsen [synonymous to *T. sublilacina* (Ellis and Holw.)

Wakef.; Agerer 1996a,b], *T. ferruginea* (Pers.: Fr.) Pat. (Raidl 1998; Raidl and Müller 1996), *T. pilosa* (Burt) Bourdot and Galzin (Jakucs and Agerer 1999; Jakucs 2002a), *T. brunneorufa* Larsen (Agerer and Bougher 2001), *T. subtestacea* (Jakucs and Agerer 2001; Jakucs 2002b), and *T. galzinii* Bourdot have been described comprehensively. The latter was first published as an unidentified EM named "*Quercirhiza fibulocystidiata*" (Jakucs et al. 1997, 1998) but it has subsequently been identified using DNA sequence analysis (Köljalg et al. 2001). In addition, several unidentified EM, presumably *Tomentella* species, have been described (Gronbach 1988; Brand 1991; Haug and Pritsch 1992; Agerer et al. 1995; Fischer and Agerer 1996; Palfner and Agerer 1996; Montecchio and Agerer 1997; Golldack et al. 1998, 1999; Wöllecke et al. 1999; Román et al. 2002a,b).

Molecular methods (e.g., DNA sequence analysis) are important tools with which to distinguish unknown individual mycorrhizal root tips (Gardes and Bruns 1993; Bruns et al. 1998) and to identify them by comparing their sequences with those obtained from sporocarps (Bruns et al. 1998). Köljalg et al. (2000, 2001, 2002) constructed an nrDNA-ITS sequence database from sporocarps of tomentelloid fungi that can help to identify *Tomentella* EM. The use of molecular data confirmed the occurrence of five *Tomentella* and one *Pseudotomentella* species in the EM communities of Swedish coniferous forests (Köljalg et al. 2000). The specific morphological and anatomical characteristics of tomentelloid EM are essential for selection of anatomotypes in ecological studies. The combination of the microscopical approach and molecular investigation of EM gives more accurate information in both taxonomical (Köljalg et al. 2001) and ecological studies (Dahlberg et al. 1997).

During a 4-year investigation in broad leaved forests of the Great Hungarian Plain, tomentelloid EM were revealed to be common members of the forest communities. More than 12 different tomentelloid EM morphotypes have been distinguished (Jakucs 2002c). The aim of this study was to characterize and identify the most abundant representatives of these. Ten EM isolates determined as *Tomentella stuposa* and a closely related taxon are characterized by morphological-anatomical methods and nrDNA-ITS sequence analysis.

Materials and methods

Sampling

Ectomycorrhizae were isolated from soil samples collected in two regions of the Great Hungarian Plain (Tompa and Püspökladány) and in Germany (Höglwald).

The *Populus alba* L. stand (Tompa) is in the southern part of Hungary near the Serbian border, at 120–130 m altitude with 35- to 40-m-high trees mixed with *Acer negundo* L., *Celtis occidentalis* L., *Fraxinus excelsior* L., *Robinia pseudoacacia* L., *Sambucus nigra* L. and *Ulmus laevis* Pall. The average rainfall in the region is 550 mm/year. The soil is sandy, mixed with loess and with a quite thick (about 10 cm) moder-type humus layer containing 0.4% lime, pH 7.1–7.6.

The 67-year-old *Quercus cerris* L. stand (Püspökladány) in the center of the Hungarian Plain has *Prunus spinosa* L., *Fraxinus* sp. and *Rosa* sp. in the understorey. The forest is established on limed solonetz soil, pH 6.5. The average rainfall in the region is 526 mm/year.

One EM sample originated from a 95-year-old pure Norway spruce [*Picea abies* (L.) Karst.] stand in Höglwald, Bayern, based on a dystric Cambisol derived from pleistocene loess over tertiary sediments with average rainfall of 800 mm/year (Kreutzer and Bittersohl 1986).

At all sites, 20×20×20 cm soil cubes were taken with a sharp knife from the upper layer of the forest soil. In the laboratory, soil samples were stored in a refrigerator at 4°C for a period of less than a week. Soil was washed from the root samples with tapwater over a sieve and ectomycorrhizae were separated and sorted in water under a stereomicroscope. Ectomycorrhizal tips were fixed in FEA [formaldehyde:70% ethanol:concentrated acetic acid, 5:90:5 (v/v)] for further light microscopy. For DNA analysis, three EM tips from each type were fixed in CTAB buffer (2% CTAB, 20 mM EDTA pH 8, 100 mM Tris-HCl pH 9, and 1.4 mM NaCl). EM specimens fixed in FEA have been preserved in the collection of E. Jakucs and R. Agerer and voucher specimens deposited in the Hungarian Natural History Museum, Budapest (BP) and in the Botanische Staatsammlung München (RA) (Holmgren et al. 1990).

Herbarial numbers, collection data, and GenBank accession numbers of the EM investigated are listed in Table 1.

Morphological-anatomical characterization

Ectomycorrhizae were characterized and described using the widely accepted morphological, anatomical and histochemical methods of Agerer (1991). The mycorrhizal system was examined by stereomicroscopy. The mantle structure, the hyphal and rhizomorphal characteristics were studied by DIC (Nomarski) microscopy, including microscopic drawings and digital photodocumentation.

DNA isolation

DNA isolation and amplification methods were described previously (Kovács et al. 2001). Mycorrhizal tips were thoroughly ground with micropestles and sand in Eppendorf tubes in CTAB buffer. After incubation at 65°C for 45 min the samples were extracted twice with an equal volume of chloroform and centrifuged for 15 min at 12,000 *g* after both extractions. The DNA was precipitated with two volumes of ethanol and, after storing at least 8 h at −20°C, it was pelleted by centrifugation at 12,000 *g* for 30 min. The pellet was washed with 70% ethanol, dried and redissolved in 30 µl sterile Milli-Q water (Millipore, Bedford, Mass.).

Table 1 Herbarial numbers, collection data and GenBank accession numbers of the mycorrhizae studied

Mycorrhizal sample	Collection sites	Collection date	Collected by	Host	GenBank number
BP 96971	Püspökladány, Hungary	8 April 1998	E. Jakucs	*Quercus cerris*	AY635168
BP 96972	Tompa, Hungary	2 June 1998	E. Jakucs	*Populus alba*	AY635169
BP 96973	Tompa, Hungary	2 June 1998	E. Jakucs	*Populus alba*	AY635170
BP 96974	Tompa, Hungary	2 June 1998	E. Jakucs	*Populus alba*	AY635171
BP 96978	Tompa, Hungary	8 October 1998	E. Jakucs	*Populus alba*	AY635172
BP 96979	Tompa, Hungary	8 October 1998	E. Jakucs, Z. Bratek, G. M. Kovács	*Populus alba*	AY635173
BP 96980	Tompa, Hungary	8 October 1998	E. Jakucs, Z. Bratek, G. M. Kovács	*Populus alba*	AY635174
BP 96981	Tompa, Hungary	8 October 1998	E. Jakucs, Z. Bratek, G. M. Kovács	*Populus alba*	AY635175
BP 96985	Tompa, Hungary	7 July 1999	E. Jakucs	*Populus alba*	AY635176
RA 12939	Höglwald, Bayern, Germany	8 April 2000	R. Agerer	*Picea abies*	AY635177

DNA amplification and sequencing

The PCR amplification of the ITS region used primers ITS1F and ITS4 according to Gardes and Bruns (1993), with some modification. The reaction mixture contained 0.1 volume 10× PCR buffer (Red-*Taq*; Sigma, St. Louis, Mo.), 200 µM each dATP, dCTP, dGTP and dTTP (Sigma), 0.5 µM each primer, 1 U *Taq* DNA polymerase (Sigma Red *Taq*) and the DNA extract. The amplifications were performed with a DNA-Engine thermo cycler (MJ Research, Waltham, Mass.), programmed for a denaturation step at 93°C for 3 min, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 52°C, and extension for 1 min at 72°C; the thermal cycling was terminated by a final extension for 10 min at 72°C. For direct sequencing, PCR products were purified with a PCR Clean up-M kit (Viogene, Sunnyvale, Calif.). For cycle sequencing, an ABI PRISM 3.1 BigDye Terminator Kit (Perkin Elmer; Perkin Elmer-Applied Biosystems, Foster City, Calif.) was used and the electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer according to the manufacturer's instructions.

Phylogenetic analysis

DNA sequences were processed and analyzed with the Staden Program Package (Staden 1996). The sequences were identified and checked with the BLAST homology search program. Multiple alignments of homologous ITS sequences obtained from GenBank were made using the MultAlin program (Corpet 1988) at the INRA server with a DNA-5-0 score matrix. Alignments were checked and manually edited if necessary. During phylogenetic analyses, distances of sequences were calculated by the MEGA2.1 program (Kumar et al. 2001) with the Kimura's two-parameter model and with pairwise deletion of gaps. Phylogenies were inferred by the neighbor-joining method (Saitou and Nei 1987) on the MEGA2.1 program and tested by bootstrapping (Felsenstein 1985) using 1,000 replicates. Phylogenetic trees were visualized and edited using the Tree Explorer of the MEGA2.1 program.

Results

The ten ectomycorrhizal samples belong to two, highly similar, anatomotypes (At I and At II). Samples BP 96981/BP 96972, and sample RA 12939, are representative of At I and At II, respectively, and have been described and documented in detail.

Morphological-anatomical description of At I ectomycorrhiza

Main features

Ectomycorrhizae are dark brown when young and brown to black at maturity, monopodial, pinnate to pyramidal. Mantle pseudoparenchymatous, with angular cells in the outer layer and groups of globular cells on the surface (Figs. 1, 3, 8). Middle mantle layers angular, with somewhat elongated cells organized in star-like nests (Fig. 2). Inner layer of mantle plectenchymatous (Fig. 3). Undifferentiated rhizomorphs are present (Figs. 3, 9). Emanating hyphae clamped, hyaline, yellow or brown and somewhat wavy, originating in groups from a few, distinct patches of the mantle (Figs. 3, 4).

Morphological characters

Ectomycorrhizal systems abundant, dense, monopodial, pinnate to pyramidal. Main axes 7–9 mm long and 0.5 mm in diameter, straight or slightly bent. Unramified ends 1–4 mm long and 0.3–0.4 mm in diameter, straight, cylindric or slightly tapering; brown, tips somewhat lighter than at other parts, older parts dark-brown to black; surface densely grainy caused by groups of globular cells. Rhizomorphs infrequent, slightly differentiated in structure.

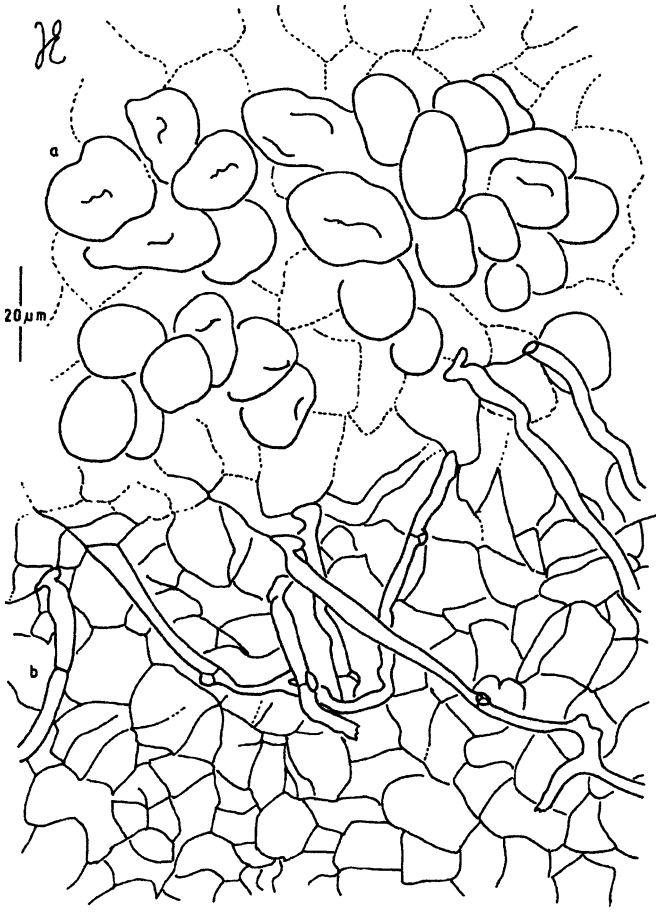

Fig. 1a, b At I. **a** Surface view of mantle with mounds of globose cells. **b** Pseudoparenchymatous-angular outer mantle layer and emanating hyphae

Anatomical characters of mantle in plan view

Mantle pseudoparenchymatous, with groups of globose cells and at some places with yellow-brown amorphous matrix on surface (Figs. 1a, b; 8c). Groups of globose cells with conical wall structures (Fig. 3a) formed by 5–10 cells, cells 20–30 µm diameter, cell walls 0.2–04 µm thick. Outer mantle layer cells angular (mantle type K, Agerer 1991) (Fig. 8d), brown; surface of cells smooth, a few places with amorphous yellow-brown material on surface sticking soil particles; cell walls 0.2–0.5 µm thick; 6–7 cells in a square of 20×20 µm. Middle mantle layers pseudoparenchymatous, angular; cells somewhat elongated and organized in star-like nests (Figs. 2a, 9d); cell walls 0.2 µm thick, brown. Inner mantle layers plectenchymatous (Fig. 2b), cell walls brown. Very tip pseudoparenchymatous, cells angular, like other parts of mantle, but cells smaller, 8–9 cells in a square of 20×20 µm.

Anatomical characters of emanating elements

Hyphae with clamps (Figs. 1b, 3b, 4), backwards oriented ramifications and reversed clamps not observed, anastomoses with contact clamp present. Emanating hyphae straight or slightly wavy, at septa even, distance between septa 50–150 µm, ramification Y-shaped, with one side branch and that at two hyphal diameters below septum; clamps present, simple septa infrequent; hyphae 1–3 µm thick; cell walls mostly 0.2–0.8 µm thick, even in thickness or at septa slightly constricted, some emanating hyphae have globose basal cells of 10–15 µm diameter, with walls up to 1.2 µm thick; apical ends simple, walls at tips as thick as remaining walls; thin cell walls hyaline, walls of thick-walled hyphae membranaceously yellow or brown; surface smooth, without any crystals and appositions; some clamps with a hole, in lateral view as broad as their hypha, shaped as a semicircle, in dorsal view oval. Rhizomorphs slightly differentiated in structure (Figs. 3c; 9a, b), nodia and conical young side-branches present, ramifications with one or two side branches at nodia; rhizomorph internally not differentiated (type B, Agerer 1991). Cystidia lacking. Sclerotia lacking.

Material studied

Hungary, Tompa, *Populus alba* stand. leg. E. Jakucs, Z. Bratek and G. M. Kovács 2 June 1998 (BP 96972) and 8 October 1998 (BP 96981) (in Budapest).

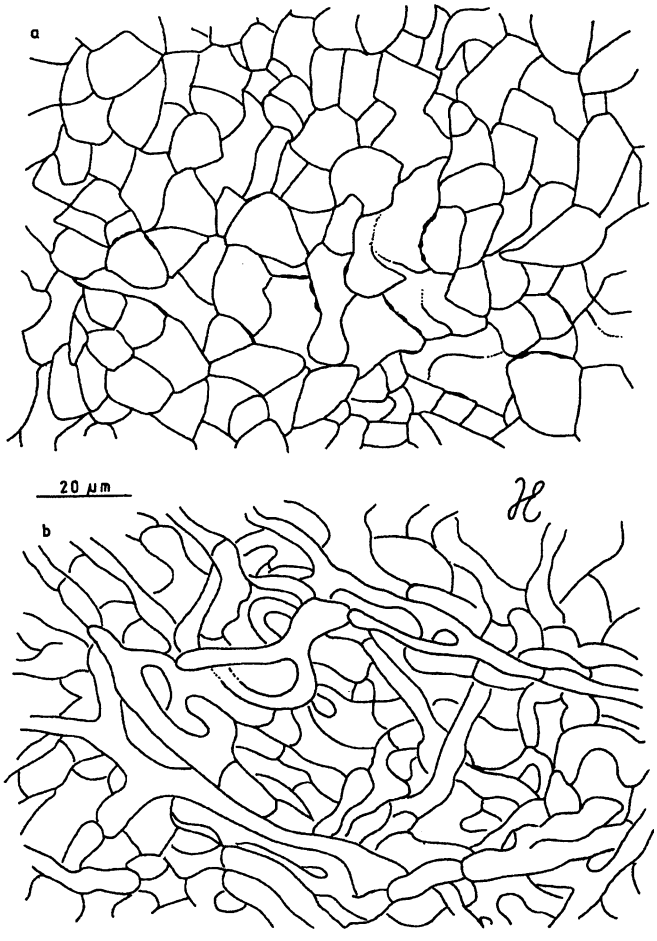

Fig. 2a, b At I. **a** Outermantle layer with angular cells. **b** Plectenchymatous inner layer of mantle

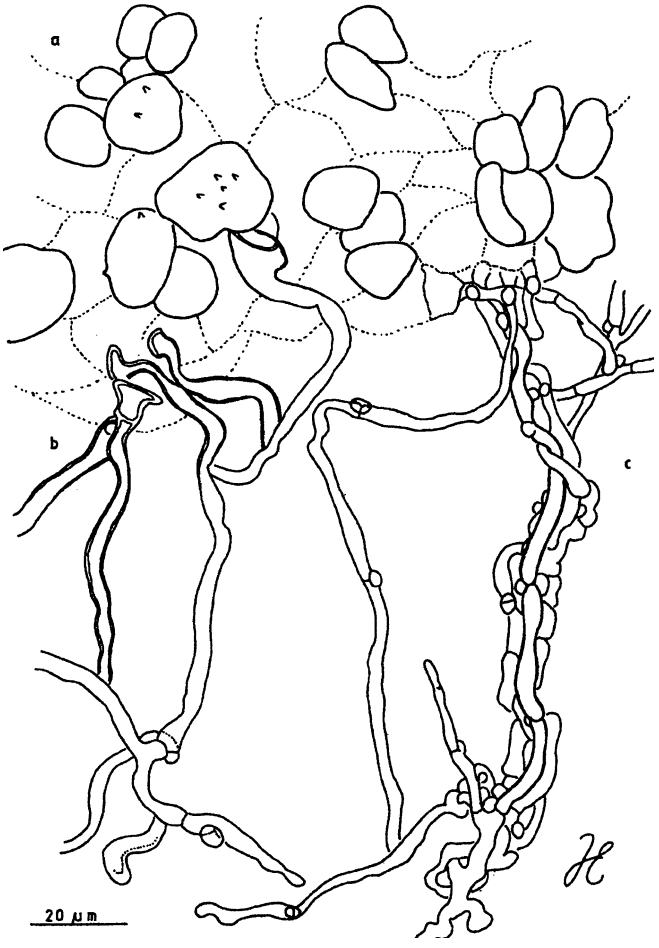

Fig. 3a–c At I. **a** Surface view of mantle with mounds of globose cells laying on a pseudoparenchymatous outer mantle layer; cells of mounds occasionally with short conical cell wall protuberances. **b** Emanating hyphae originating fom mantle. **c** Origin of a young rhizomorph with nodia

Morphological-anatomical description of At II ectomycorrhiza

Main features

Ectomycorrhizae are monopodial, pinnate to pyramidal, dark-brown when young and dark-brown to black at maturity, with blackish-brown dots caused by densely packed blue granules. Mantle pseudoparenchymatous, with angular cells in the outer layer, and groups of globose cells on the surface (Fig. 5). Middle mantle layer cells angular, without pattern or indistinctly star-like (Fig. 6), more distinct close to mantle surface. Inner layer of mantle plectenchymatous, with ring- to star-like pattern and a pseudoparenchymatous nest of cells in places (Fig. 7). Emanating hyphae clamped, simple septa present, brown and somewhat wavy emanating hyphae patchily arranged (Figs. 6, 8a). Undifferentiated rhizomorphs present (Figs. 6, 9c).

Morphological characters

Ectomycorrhizal systems abundant, dense, monopodial, pinnate to pyramidal (Fig. 5a). Main axes up to 20 mm long and 0.3–0.5 (0.6) mm diameter, straight or slightly bent, 0–2 orders of ramifications, 8–15 side-branches per 10 mm. Unramified ends up to 2 (3) mm long and 0.25–0.3 mm diameter, straight to bent, infrequently slightly sinuous, cylindric, brown, at tips somewhat lighter than at other parts, older parts dark brown to black; surface densely grainy caused by groups of globose cells, loosely woolly, mostly shiny. Rhizomorphs infrequent, not differentiated in structure, organized as loosely to densely woven bundles of hyphae (uniform loose to uniform compact, according to Agerer 1999).

Anatomical characters of mantle in plan views

Mantle pseudoparenchymatous, with groups of globose cells, or infrequently cells triangular or with one end torn out (Fig. 5c). Globose cells in groups, cells 10–21×7.5–12 µm, cell walls ca. 0.5 µm thick, with conical wall structures. Outer mantle layer cells angular (mantle type K, Agerer 1991), brown, with dense aggregations of bluish granules,

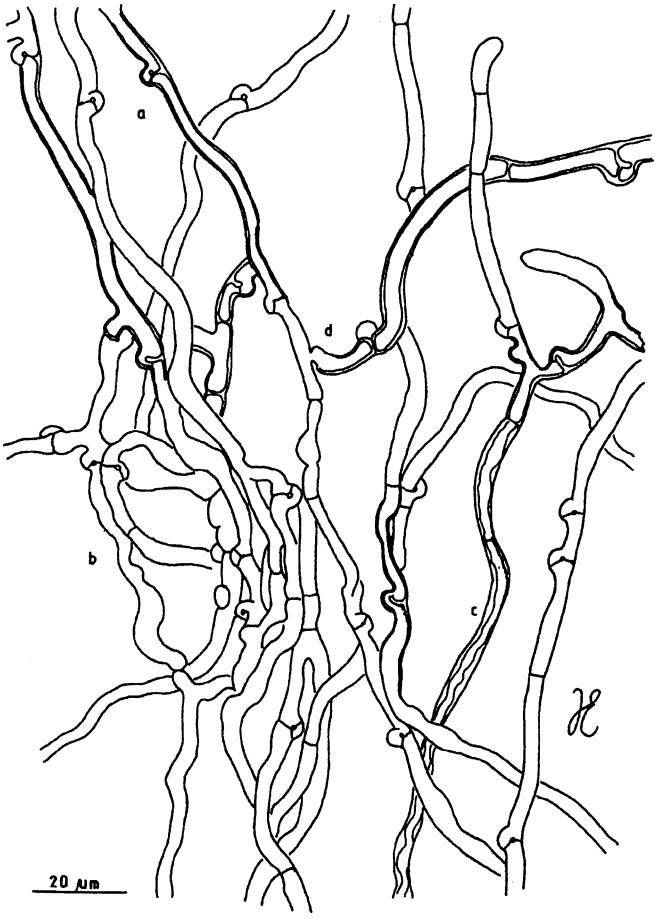

Fig. 4a–d At I. **a** Emanating hyphae with clamps and thickened cell wall. **b**, **d** Ramifications of hyphae of differentially thick cell walls. **c** Intrahyphal hyphae

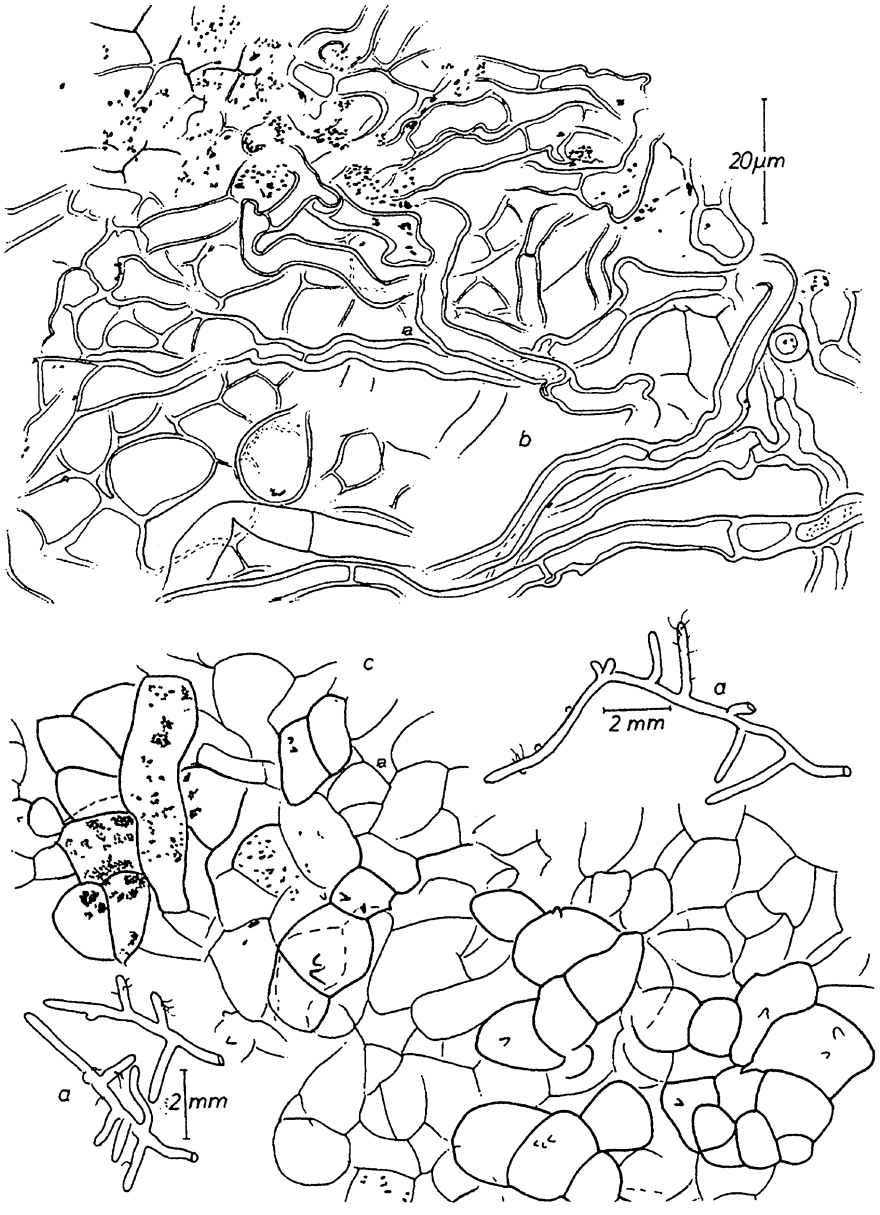

Fig. 5a–c At II. **a** Habit. **b** Surface view of mantle with globose cells and a patch of emanating hyphae; intracellular blue granules, some lying on the cell surface. **c** Surface view of mantle with mounds of globose cells lying on a pseudoparenchymatous outer mantle layer; cells of mounds occasionally with short triangular cell wall protuberances; blue granules within mound cells and within cells of outer mantle layer

mostly within cells but also at the surface, causing the black patches of the habit (Figs. 5, 6b, 7a); surface of mantle cells smooth; cell walls 0.3–0.5 µm thick; 7–12 cells in a square of 20×20 µm, cells 10–20(25)×7–13 µm. Middle mantle layers pseudoparenchymatous, angular; cells somewhat elongated, indistinctly arranged in star-like nests (Fig. 6a); cell walls thin, brownish; dimensions as in outer layers. Inner mantle layers plectenchymatous, only one hyphal layer thick, ring- or star-like, sometimes with nests of pseudoparenchymatous cells, cell walls brownish (Fig. 7). Very tip: pseudoparenchymatous, cells angular, like other parts of mantle, but cells smaller.

Anatomical characters of emanating elements

Clamps, backwards-oriented ramifications and reversed clamps not observed, anastomoses with contact clamps lacking. Emanating hyphae (Fig. 6d) straight or slightly wavy, at septa even, distance between clamps (70) 130–200 (235) µm, ramification rectangular to Y-shaped, with one side branch a considerable distance from the septum; clamps present, simple septa infrequent or sometimes frequent, evenly distributed; hyphae 4–5.5 µm thick, elbow-like protrusions very infrequent, membranaceously yellow or brown; surface smooth, without any crystals and appositions; cell walls mostly (0.5) 1 (1.5) µm thick, even in thickness; distal ends simple or ramified, walls at tips thinner than remaining walls; clamps without a hole, in lateral view thinner than their hypha, less than a semicircle in shape, in dorsal view cylindric; anastomoses open, with a short bridge, walls as thick as remaining walls, bridge thinner than hyphae. Rhizomorphs (Fig. 6c) (type B, Agerer 1991) uniform loose to uniform compact (Agerer 1999), i.e., undifferentiated, as loosely or densely woven

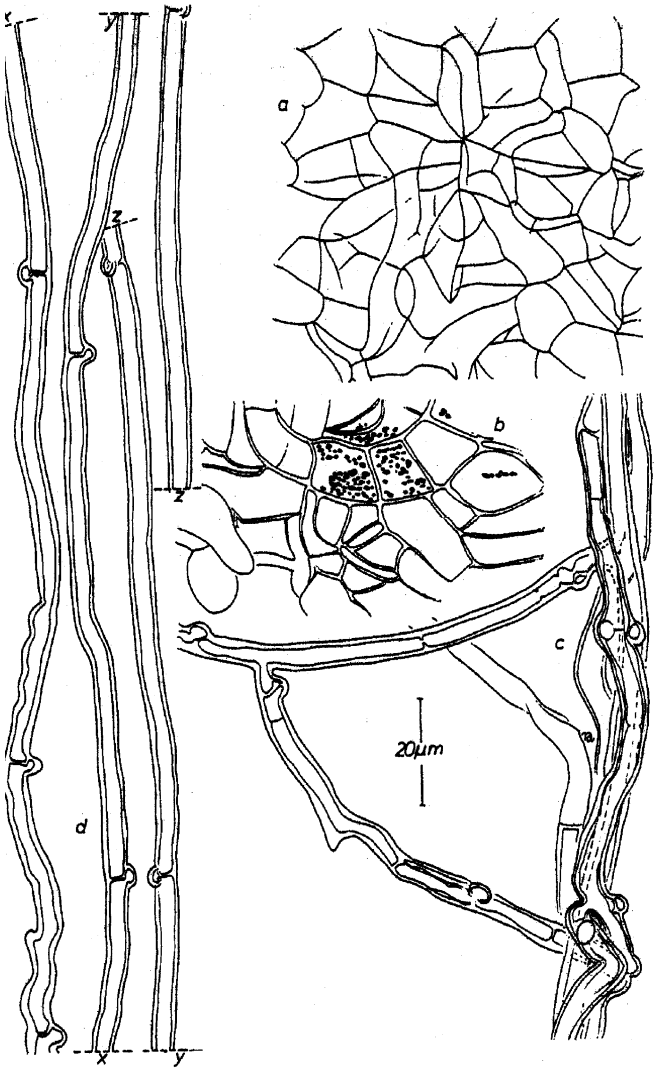

Fig. 6a–d At II. **a** Outer mantle layer, occasionally with rosette-like arranged cells. **b** Plan view of outer mantle layer; some cells with blue granules; cells with blue granules revealing thicker walls than remaining cells. **c** Some emanating hyphae form a thin bundle; note intracellular hyphae. **d** One emanating hypha drawn in several portions; point of continuation in *x*, *y*, and *z*

hyphal bundles, nodia lacking. Cystidia lacking. Sclerotia lacking.

Material studied

Germany, Bayern, Aichach-Friedberg district, between Odelzhausen and Mering, in the forest Höglwald near Tegernbach; close to the forest road near Zillenberg. leg. R. Agerer, 8 April 2000, RA 12938 and RA 12939 (all in Munich; as *Piceirhiza nigripunctata*: Agerer et al. 2002).

Comparison of the two anatomotypes

The common diagnostic characteristics of At I and At II anatomotypes are the dark-brown, angular mantle cells and globose cell-groups on the surface (mantle type K, Agerer 1991), the latter with conical warts on the cell wall, the ring- to star-like structure of the inner plectenchymatous mantle layer, the wavy, clamped, brown emanating hyphae and the scarcely differentiated rhizomorphs (type B, Agerer 1991). However, some slight anatomical differences between the two anatomotypes are as follows:

1. The presence of blackish-brown dots of the brown mantle caused by densely packed blue granules are characteristic of At II but are not conspicuous, although observed, in At I.
2. The angular mantle cells and emanating hyphae of At II have definitely thicker walls [0.3–0.5 µm and (0.5) 1 (1.5) µm, respectively] than those of At I (0.2–0.4 and 0.2–0.8 µm, respectively).
3. The rhizomorphs of At I, although internally undifferentiated, are somewhat differentiated in structure, having nodia and conical young side-branches; while rhizomorphs of At II are undifferentiated, organized as loosely or densely woven hyphal bundles without nodia.

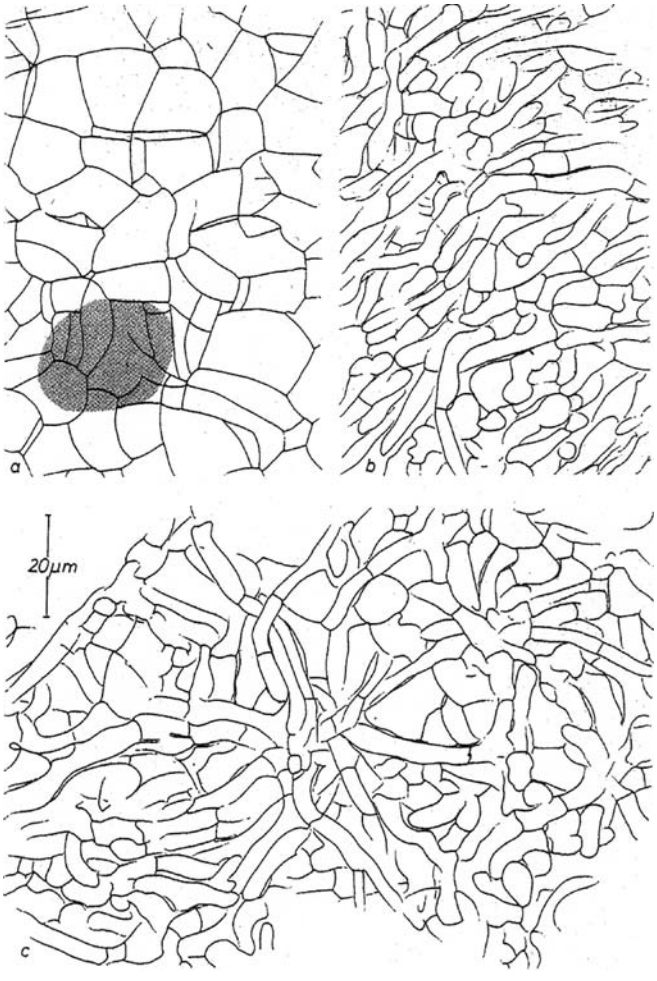

Fig. 7a–c At II. **a** Plan view of middle mantle layer with angular cells; the dotted area indicates that portion of the outer mantle layers where the cells are filled with blue granules. **b** Plan view of inner mantle layer; hyphal arrangement irregular. **c** Plan view of inner mantle layer; hyphae form star- to ring-like structures with pseudo-parenchymatous nests at some places

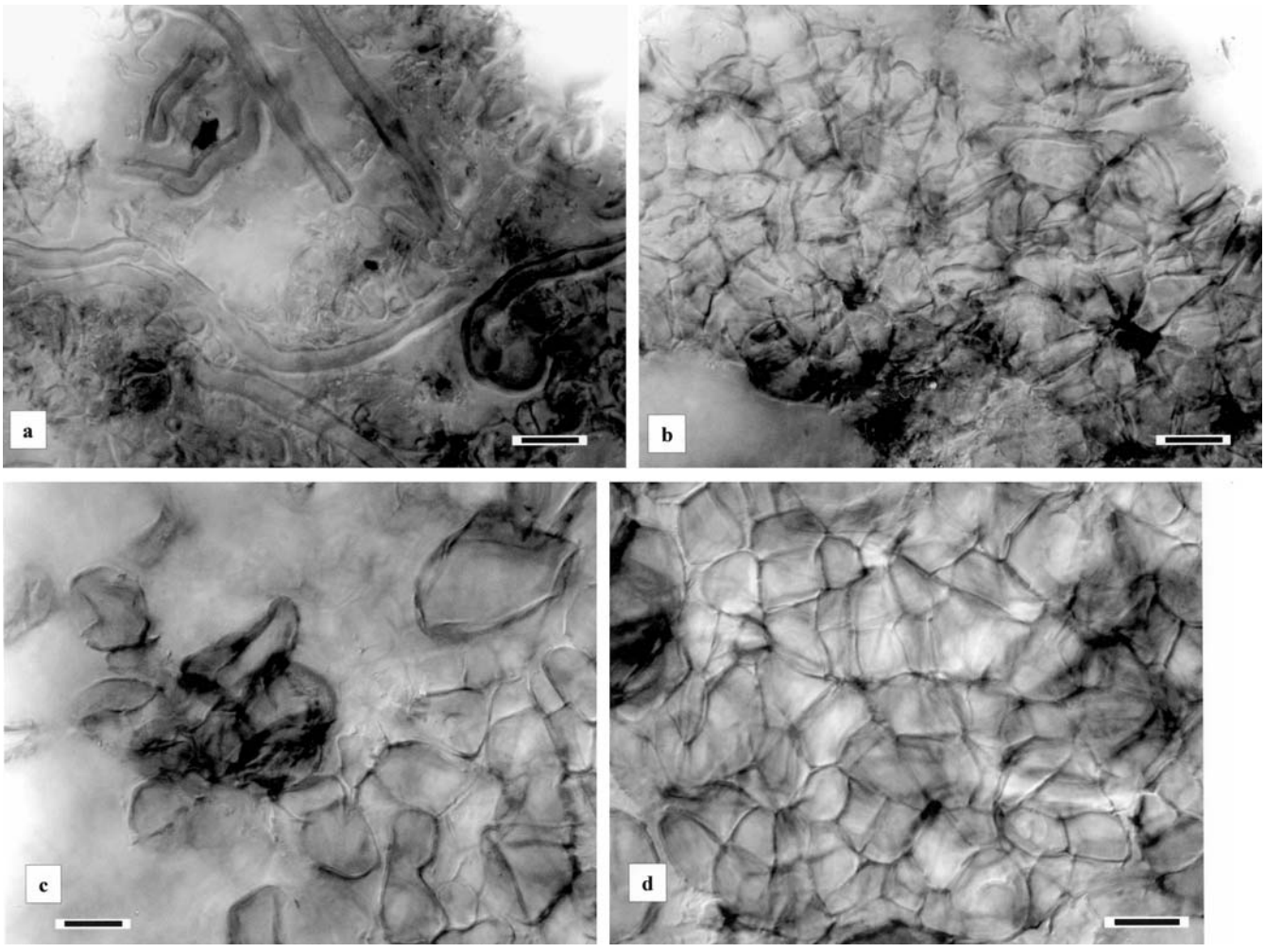

Fig. 8 a Mantle surface and emanating hyphae of At II. **b** Angular outer mantle layer of At II. **c** Group of globose cells on mantle surface of At I. **d** Outer mantle layer with angular cells of At I

Molecular identification

During the phylogenetic analysis of nrDNA-ITS sequences, including the 39 known database nrDNA-ITS sequences of *Tomentella* species obtained from the GenBank (Fig. 10), the ten ectomycorrhizal samples grouped together. Generally, the inter- and intra-specific distances vary in a wide, overlapping range.

The EM examined form two clades of one monophyletic group. One of these clades (represented by the BP 96971 and RA 12939 samples collected from different geographic regions and hosts) corresponds to At II. These EM cluster together with sequences originating from fruitbodies of *Tomentella stuposa*. Although there is some inhomogeneity within this clade, distances are within the range of the intraspecific differences, therefore At II can be regarded as identified as the EM of *T. stuposa*.

The clade containing BP 96972, BP 96973, BP 96974, BP 96978, BP 96979, BP 96980, BP 96981 and BP 96985 corresponds to At I. These anatomically similar samples, collected from the same region (Tompa) and host (*P. alba*), form a homogenous clade and may be a different but very close taxon to *T. stuposa*, perhaps a distinct species that can be identified later if fruitbody sequences become available. Based on the intraspecific distances seen in the other parts of the tree (like, e.g., *Tomentella badia*) it cannot be excluded that the samples of At I still belong to *T. stuposa*. In this case At I could represent a subspecific taxon of another host and geographic region (Fig. 10).

Discussion

EM are defined as 'tomentelloid' if they have melanized hyphae and are characterized by one or more of the following criteria: clamp connections, cystidia and a greenish-blue color reaction in KOH (Köljalg et al. 2000). Five of the six identified *Tomentella* EM described so far (except *T. brunneorufa*) have yellow, ochre or brown mantles, clamped hyphae and rhizomorphs. However, several blackish-brown unidentified EM with a diversity of character-patterns are also thought to be tomentelloid. The RA 12939 sample, identified in this work as *T. stuposa*, has also been mentioned previously as unidentified, named "*Piceirhiza nigri-*

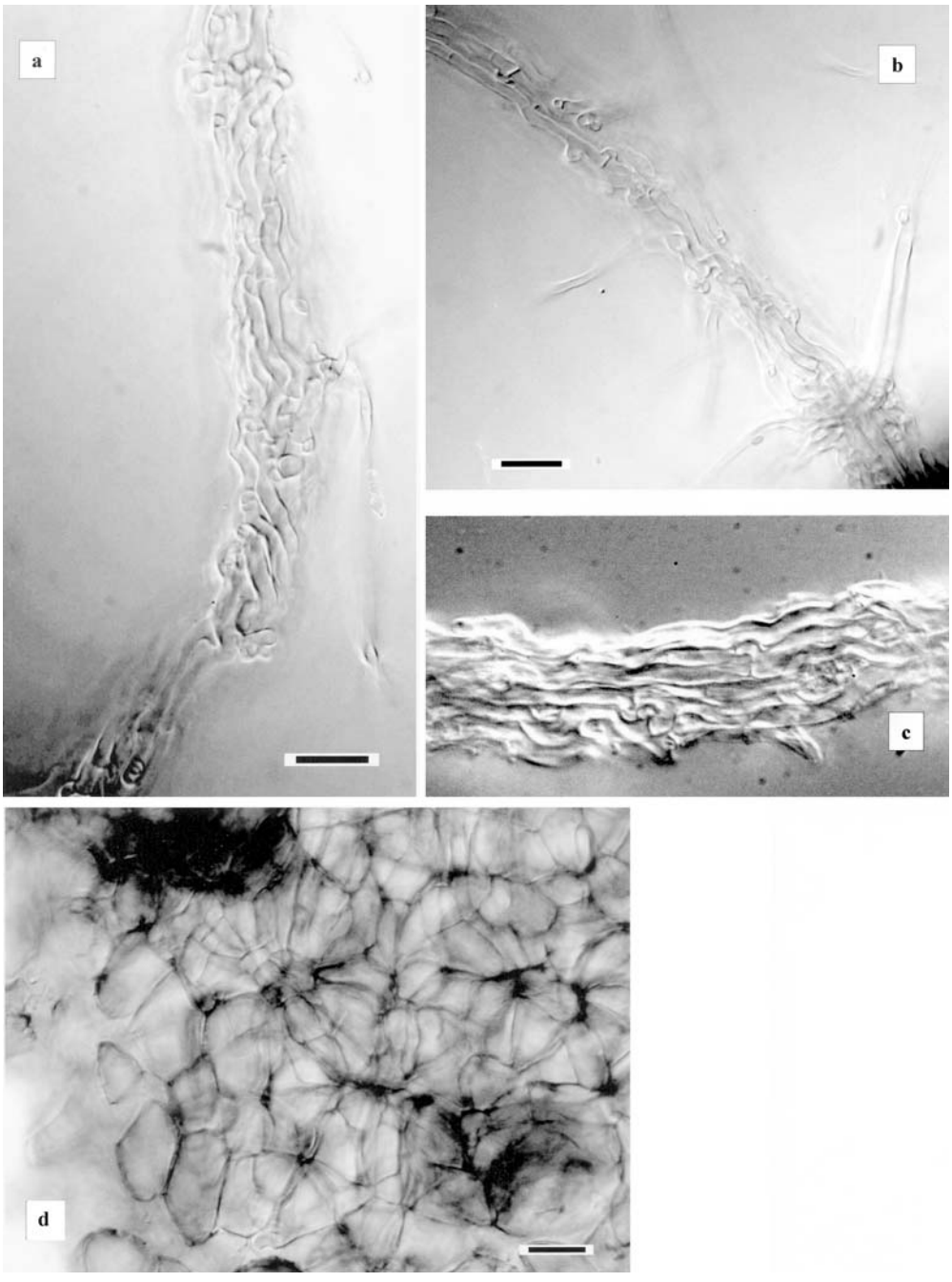

Fig. 9 a Rhizomorph of At I. **b** Ramifying, young rhizomorph of At I with nodia of At II. **c** Undifferentiated rhizomorph of At II. **d** Medial layer of mantle of At I with star-like organized angular cells

punctata". This name referred to the characteristic dark pigment dots of mantle (Agerer et al. 2002).

The EM of *T. stuposa* and its related anatomotype differ from all previously described blackish-brown tomentelloid morphotypes. "*Piceirhiza nigra*" was the first of these to be studied in detail (Gronbach 1988, Haug and Pritsch 1992). Agerer et al. (1995) concluded that, based upon its thelephoric acid content, "*Piceirhiza nigra*" belongs to the Thelephoraceae and its pseudoparenchymatous mantle structure places it in the genus *Tomentella*. Köljalg et al. (2000) mention that "*P. nigra* is very similar to...the mycobiont identified as *T. stuposa*." We confirm that this common, unidentified tomentelloid EM is closely related to *T. stuposa*. Although its angular mantle with globose cells on the surface and the brown, clamped hyphae are identical with those of *T. stuposa*, "*Piceirhiza nigra*" must be a different species because its inner mantle layer is not plectenchymatous and it has cystidia and warty hyphae. Unfortunately, molecular data for "*P. nigra*" are lacking. "*Quercirhiza cumulosa*" described by Román et al. (2002a) is also morphologically similar to *T. stuposa* due to its brown, angular mantle cells and globose surface cells, clamped hyphae and lack of cystidia, but it differs in the epidermoid structure of the inner mantle layers, its rhizomorphs with thick central hyphae (type C, Agerer 1991) and the presence of warty hyphae.

Several additional unidentified brown tomentelloid EM have been described but these differ far more from *T. stuposa*

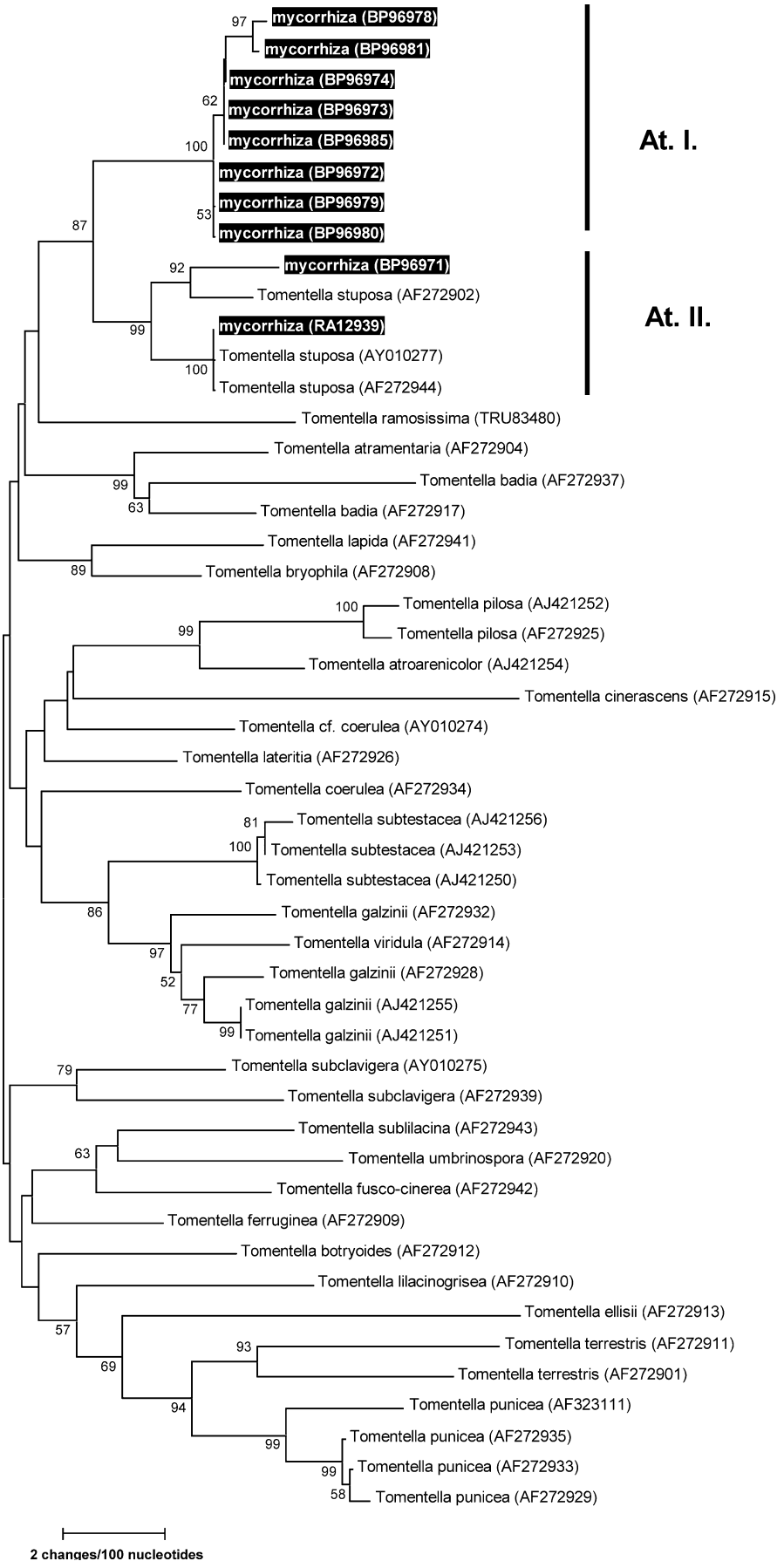

Fig. 10 The unrooted neighbor-joining tree of 49 *Tomentella* ITS sequences constructed using the program MEGA2.1. Kimura's two-parameter model was used with pairwise deletion at gaps in the analysis of the 514-character-long alignment. Bootstrap values are indicated as percentages and shown only above 50%

than the former two. "*Pinirhiza dimorpha*" (Golldack et al. 1999) differs in the lack of surface globose cells, rhiromorphs and clamped hyphae, and in the presence of bottle-shaped cystidia. "*Pinirhiza cyaneoviridis*" (Golldack et al. 1998) lacks rhizomorphs and clamped hyphae. "*Pinirhiza hyphocystidiata*" (Wöllecke et al. 1999) has roundish surface cells, but its mantle is plectenchymatous and it has unclamped hyphae. "*Quercirhiza nodulosomorpha*" (Azul et al. 1999) lacks globose cell groups, possesses cystidia and has differentiated (C-type) rhizomorphs.

Tomentelloids are among the most abundant members of EM communities in temperate forests. Brand et al. (1994) reported a 28% abundance of "*Piceirhiza nigra*" in limed spruce stands. According to Köljalg et al. (2000) "*Piceirhiza nigra*" forms about 20% of EM in limed spruce plots in South Sweden. Gardes and Bruns (1996) found *Tomentella sublilacina* to colonize ca. 15% of the EM root tips in a *Pinus muricata* stand in the United States. In drought-adapted *Populus alba* forests of the Hungarian Plain, blackish-brown tomentelloids were in a minority (<10%) or were a minority codominant (10–50%) component of EM associations (Jakucs 2002c). Köljalg et al. (2000) demonstrated six tomentelloid taxa in Swedish boreal forests that represent about 1–8% of the EM community. Besides *Pseudotomentella tristis*, they confirmed the presence of *T. sublilacina*, *T. bryophila*, *T. atramentaria*, *T. badia* and *T. stuposa* by ITS sequences. *Tomentella stuposa* was present in three out of the six sites examined. According to our present investigations, in some soil samples of Püspökladány, *T. stuposa* (At II) was present on more than 70% of the *Quercus cerris* roots. The average abundance of the closely related At I on *Populus alba* in Tompa and Kelebia was between 5–20% in a 4-year-long investigation (E. Jakucs, unpublished data).

The host relations within the *T. stuposa*-group are not yet clear. Although previous data refer mainly to coniferous hosts (Brand et al. 1994; Agerer et al. 1995; Gardes and Bruns 1996; Al Sayegh-Petkovsek and Kraigher 1999; Köljalg et al. 2000), investigations in broad-leaved forests of central and south Europe confirmed its affinity with angiosperm hosts, such as poplar (Jakucs 2002c) or oak (Román et al. 2002a).

In our present work, one EM sample of At II was collected from *Picea abies* in Germany, the other from *Quercus cerris* in Hungary (Fig. 10). These two samples (although both identified as *T. stuposa*) are segregated in the phylogenetic tree, which may be related to geographic distance or host specificity. The eight samples belonging to At I, forming a distinct, homogeneous clade, were collected from *Populus alba*. This pattern may be the consequence of separation of these anatomotypes by hosts. Similar geographic and host differences have been reported earlier, e.g., in the case of *Pisolithus* (Díez et al. 2001). The study of this host specificity with molecular-ecological markers is the subject of further work.

It can be concluded that *T. stuposa* and its relatives (like "*Piceirhiza nigra*") represent a significant proportion of EM communities in both temperate coniferous and broad-leaved forests. However, answering the question of specificity between the mycobionts and their host trees would seem to be essential to understand their function in forests.

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