Identification of *Picea*-ectomycorrhizae by comparing DNA-sequences

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Identification of the fungi forming ectomycorrhiza is still a great challenge. Ectomycorrhizae of *Picea abies*, collected in southwest Germany during several years and described as morphotypes, were identified using LSU and ITS sequences. To this the fungal sequences amplified from the mycorrhizae were compared with sequences from identified sporocarps. The fungal partner of *Piceirhiza gelatinosa* was identified as *Hygrophorus olivaceoalbus*, the fungal partner of *Piceirhiza rosa-nigrescens* was identified as *Dermocybe cf. semisanguinea*, and the fungal partner of a white mycorrhiza, described here for the first time, belongs to the *Hebeloma velutipes* group (*Hebeloma crustuliniforme* complex). Identification to genus level was possible for *Piceirhiza lanuginosa* where a *Cortinarius*-species is the fungal partner. A *Tomentella*-species forms a newly described light-brown mycorrhiza. Identification to family and to order-level was achieved for a milky-dull silvery mycorrhiza (Cortinariaceae), and *Piceirhiza globulifera* (Aphyllophorales), respectively. Ten samples of white, slightly bent mycorrhizae were formed by 8 different *Cortinarius*-species, including among others *Cortinarius traganus, C. delibutus*, and *C. brunneus*. The fungal partners of several brown, smooth mycorrhizae displaying only a Hartig net are Ascomycetes, among these are *Wilcoxina* cf. *mikolae* and *Hymenoscyphus* cf. *ericae*.

During recent years, several investigations of mycorrhizal communities have been published. All authors tried to gather information about the identity of the fungal partners. Only a small portion of ectomycorrhizal types could so far be identified on the basis of anatomical characters (FRANSSON et al. 2000: 68 morphotypes, 18 identified). Many researchers (JONSSON et al. 1999, DAHLBERG et al. 1999, ERLAND et al. 1999, PRITSCH et al. 1997, KERNAGHAN 2001) used the method of NYLUND et al. (1995), proving the identity of the fungal partners by matching the ITS-RFLP-patterns of mycorrhizas and fruitbodies. However, NYLUND et al. (1995) already noticed the disadvantage of unmatched RFLP-patterns which give no hint to the group of fungi the fungal partner belongs to. Additionally, closely related species are likely to coincide in their restriction patterns, making species differentiation impossible. The third disadvantage of this method is that intraspecific variation, like single-site-mutations, can lead to different RFLP patterns and mislead or prevent the recognition of a species. BRUNS et al. (1998) started to assemble a sequence database of Hymenomycetes (Basidiomycota) using a small region of the mitochondrial large subunit rRNA gene. The database has been expanded to 175 taxa (HORTON 2000). The most obvious omission of the database is the lack of Ascomycetes (BRUNS et al. 1998, TAYLOR & BRUNS 1999).

In the following study, DNA sequences of the D1/D2 region of the nuclear-encoded large subunit RNA gene (LSU) and of the ITS region were obtained from mycorrhizae and fruitbodies collected on the same plots. Additional sequences from other herbarium material and GenBank were included. Analyses of sequence data were used to identify the ectomycorrhiza forming fungi of known or newly described morphotypes.

Materials and methods

Sampling of mycorrhizae and sporocarps

Mycorrhizae were sampled at the ARINUS forest sites Villingen (V) and Schluchsee (S) in southwestern Germany and near Tübingen (Schönbuch). Data about the sites are presented in RASPE et al. (1998) and EINSELE (1986). Sporocarps of epigeous ectomycorrhizal fungi on the Villingen and Schluchsee plots were collected in October 1998. The investigated samples and supplementary herbarium material are listed in Tab. 1.

Morphological classification of ectomycorrhizae and description of new types

Samples were taken with a cylindrical steel corer of 4.5 cm diameter down to the mineral soil. Mycorrhizae were examined and classified into morphotypes on the basis of macroscopic and microscopic features (HAUG & PRITSCH 1992, AGERER 1986-1998). Colour pictures of all mentioned ectomycorrhizal

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Tab. 1. List of investigated samples

a) Ectomycorrhizae

Name	Origin§	LSU-region	ITS-region	Identification
Piceirhiza gelatinosa	S15	AF430274	AF430253	*Hygrophorus olivaceoalbus* (Fr. ex Fr.) Fr.
Piceirhiza rosa-nigrescens	V10	AF430281	AF430260	*Dermocybe* cf. *semisanguinea* (Fr.) Mos.
Piceirhiza lanuginosa	S12	AF430288	AF430288	*Cortinarius* sp.
Piceirhiza obscura	S68	AF430277	AF430259	Thelephoraceae
Piceirhiza echinata	S62	AF430276	AF430258	Aphyllophorales/Agaricales (?)
Piceirhiza globulifera	V50	AF430284	n.s.	Aphyllophorales
P.abies – H. velutipes group	V24	AF430291	AF430291	*Hebeloma crustuliniforme*-complex *Hebeloma incarnatulum* Smith
P.abies – H. velutipes group	S21	AF430275	AF430254	*Hebeloma crustuliniforme*-complex *Hebeloma velutipes* Bruchet
Piceirhiza cortinacearum	V15	AF430290	AF430290	*Cortinarius/Dermocybe*
Piceirhiza tomentellarum	S69	AF430289	AF430289	*Tomentella* sp.
Cortinarioid 1	S39	n.s.	AF430255	*Cortinarius/Dermocybe*
Cortinarioid 2	S48	n.s.	AF430256	*Cortinarius delibutus* Fr.
Cortinarioid 3	V54	n.s.	AF430264	*Cortinarius*, subgenus Telamonia
Cortinarioid 4	V34	n.s.	AF430262	*Cortinarius*, subgenus Telamonia
Cortinarioid 5	V14	AF430282	AF430261	*Cortinarius brunneus* Fr.
Cortinarioid 6	S54	n.s.	AF430257	*Cortinarius/Dermocybe*
Cortinarioid 7	V47	n.s.	AF430263	*Cortinarius*, subgenus Telamonia
Cortinarioid 8	V66	n.s.	AF430265	*Cortinarius*, subgenus Telamonia
Cortinarioid 9	V82	n.s.	AF430266	*Cortinarius traganus* Fr.
Cortinarioid10	V30	AF430292	AF430292	*Cortinarius brunneus* Fr.
Brown 1	V73	AF430286	n.s.	*Wilcoxina* cf. *mikolae*
Brown 2	V37	AF430283	n.s.	*Wilcoxina* cf. *mikolae*
Brown 3	V70	AF430285	n.s.	*Wilcoxina* cf. *mikolae*
Brown 4	T7/2	AF430278	n.s.	*Hymenoscyphus* sp.

b) Fruitbodies of fungi

Name	Collection-No.*	Collection-site #	LSU-region	ITS-region
Cortinarius brunneus Fr.	IH-P17	V1	AF430287	AF430287
Cortinarius traganus Fr.	IH-P16	V1	AF430268	AF430251
Hebeloma velutipes Bruchet	IH-P10	V1	AF430267	AF430250
Hygrophorus agathosmus(Fr. ex Secr.) Fr.	IH-P5	V1	AF430269	n.s.
Hygrophorus leucophaeus (Scop. ex Fr.) Fr.	US97/159	GöW	AF430280	n.s.
Hygrophorus eburneus (Bull. ex Fr.) Fr.	US97/138	GöW	AF430279	n.s.
Hygrophorus capreolarius Kalchbr.	KR6905	H	AF430272	n.s.
Hygrophorus discoideus (Pers. ex Fr.) Fr.	KR6931	L	AF430273	n.s.
Hygrophorus olivaceoalbus (Fr. ex Fr.) Fr.	KR6419	BWs	AF430271	AF430252
Hygrophorus pustulatus (Pers. ex Fr.) Fr.	KR4892	T	AF430270	n.s.

n.s.: = not sequenced; no attempt was made to sequence this region
AF: = GenBank Accession-No.
§ Origin: V = Villingen, S = Schluchsee, T = Schönbuch
* Collection-Numbers refer to sporocarp material in the Herbarium of I.Haug (IH-P), K.-H. Rexer Marburg (KR) or Ursula Sittig Göttingen (US)
\# Collection sites: V1= Villingen, GöW= Göttinger Wald, H= Heiligenbronn bei Horb, L= Loßburg, BWs= Bad Waldsee, T= Schönbuch bei Tübingen

types can be viewed at the following site on the World Wide Web: http://www.mycological-progress.com.

Unidentified types and other types of interest were fixed in 2% glutaraldehyde (cacodylate buffer, pH 7.2) for light microscopy. Semithin longitudinal sections were stained with neofuchsin-crystal violet. For DNA analysis 2 to 5 tips of the same system were frozen in Eppendorf-cups and stored at –20 °C.

Several little systems (2-5 tips) of white, slightly bent mycorrhizae with varying numbers of rhizomorphs and emanating hyphae were frozen, because they looked similar, but not identical. The same was done with sheathless, brown mycorrhizae, where a morphological differentiation was not possible.

DNA extraction, PCR, and sequencing

DNA was isolated according to the manufacturer's instructions from frozen or dried sporocarp material (lamellae), and frozen mycorrhizal samples using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany). Part of the LSU was amplified by the polymerase chain reaction (PCR) with the primerpairs NL1-NL4 or NL1-LR6 (O'Donnell 1993). The internal transcribed spacer (ITS) within the ribosomal RNA genes was amplified using the primers ITS1 or ITS1F and ITS4 or ITS4B (White et al. 1990, Gardes & Bruns 1993).

PCR reaction volume was 50μl, with concentrations of 1.5 mM $MgCl_2$, 200 μM of each dNTP (Life Technologies, Eggenstein, Germany), 0.5 μM of each of the primers (MWG-Biotech, Ebersberg, Germany), 1U Taq-polymerase (Life Technologies, Eggenstein, Germany), 10% amplification buffer (Life Technologies, Eggenstein, Germany), and an empirically determined dilution of the DNA extract. Good results were achieved at diluting the DNA-extract (200μl) 1:25 or 1:100. PCR conditions were chosen as follows: a touch-down profile with annealing temperatures between 60 and 50 °C for the LSU and between 55 and 45 °C for the ITS. After initial denaturation at 94 °C for 3 min, 10 cycles were run with variable annealing temperatures ranging from 60 °C (LSU) respectively 55 °C (ITS) in the first cycle to 51 °C (LSU) respectively 46 °C (ITS), in each cycle decreasing by 1°C, followed by 25 (LSU) respectively 35 (ITS) cycles with a constant annealing temperature of 50 °C (LSU) respectively 45 °C (ITS). Each of the cycles consisted of an annealing step of 0.5 min, an elongation step of 72 °C for 1 min, and a denaturation step of 94 °C for 0.5 min. The PCR was finished with a final elongation phase at 72 °C for 7 min, after which the samples were stored at 4 °C. Controls without template were run for every PCR reaction. The PCR products obtained were purified using the QIAquick protocol (Qiagen, Hilden, Germany) or with Nucleospin (Macherey-Nagel). Direct sequencing of PCR products was performed using the PCR primers as sequencing primers. Cycle sequencing was conducted using the ABI PRISM Dye-Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California) followed by electrophoresis on an automated sequencer (ABI 373A Stretch, Applied Biosystems, Foster City, California). Sequencing was carried out according to the protocols supplied by the manufacturer, but reducing the cycle sequencing reaction volumes by half. Both strands of DNA were sequenced. Sequence editing was performed using Sequence Navigator (version 1.0, Applied Biosystems, Foster City, California).

Molecular identification of ectomycorrhizal types

The mycorrhizal LSU-sequences were assigned to taxonomic groups by constructing sequence alignments with sequence data of M. Weiß (Weiß et al. 1998, Weiß & Oberwinkler 2001, partially unpublished; Department of Systematic Botany and Mycology of the University of Tuebingen). For this approach we used the MegAlign module of the Lasergene software package (DNASTAR, Inc.). This program performs multiple sequence alignments according to the ClustalW algorithm (Thompson, Higgins & Gibson, 1994) and constructs phylogenetic hypotheses using the neighbor-joining distance method (Saitou & Nei, 1987). The placement of the mycorrhizal sequences was analysed. Putatively allied species (sporocarps, same genus/family) sampled on the same plot or from other collections (Tab.1b) were sequenced and directly compared with the mycorrhizal sequences. BLAST searches (Altschul et al., 1990) with LSU- and ITS-sequences were performed on the GenBank database. For species identification the ITS-region was chosen, since interspecific variability of this region was shown to be fairly high compared with its intraspecific variation (Eberhardt et al. 1999, Gardes et al. 1991, Henrion et al. 1994, Karén et al. 1997, Pritsch et al. 1997).

DNA sequences determined for this study were deposited in GenBank, and accession numbers are given in Tab. 1.

Results

Short descriptions of new ectomycorrhizae

Picea abies – Hebeloma velutipes group, *Hebeloma crustuliniforme* complex

Photographs and Figures in Colour-Atlas of Ectomycorrhizae (Agerer 1987-1998, in press)

Macroscopic characteristics:
Colour: white, or with white patches of hyphae over translucent orange-brown to brown cortical cells;
Surface: smooth to fine fibrous, covered in patches with densely interwoven and emanating hyphae or rhizomorphs;
Ramification: monopodial-pinnate, extensive systems;
Emanating hyphae and rhizomorphs:frequently present, white, hyphal fans often connect several root tips, rhizomorphs with many emanating hyphae.

Microscopic characteristics:
Emanating hyphae: thin-walled, with clamps, with verrucose incrustations (note: incrustation is lost after fixation and embedding!), hyphal diameter: 3–4 μm;
Rhizomorphs: up to 150 μm in diameter, consist of a bundle of tightly interwoven, undifferentiated hyphae, hyphae are thin-walled, with or without incrustations, with clamp connections, 3–5 μm broad;
Hyphal mantle: outer layer consisting of a loose prosenchymatous web, hyphal clamps are present; inner layer densely prosenchymatous;
Thickness of hyphal mantle: 5–40 μm thick and irregularly layered.

***Piceirhiza cortinacearum* AF430290**

Macroscopic characteristics:
Colour: milky dull, hyphal mantle transparent with silvery glossy patches;
Surface: woolly;
Ramification: monopodial-pinnate, extensive systems, completely enveloped in mycelium;
Emanating hyphae: mycorrhizae and long roots densely enveloped in milky dull hyphae;
Rhizomorphs: not observed.

Microscopic characteristics:
Emanating hyphae: septate with clamps, smooth walls, hyphal diameter 4,5–5 μm (Fig. 1a, 2a);
Hyphal mantle: outer layer consisting of a loose prosenchyma, hyphae septate with or without clamps, hyphal diameter 5–6 μm (Fig. 1b,c, Fig. 2b); followed by a irregular to puzzle-like synenchyma with matrix material, hyphae septate without clamps (Fig. 1d, Fig. 2c);
Thickness of hyphal mantle: 15–30 μm.

***Piceirhiza tomentellarum* AF430289**

Macroscopic characteristics:
Colour: light-brown, at the tips nearly colourless with dark-brown spots, at the bases dark-brown;
Surface: smooth to grainy, glossy;
Ramification: monopodial-pinnate, unramified ends straight and short;
Emanating hyphae: surrounding the mycorrhizae, hyaline to light-brown;
Rhizomorphs: not observed.

Microscopic characteristics:
Emanating hyphae: septate with clamps, with smooth walls, straight, diameter 3 μm (Fig. 3b);
Hyphal mantle: outer layer consisting of a net prosenchyma, hyphae septate with clamps or without clamps (Fig. 3a); followed by a polygon-synenchyma (Fig. 3c,d, Fig. 4a,b); change to a compact prosenchyma near the cortical tissue (Fig. 4c);
Thickness of hyphal mantle: 30–35 μm.

Identification of ectomycorrhizal morphotypes using LSU- and ITS-sequences

Six *Piceirhiza*-ectomycorrhizae, three newly described mycorrhizal types, and two groups of morphologically similar types (white, with rhizomorphs, slightly bent; brown, glossy, nearly no hyphal sheath but Hartig-net) were analysed (Tab. 1a).

The LSU sequence of the *Piceirhiza gelatinosa*-mycorrhizae was clearly placed in the genus *Hygrophorus* (data not shown). Additional sequencing of 7 *Hygrophorus* species (Tab. 1b) produced a match: 622/622bp of the LSU and 564/564 bp of the ITS of *Hygrophorus olivaceoalbus* and *Piceirhiza gelatinosa* were identical. Thus, it can be concluded that the fungal partner of *Piceirhiza gelatinosa* is *Hygrophorus olivaceoalbus*.

The LSU-sequence of *Piceirhiza rosa-nigrescens* (Haug & Pritsch 1992) was placed in the genus *Cortinarius*, subgenus *Dermocybe* (data not shown). Based on the ITS-sequence the BLAST-procedure revealed great similarity with several vouchers of *Dermocybe semisanguinea*. Three American strains (U56067, U56066 and U56063) showed 627 of 630 bp identical. A Norwegian strain (AJ236061) of *Dermocybe semisanguinea* revealed 609 of 618 bp identical. Thus, it can be assumed that the fungal partner of *Piceirhiza rosa-nigrescens* is *Dermocybe* cf. *semisanguinea*.

The LSU-sequence of *Piceirhiza lanuginosa* (Haug & Pritsch 1992) was placed in the genus *Cortinarius* (data not shown) and the ITS-sequence was related to the ITS-sequence of *Cortinarius obtusus* AJ238035 (identities : 632/642 = 98%).

The fungal partner of *Piceirhiza obscura* (Gronbach 1988) belongs to the Thelephoraceae (LSU-tree, data not shown). Neither with the LSU- nor with the ITS-sequence identification to genus level was possible. The best agreement in the BLAST results was achieved with the ITS-sequence U83469 (identities : 611/659 = 92%), a member of the Thelephoraceae, amplified from orchid mycorrhizae (Taylor & Bruns 1997).

The LSU-sequence of *Piceirhiza echinata* (Haug & Pritsch 1992) clustered in the LSU-tree (data not shown) near *Lentaria sp*., but the identities were not very high (576/639 = 90%). A BLAST-search with the LSU had the following results: 92% identities (529/574) with *Heterobasidion annosum* AF287866, 91% identities (529/580) with *Oligoporus rennyi* AF287876, 91% identities (519/566) with *Hygrocybe citrinopallida* U66435, 89% identities (549/615) with *Lentaria albovinacea* AJ406552. Based on the ITS-sequence the BLAST-procedure revealed no great similarity to an available sequence. The best alignment was achieved with *Tylospora fibrillosa* AF052560: base 19-119 (ITS1-region), identities: 89/105 (84%); base 145-396 (5,8S rRNA, ITS2-region), identities : 247/253 (97%).

The fungal partner of *Piceirhiza globulifera* belongs to the Aphyllophorales: the LSU-sequence of *P. globulifera* clustered near *Clavulina cinerea* (data not shown) and shows similarities with *Clavulina cinerea* AJ406433 (identities:

670/706 = 94%), *Sistotremastrum niveocremeum* AJ406429 (identities: 659/701 = 94%), *Sistotrema brinkmannii* AJ406430 (identities: 652/692 = 94%) and *Multiclavula mucida* AF287875 (identities: 678/725 = 93%).

The *Hebeloma velutipes* group mycorrhizae were very common on the limed ARINUS-plots. Their LSU sequence was placed in the genus *Hebeloma* (data not shown). The identical sequence (542bp) was found in the fungus P10 (Tab. 1b) belonging to the *Hebeloma crustuliniforme* complex (VESTERHOLD 1995, AANEN et al. 2000), and U11918 *Hebeloma crustuliniforme* has a very similar sequence (541/542 identities). The ITS-sequences of the carpophore (P10) and two mycorrhizal samples (S21, V24) were obtained to get more information on the classification of these specimens within the *H. crustuliniforme* complex. The ITS-data clearly show that all three sequences belong to the *H. velutipes* group (AANEN et al. 2000): The carpophore sequence shows polymorphisms at 12 positions, with the BLAST procedure the *H. velutipes* sequences are most similar (AF124677 identities: 562/576 = 97%; AF124686 identities 563/580 = 97%; these sequences are monomorphic, the polymorphic sequences are not in the GenBank). The mycorrhizal samples S21 and V24 show unambiguous non-polymorphic ITS-sequences: The sequence of V24 is nearly identical with *H. incarnatulum* AF124684 (identities: 610/611), the sequence of S21 is identical with *H. velutipes* AF124679 (identities: 607/607).

The LSU-sequence of *Piceirhiza cortinacearum* (V15) was placed in the Cortinariaceae (data not shown). The ITS-sequence did not match any known sequence. The best BLAST results were a similarity of 90% (586/647) with *Protoglossum sp*. AF325561 and of 89% (605/677) with *Dermocybe olivaceopicta* U56050. Therefore, this newly described woolly type with puzzle-like synenchyma was given the name *Piceirhiza cortinacearum* AF 430290.

The fungal partner of *Piceirhiza tomentellarum* (S69) belongs to the Thelephoraceae (LSU-tree, data not shown). The ITS-sequence of *Piceirhiza tomentellarum* AF430289 showed 98 % identities (548/559) with *Tomentella coerulea* AF272934. Therefore, the fungal partner of this mycorrhiza is a *Tomentella* species.

To get an impression of the species diversity within the white types in a *Picea*-stand, the ITS-region of ten samples (little systems with 2-5 tips) of white, slightly bent mycorrhizae with more or less abundant rhizomorphs and prosenchymatous sheaths (Cortinarioid 1-10,Tab. 1a) were sequenced and compared. All the sequences were placed in the genus *Cortinarius*/*Dermocybe*. Two sequences were nearly identical (V14/V30: 523/528bp identical), two samples (V34,V47) were identical (551bp). Four sequences matched to identified *Cortinarius* sequences: V14 and V30 with *Cortinarius brunneus* (V14/P17: identities 523/528 = 99%, V30/P17: identities 684/686 = 99,7%), S48 with *Cortinarius delibutus* (U56025: identities 652/656 = 99%), and V82 with *C. traganus* (541/541bp identical). Three further sequences (V54, V66, V34=V47) were included in the subgenus Telamonia (based on ITS-alignment, GARNICA, in prep., Department of Systematic Botany and Mycology of the University of Tuebingen). The sample S54 could not be identified to species level, because the similarity with known sequences (BLAST: *Dermocybe austrovenata* AF112147 328/377 = 87%) was too small.

Four samples of brown smooth fine roots (Brown 4-7, Tab. 1b) with no distinct hyphal sheath but Hartig-net were investigated for their fungal partners. Because of the missing hyphal sheath characteristics, there are nearly no possibilities for a morphological differentiation of the involved fungi. All sequenced fungi belonged to the Ascomycetes. The LSU-sequence of sample T7/2 showed great similarities to the sequence of *Hymenoscyphus ericae* AF284122 (identities: 483/492 = 98%). The other three were similar and the LSU-sequences were placed in the genus *Wilcoxina* with 94 to 96% similarities to AF127119 (*W. mikolae*) respectively AF156926 (*Wilcoxina sp*. RPC10).

Discussion

The mycorrhizae of *Piceirhiza gelatinosa* were first described by GRONBACH & AGERER 1986. Photographs are included in the Colour-Atlas of Ectomycorrhizae (AGERER 1987-1998, plate 30) and in the microscopical atlas of ectomycorrhizal types (HAUG & PRITSCH 1992). The hyphal mantle and the pileipellis of *Hygrophorus olivaceoalbus* both have hyphae that are embedded in a gelatinous substance, but the characteristic labyrinthic arrangement of the hyphae in the hyphal sheath can not be recognized in the drawings of the pileipellis given by BAS et al. (1990, p.129, Fig. 113). DAHLBERG et al. (1997) identified mycorrhizae by ITS-RFLPs using a sporocarp based reference database including *Hygrophorus olivaceoalbus*. The authors identified the mycorrhizae of *H. olivaceoalbus*, but because of coarse morphotyping, they could only say that the mycorrhizae are white and cottony, as are the mycorrhizae of *Cortinarius, Hebeloma*, and seven other unidentified RFLP-taxa. The results confirm that the mycorrhizae of *Hygrophorus olivaceoalbus* are white, however, they are never cottony but have a smooth, waxy surface. During the 5-year-investigation of mycorrhizal types on the ARINUS-sites (HAUG et al. 1992), several strikingly long or fingerlike branchings of *Piceirhiza-gelatinosa*-mycorrhizae were found (HAUG 1989). KAREN et al. (1997) found intraspecific restriction site polymorphisms for *H.olivaceoalbus*. The length of the ITS-region of three vouchers from Sweden, Finland and Norway appeared to be the same (600 bp), but all three vouchers were polymorphic for HinfI. The HinfI-RFLP-pattern calculated from the ITS-sequence of KR6419 (~300, 197, 87, 8) is again different from the nordic ones. Thus, an identification of the *P. gelatinosa*- mycorrhizae with the RFLP-method would not have been possible. This example shows that RFLP´s are limited in their broad scale utility, but are useful at a local scale.

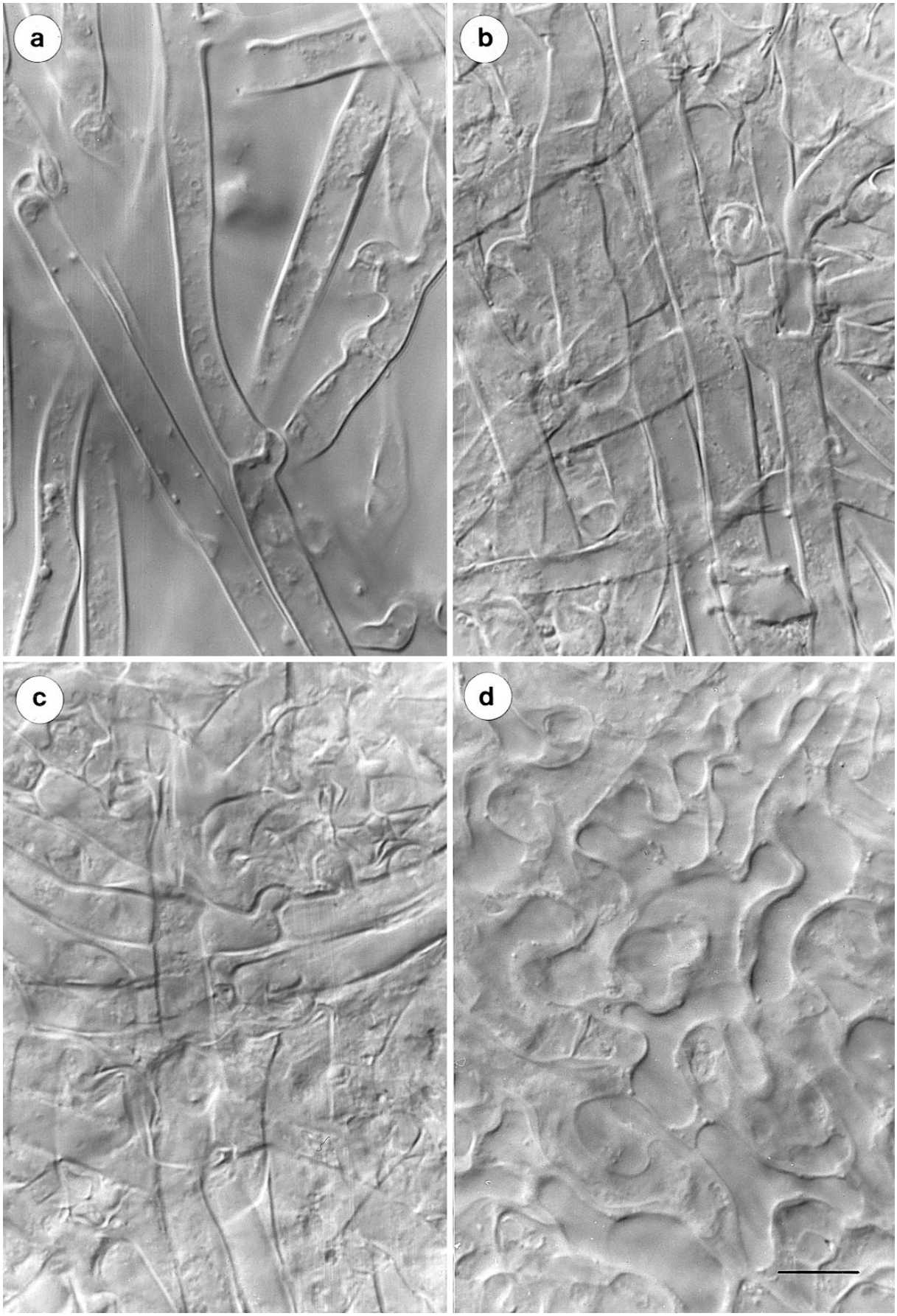

Fig. 1. *Piceirhiza cortinacearum* AF430290 – Photographs of hyphal mantle preparations with Normarsky interference microscopy; a. emanating hyphae, b., c. surface view of mantle consisting of a loose prosenchyma, d. inner surface of mantle: irregular to puzzle-like synenchyma (scale 10 μm)

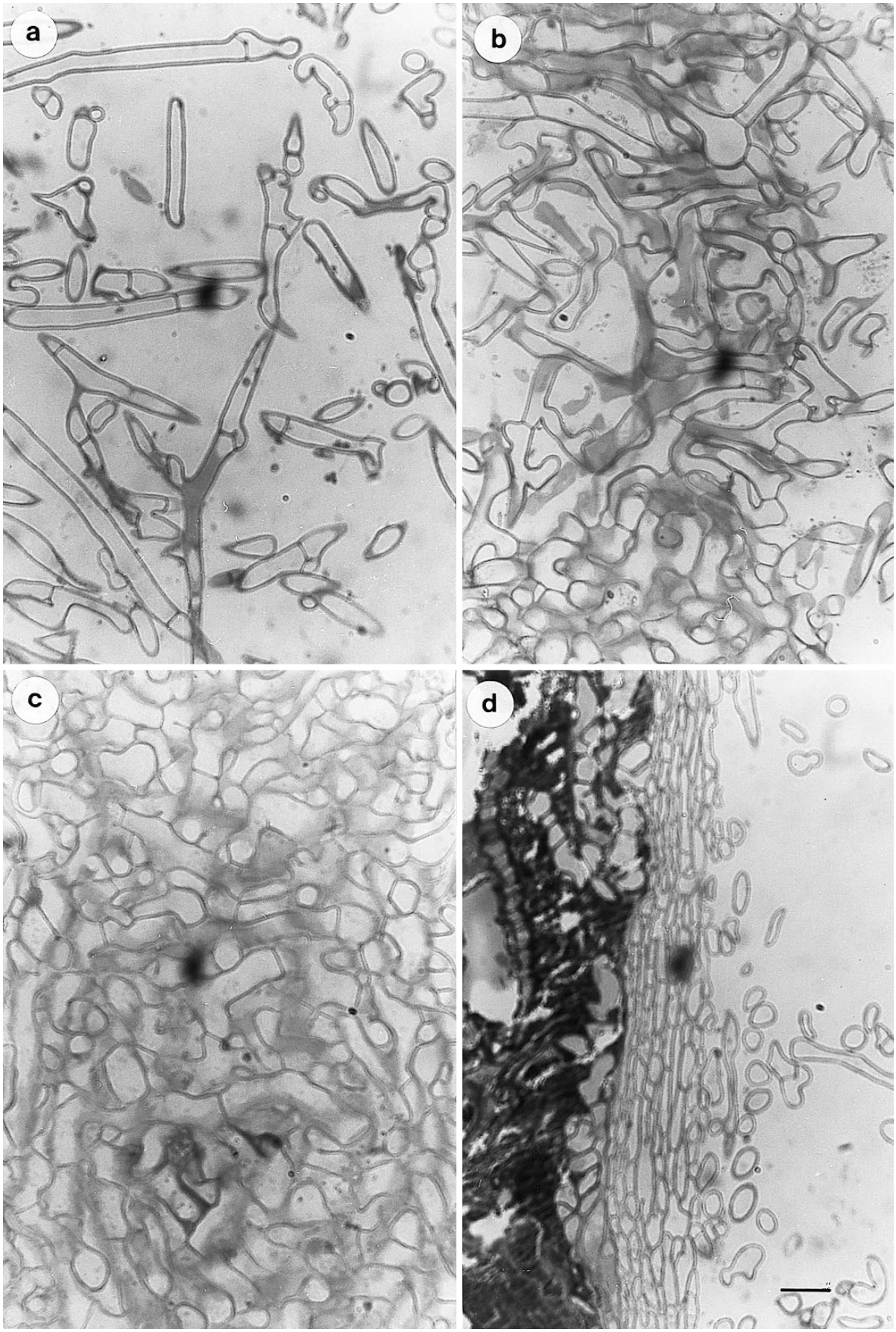

Fig. 2. *Piceirhiza cortinacearum* AF430290 – Photographs of longitudinal sections; a. emanating hyphae, b. outer hyphal mantle: loose prosenchyma, c. inner hyphal mantle: irregular to puzzle-symenchyma, d. median section through mycorrhiza: hyphal mantle and cortical cells with Hartig-net (scale 10 μm)

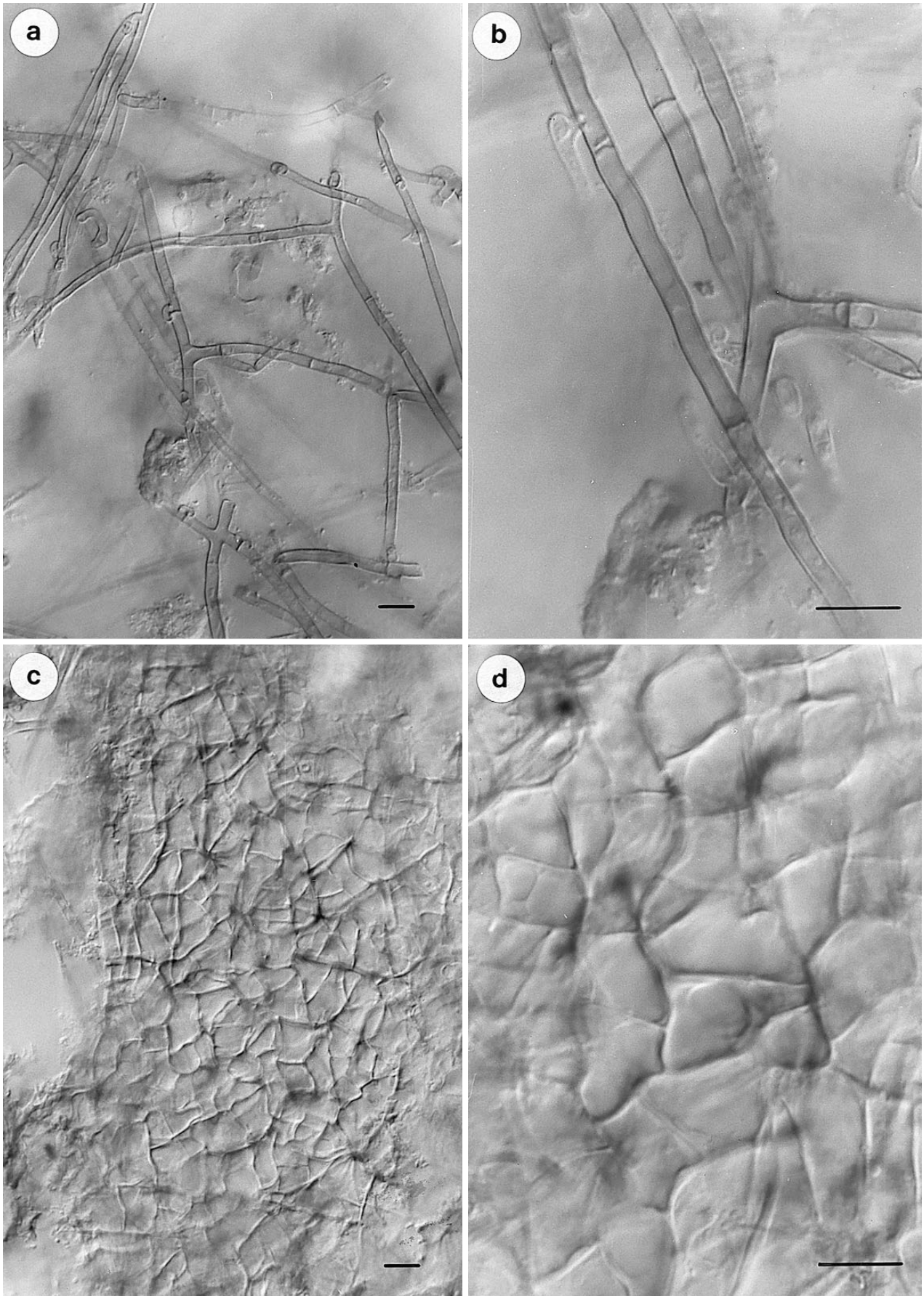

Fig. 3. *Picea abies – Tomentella sp*. AF430289 – Photographs of hyphal mantle preparations with Normarsky interference microscopy; a. surface view of mantle: net-prosenchyma, b. emanating hyphae, c., d. middle layer of mantle: polygon synenchyma (scale 10 μm)

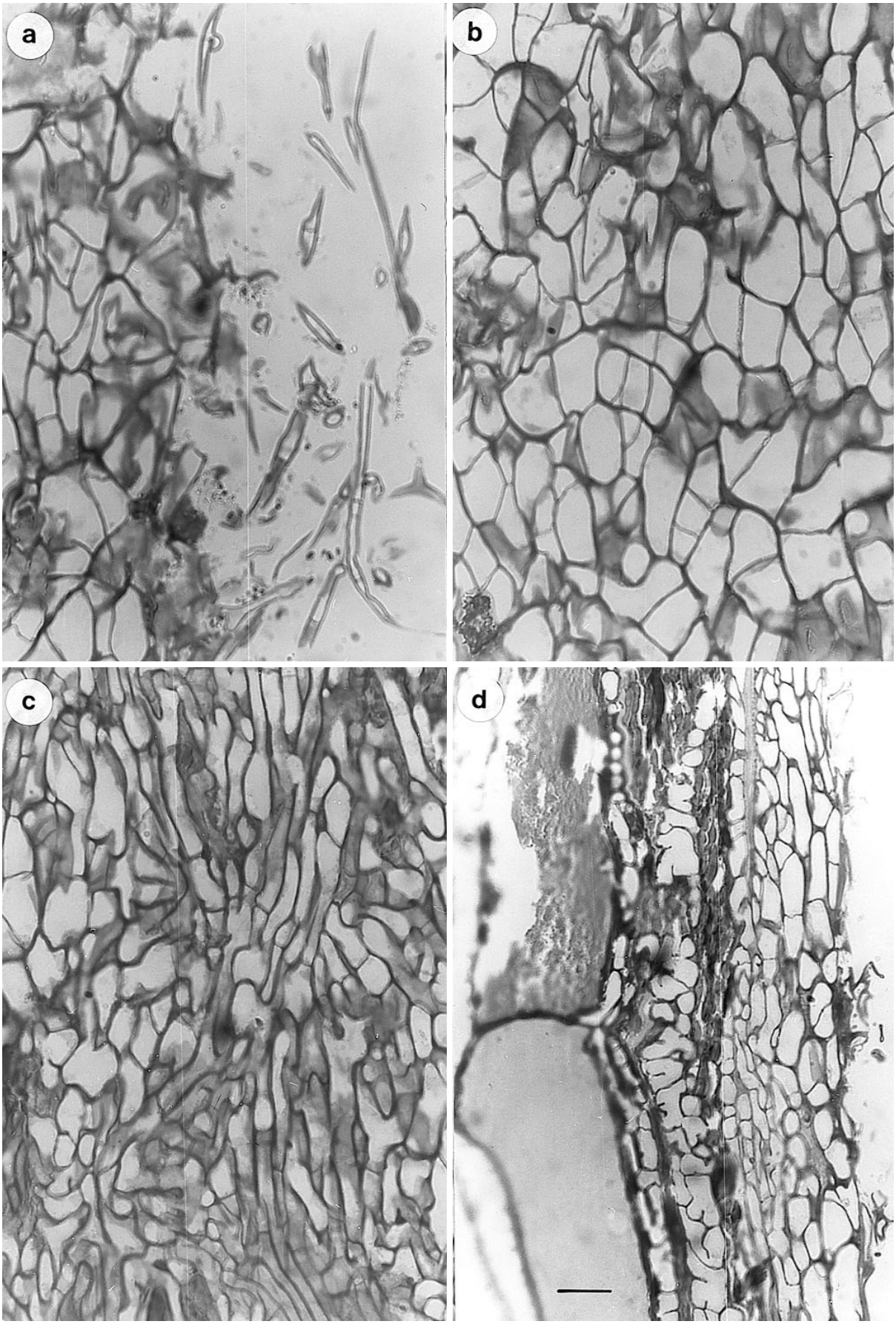

Fig. 4. *Picea abies – Tomentella sp*. AF430289 – Photographs of longitudinal sections; a. emanating hyphae and polygon-synenchyma, b. middle layer of mantle: polygon-synenchyma, c. inner hyphal mantle: compact prosenchyma, d. median section through mycorrhiza: hyphal mantle and cortical cells with Hartig-net (scale 10 μm)

It is unclear why the European mycorrhizal sequence of *Piceirhiza rosa-nigrescens* shows more similarities to the American *Dermocybe semisanguinea* collections (U56067, U56066, U56063) as to the European collection (AJ236061). Normally a greater genetic similarity will be supposed to the European material. No basidiocarp voucher from the mycorrhizal plot was available. Thus, it remains a rest of uncertainity: *Dermocybe* cf. *semisanguinea*. The mycorrhizae of *Dermocybe semisanguinea* were described by ZAK (1971) from *Pseudotsuga menziesii* and by UHL (1988) from *Pinus sylvestris*. The morphological and anatomical characteristics of these two descriptions and of *Piceirhiza rosa-nigrescens* are in agreement. UHL (1988) described the mycorrhizae as yellow to yellow-white and did not mention a reaction with KOH, ZAK (1971) and HAUG & PRITSCH (1992) noticed that KOH applied to the hyphal sheath and emanating hyphae produced a distinctive colour reaction from rose-red to deep purple respectively bluish-black. The description of ZAK (1971) is very short, specifications to the colour and the structure of the hyphal sheath and the rhizomorphs are missing. Therefore HAUG & PRITSCH (1992) described the rose-red mycorrhizae as *Piceirhiza rosa-nigrescens*. Whether the fungal partner of the yellow-white mycorrhizae with no KOH-reaction of UHL (1988) is the same as for *P. rosa-nigrescens* can not be checked because there is no material for sequencing.

In the species-rich genus *Cortinarius* close species show only small but constant differences in the ITS-region (GARNICA, pers. comm.). The difference of 10 bp between the ITS-sequence of *P. lanuginosa* and *Cortinarius obtusus* make a classification uncertain, because this is within the overlap between inter- and intraspecific variation in the genus *Cortinarius* (GARNICA, pers. comm.). Therefore the mycorrhizae of *Piceirhiza lanuginosa* are not recognized to be formed by *Cortinarius obtusus* but by a near kindred species. Morpho-anatomically, the mycorrhizae of *Cortinarius obtusus* are described as white with rhizomorphs (AGERER 1987-1998, plate12) and the *Piceirhiza lanuginosa*-mycorrhizae are milky dull without rhizomorphs.

The membership of the fungal partner of *Piceirhiza echinata* remains unclear. According to the results of the BLAST-search it belongs to the Aphyllophorales or Agaricales. The ITS-BLAST-results contain no great information because the main part of the alignment is in the 5,8SrRNA-region. Thus, the fungal partner of *Piceirhiza echinata* belongs to a fungal group where at the moment no sequences are available in the GenBank.

The morphological and anatomical characteristics of *Piceirhiza globulifera* fit the description of *Fagirhiza globulifera* (BRAND 1991) and *Pinirhiza globulifera* (MLECZKO 1998). The most distinctive feature of these mycorrhizae is the presence of capitate cystidia which grow perpendicularly to the mantle surface. No identified ectomycorrhiza with the features of *P.globulifera* has been published so far. BRAND (1991) supposed the fungal partner in genera possessing this kind of cystidia: *Grandinia* (Corticiaceae), *Tomentella* (Thelephoraceae), *Galerina* (Cortinariaceae). The sequence data analysis exclude the Thelephoraceae and Cortinariaceae but hint towards an aphyllophoraceous fungus. The similarities (94 %) to *Clavulina, Sistotrema, Sistotremastrum* and *Multiclavula* are noteworthy. None of the species are identified as mycorrhizal partners up to now. It is very unlikely that *Grandinia granulosa* (syn. *Hyphodontia aspera* AJ406458) is the fungal partner of *P.globulifera*, because there are great differences between the two sequences (V50/AJ406458: 421/499, 84 %).

The placement of the ITS-sequences of the white mycorrhizae in the *H. velutipes* group is in good agreement with the results of AANEN et al. (2000), who found that this group (clade I) is dominated by species growing with non-Salicaceae-hosts. AANEN et al. (2001) observed sequence polymorphism in the ITS of sporocarps of *H. velutipes* from different provenances. The same applies for sporocarp P10. The morpho-anatomical characteristics (cylindrico-clavate, non-capitate cheilocystidia, dextrinoid spores) confirm the classification with *H.velutipes* (AANEN et al. 2001, KUYPER, Department of Environmental Sciences, Wageningen, The Netherlands, pers. comm.). No morpho-anatomical differences were observed with the description of *Picea abies - Hebeloma crustuliniforme* ectomycorrhizae (BRUNNER et al. 1991). It might be that a molecular investigation will reveal that the fungal partner of these mycorrhizae also belongs to the *Hebeloma velutipes* group. KUYPER (Department of Environmental Sciences, Wageningen, The Netherlands; pers. comm.) believes, that most of the ectomycorrhizal work where *H. crustuliniforme* was implicated, was in fact done with *H. velutipes*, which grows almost with conifers (*Pinus, Picea, Pseudotsuga*) in contrast to *H. crustuliniforme* which prefers *Populus, Salix* or *Tilia*.

The mycorrhizae of *Piceirhiza cortinacearum* are similar to the *Piceirhiza lanuginosa*-mycorrhizae, but they show the following morpho-anatomical differences: silvery patches on the sheath surface, puzzle synenchyma instead of compact prosenchyma as inner hyphal mantle layer.

It must be assumed that the white, slightly bent mycorrhizae with more or less abundant rhizomorphs and prosenchymatous sheaths belong to the Cortinariaceae (AGERER 1995), especially to the genera *Cortinarius* and *Dermocybe*. A great number of species have to be considered as likely candidates. The molecular analyses showed that 8 species were encountered in the10 samples. Analysing more samples will certainly reveal even further species. Up to now, in community investigations, only two *Cortinarius* species were identified by KAREN & NYLUND (1997), MAHMOOD et al. (1999), JONSSON et al. (1999b), HORTON & BRUNS (1998). JONSSON et al. (1999a) identified at least 8 species. In the study of DAHLBERG et al. (1997) at least 4 species were encountered. The investigations of DAHLBERG et al. (1997) and JONSSON et al. (1999a) show that it is impossible to seperate all *Cortinarius* species by RFLP-patterns by using only three enzymes. The result are *Cortinarius*-groups (*Cortinarius* species with

the same RFLP-patterns) which include up to 14 species. Therefore, correct knowledge on the number of the *Cortinarius*-species is not possible by the RFLP-method. The number of *Cortinarius*-species is usually underestimated owing to morphological and sequence similarity.

The identification of an ectomycorrhizal fungal partner of the *H. ericae*-aggregate supports the results of VRALSTAD et al. (2000), who amplified the ITS1 region of *Piceirhiza bicolorata* samples which shared approx. 95% ITS1 sequence identity with the ericoid mycorrhizal fungus *Hymenoscyphus ericae*. *Wilcoxina* is a well-known partner of ectomycorrhizae especially from disturbed stands (BAAR et al. 1999) or in pot-cultures (TAYLOR & BRUNS 1999).

Conclusion

For identification and re-identification of ectomycorrhizae detailed anatomical descriptions combined with sequence analysis are used in this study. The number of mycorrhizae identified to species level is still limited because of lacking fungal reference, but the comparison of DNA-sequences is a promising method for identifying ectomycorrhizal fungal partners.

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