Mycorrhiza (2000) 10:87–93

ORIGINAL PAPER

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Identification and differentiation of mycorrhizal isolates of black alder by sequence analysis of the ITS region

Accepted: 25 May 2000

Abstract Twenty isolates of black alder ectomycorrhizas were characterized on the basis of internal transcribed spacer (ITS) DNA sequences and colony morphology in pure culture. The isolates were obtained from individual, surface-sterilized mycorrhizas morphologically identified as the mycorrhizal type "*Alnirhiza cystidiobrunnea*". Analysis of ITS sequences allowed differentiation into four groups; three were closely related, while one isolate (BEh-Uw1) was separated by high sequence dissimilarity within the ITS1 and ITS2 spacer regions. Culture morphology was not a satisfactory differentiating feature for these four groups. An Ncbi GenBank DNA database search revealed that isolates within the three closely related ITS groups displayed high homology to ITS sequences of *Tomentella sublilacina* and *Thelephora terrestris*, whereas BEh-Uw1 had the highest sequence similarity to an ITS DNA sequence of a basidiomycete DNA isolated from bamboo leaves.

Key words ITS sequence · Ectomycorrhizal isolates · "*Alnirhiza cystidiobrunnea*" · *Tomentella sublilacina* · *Thelephora terrestris*

Introduction

Although identification of many field-sampled ectomycorrhizas (EM) has been achieved, a considerable number of mycorrhizal types described on the basis of morphological and anatomical features remain unidentified (Agerer 1998). In the framework of the German program "Ecosystem research in the Bornhöved Lakes district" (Northern Germany), 16 different EM morphotypes of black alder were characterized by morphological-anatomical studies and PCR/RFLP patterns of the rDNA internal transcribed spacer (ITS) region (Pritsch et al. 1997a,b). During these studies, eight morphotypes were identified by corresponding fruit body RFLP patterns, while eight isolates remained unidentified. Of these eight unidentified morphotypes, "*Alnirhiza cystidiobrunnea*" (Pritsch et al. 1997b) was one of the EM most regularly occurring at all investigated plots, with an estimated proportion of 3–10% per year of all mycorrhizal types at the two main experimental locations at Lake Belau (Pritsch 1996). This morphotype showed some color variation with mostly light to dark brown but also with almost grayish and whitish forms. As a precondition for physiological studies, isolation of the fungal partner into pure culture was successfully performed from mycorrhizal roots of "*Alnirhiza cystidiobrunnea*". Colony morphology varied among different isolates, again indicating some genetic variability within this morphotype. Because of the varying culture morphology and color of collected mycorrhizal tips, a detailed investigation was started of whether the isolates of "*Alnirhiza cystidiobrunnea*" represent a single species or a species group.

The ITS region of the ribosomal DNA was used as a target sequence because the variability of this region is suited for phylogenetic differentiation at the species and intraspecies level (Bruns et al. 1991). Although the number of published ITS sequences is still restricted, a range of sequence data is available from EM fungi. In this paper, a comparison of sequences from mycorrhizal isolates with the databases is presented.

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Materials and methods

Sampling sites

Samples of mycorrhizal roots were taken from an alder forest at Lake Belau (30 km south of Kiel, Schleswig-Holstein, Germany)

and a second stand approximately 300 km away at Lammer Holz (Braunschweig, Lower Saxony, Germany). The alder stand at Lake Belau with a size of ca. 1 ha is situated on the western shore. Black alder [*Alnus glutinosa* (L.) Gaertn.] is the dominant tree species (ca. 60 years old) growing intermixed with birch (*Betula pubescens* Ehrh.) and, in elevated parts at the margin of the stand, with oak (*Quercus robur* L.) and beech (*Fagus sylvatica* L.). Considering the lake water table, the site may be divided into a generally wet and temporarily waterlogged part adjacent to the lake (Lake Belau lakeside, BEl) and a somewhat dryer, elevated part 0.5–1 m above the lake water level (Lake Belau hillside, BEh). The distance between the sampling sites BEh and BEl was approximately 30 m.

The 2.5-ha stand at Lammer Holz (LH) is a drained alder forest with a mix of other tree species forming an *Alno-Fraxinetum*. Samples at this stand were taken from locations within the forest where black alders dominated. A more detailed description of these locations is given by Dilly and Munch (1996) and Pritsch et al. (1997a).

Sampling and processing of root samples

Samples of black alder roots were collected from the litter layer and kept at 4 °C during transport and storage. All samples from each location were pooled and cleaned by gentle shaking in tap water within 24 h. Intact and turgescent-looking mycorrhizas with a red-tipped root apex and mantle features characteristic of fully active specimens of the morphotype "*Alnirhiza cystidiobrunnea*" (Pritsch et al. 1997b) were sorted under a stereo microscope and freed of all visible adherent soil particles. The color of mycorrhizas was recorded during superficial morphological examination.

Isolation of fungal strains

Isolation from mycorrhizal tips followed a protocol modified from Erland and Söderström (1990). Ten mycorrhizal tips were transferred to a nylon 60-μm-mesh sachet and kept humid between wet filter papers soaked with a 0.2% solution of Tween 80 until sterilization in 30 ml 0.05% glutaraldehyde (v/v) in 0.05 M Na-cacodylate buffer (pH 7) under continuous shaking for 3 min. After rinsing three times in 300 ml of sterile distilled water, mycorrhizal tips were cut into pieces of approximately 4 mm length. The pieces of one mycorrhizal tip were plated together onto one petri dish containing medium *b* (Kottke et al. 1987). To inhibit bacterial growth, 300 mg l^{-1} chlorotetracycline and 300 mg l^{-1} streptomycin sulfate were added to medium *b* after autoclaving.

Outgrowing mycelia were checked microscopically for the presence of hyphae with clamps and, if clamps were present, routinely cultivated on medium *b* without antibiotics. Inoculation of isolates to sterile black alder seedlings according to the method of Wong and Fortin (1989) revealed Hartig net formation for all isolates of the present study (data not shown). Isolates are maintained in the culture collection of the Institute of Soil Ecology, GSF-National Center for Environment and Health, Neuherberg, FRG under the names given in Table 1.

PCR and sequence analysis

Fungal material for DNA extraction was obtained from the surface of growing colonies. DNA extraction and the PCR reaction followed the protocol described by Henrion et al. (1994). Cultivation, DNA extraction and PCR amplification of each isolate were carried out in two replicates. Conditions for PCR were as described by Pritsch et al. (1997a). The primers ITS1 and ITS4 (White et al. 1990) were used for PCR and sequencing. ITS-PCR products were sequenced for the forward and reverse directions in two replicates by Eurogentec (Seraing, Belgium) with an ABI prism 377 automated sequencer, Version 2.1.1. (Perkin Elmer Applied Biosystems). Sequence chromatograms were corrected by hand for errors in the automated nucleotide detection. The corrected forward and reverse DNA sequences of each isolate were aligned and the resulting consensus DNA sequences were further analyzed. The 5′ and 3′ positions of the ITS1, 5.8 S, ITS2 regions and the 5′ start of the large subunit ribosomal DNA (Table 1) were set as in the sequence data published by Taylor and Bruns (1997).

A sequence homology search in the GenBank Nucleotide database of the National Center for Biotechnology Information (NCBI), Bethesda (http://www.ncbi.nlm.nih.gov/) was performed using the program FastA 3.0 (Pearson and Lipman 1988). Sequences found during this search and which were included in this study for the purpose of sequence comparison to our data are listed in Table 1. In the case of sequence U65607 from GenBank (Zhang et al. 1997), where the authors defined the span of the ITS region slightly differently from Taylor and Bruns (1997), spans and lengths given in Table 1 were adapted for comparison on the basis of the Taylor and Bruns data.

Phylogenetic analysis

Two multiple alignments were created using ClustAl contained in ClustalW (Thompson et al. 1994). Gaps at the 5′ and at the 3′end were excluded and gaps within alignments were treated as missing data. Multiple alignments subsequently were corrected manually on a screen using LineUP. All programs are contained in the program package for HUSAR version 3.0 DKFZ Heidelberg, Germany (http://www.dkfz-heidelberg.de).

Alignment 1 was created from all 19 alignable sequences of the mycorrhizal isolates of this study and related sequences from the ncbi database as indicated in Table 1. Alignment 1 was analyzed using distance-based methods under the Jukes-Cantor model and phylogenetic trees were calculated based on these distant matrices by the neighbor-joining method (Saitou and Nei 1987). To ascertain relationships found by distance calculations, a 1000 replicate bootstrap analysis with the neighbor-joining method was performed with the program ClusTree, which is part of ClustalW (Thompson et al. 1994) contained in the package for HUSAR version 3.0.

During sequence analyses, certain groups of isolates displayed minimal sequence deviation attributable to ambiguous bases and thus revealed almost complete sequence identity over the whole alignable span. From these groups, with identical sequences, one representative sequence was submitted to the NCBI GenBank database. Accession numbers are given in Table 1.

Based on the results of these first analyses, a second alignment (alignment 2, Table 1) was created including one representative sequence as described above. The reduction of the number of sequences was a precondition to enable parsimony analysis. The phylogenetic relationships of sequences in alignment 2 (Table 1) were analyzed using maximum parsimony-based calculations without or with bootstrapping analyses from 1000 replicate samples. Parsimony-based analyses were performed under the branch and bound option using PAUP Portable version 4.0.0d55 for Unix contained in the program package of HUSAR version 3.0. Resulting trees displaying phylogenetic relationships were either edited by the graphic mode contained in the respective programs or redrawn using the program Treeview (Page 1996) (http://taxonomy.zoology.gla.ac.uk/rod/treeview/).

Results

Isolates of black alder mycobionts

A total of 20 isolates was obtained from surface-sterilized mycorrhizal roots (Table 2). Seventeen isolates originating from light to dark brown mycorrhizas of the morphotype "*Alnirhiza cystidiobrunnea*" showed two

Table 1 Internal transcribed spacer (ITS) sequences of isolates considered for sequence comparison and phylogenetic studies. References for sequences included in the present study are (1) present study, (2) Taylor and Bruns 1997, (3) Zhang et al. 1997 (*n.i.* not included into multiple alignment)

Accession number to GenBank	ITS group in present study	Name of isolate/ fungus (reference)	Sequence length (bp)	ITS1 span	ITS1 length (bp)	5.8S span	5.8S length (bp)	ITS2 span	ITS2 length (bp)	25S span	Span of multiple alignment 1	Span of multiple alignment 2
AF104992		BEh-Uw1 (1)	567	<1–186	part.	187–343	157	344–552	209	>553	n.i.	n.i.
U65607		Basidiomyte from bamboo (3)	716	35–252	218	253–409	157	410–617	208	>618	n.i.	n.i.
	1	BEh-Aw1 (1)	450	<1–103	part.	104–261	158	262–450	part.	–	1–450	n.i.
AF104987	1	BEh-Aw2 (1)	596	<1–197	part.	198–355	158	356–572	217	>573	22–572	11–572
	2	BEl-lb1 (1)	588	<1–189	part.	190–347	158	348–564	217	>565	14–564	n.i.
	2	BEl-lb2 (1)	577	<1–187	part.	188–345	158	346–562	217	>563	12–562	n.i.
	2	BEl-lb3 (1)	577	<1–187	part.	188–345	158	346–562	217	>563	12–562	n.i.
	2	BEl-lb4 (1)	577	<1–187	part.	188–345	158	346–562	217	>563	12–562	n.i.
	2	BEl-lb5 (1)	582	<1–193	part.	194–351	158	352–568	217	>569	18–568	n.i.
	2	BEl-lb6 (1)	596	<1–197	part.	198–355	158	356–572	217	>573	22–572	n.i.
	2	BEl-lb7 (1)	586	<1–187	part.	188–345	158	346–562	217	>563	12–562	n.i.
AF104989	2	BEl-lb8 (1)	593	<1–194	part.	195–352	158	353–569	217	>570	19–569	8–569
	3	BEl-db1 (1)	579	<1–188	part.	189–346	158	347–564	218	>565	13–564	n.i.
	3	BEl-db2 (1)	561	<1–176	part.	177–334	158	335–552	218	>553	1–552	n.i.
	3	BEl-db3 (1)	570	<1–184	part.	185–342	158	343–560	218	>561	9–560	n.i.
	3	BEl-db4 (1)	582	<1–193	part.	194–351	158	352–569	218	>570	18–569	n.i.
	3	BEl-db5 (1)	570	<1–185	part.	186–343	158	344–561	218	>562	10–561	n.i.
AF104988	3	BEl-db6 (1)	586	<1–195	part.	196–353	158	354–571	218	>572	20–571	9–571
	3	LH-db1 (1)	568	<1–178	part.	179–336	158	337–554	218	>555	3–554	n.i.
AF104990	3	LH-db2 (1)	578	<1–187	part.	188–345	158	346–563	218	>564	12–563	1–563
	3	LH-lb1 (1)	569	<1–184	part.	185–342	158	343–560	218	>561	9–560	n.i.
U83481		*Tomentella sublilacina* (2)	683	58–256	199	257–414	158	415–632	218	>633	81–632	70–632
U83486		*Thelephora terrestris* (2)	685	58–256	199	257–414	158	415–631	217	>632	81–631	70–631
U83483		*Tomentella cinerascens* (2)	685	58–256	199	257–414	158	415–632	218	>633	82–632	71–632
U83487		*Thelephora americana* (2)	691	58–262	205	263–419	157	420–637	218	>638	82–637	71–637
U83484		*Thelephora penicillata* (2)	687	58–261	204	262–419	158	420–636	217	>637	82–636	71–636
U83480		*Tomentella ramosissima* (2)	687	58–255	198	256–413	158	414–634	221	>635	82–634	71–634
U83485		*Thelephora regularis* (2)	685	58–261	204	262–419	158	420–631	212	>632	82–631	71–631

Table 2 Provenience and morphological characteristics of isolates obtained from the ectomycorrhizas

Isolates denomination	No. of isolates	Provenience	ITS group in present study	Color of mycelium	Hyphal growth	Air mycelium	Pigmentation of medium *b*
BEh-Uw1	1	Lake Belau, hillside	Not grouped	Whitish, grayish	Regular	Sparse, floccose	None
BEh-Aw	2	Lake Belau, hillside	1	Whitish, grayish	Regular	Sparse, floccose	None
BEl-lb	8	Lake Belau, lakeside	2	Light brown	Regular	Sparse, floccose	Light brown
BEl-db	6	Lake Belau, lakeside	3	Dark brown	Regular to fan-like	Sparse, appressed	Dark brown
LH-db	2	Lammer Holz	3	Dark brown	Regular to fan-like	Sparse, appressed	Dark brown
LH-lb	1	Lammer Holz	3	Light brown	Regular to fan-like	Sparse, appressed	Light brown

different types of culture morphology with light or dark brown mycelium and light or dark brown pigmentation of solid medium *b* (Table 2). The three isolates obtained from whitish mycorrhizas had whitish mycelium and did not excrete pigments into the culture medium. Morphology of fungal colonies was either regular with floccose air mycelium (light brown and whitish isolates) or regular to fan-like with appressed air mycelium (dark brown isolates and LH-isolates). As a common feature, all isolates grew very slowly, produced scarce aerial mycelium and grew mainly by submersed mycelium within the agar medium.

Analysis of partial sequences of the rDNA ITS

Sequences of 19 out of 20 isolates could be properly aligned in the two alignments used for sequence comparison and phylogenetic analysis (Table 1). The sequence of the whitish isolate BEh-Uw1 had to be excluded from the multiple alignment because of high sequence dissimilarities within the ITS1 and ITS2 spacer regions.

Phylogenetic analysis as displayed by the neighbor-joining based tree in Fig. 1A revealed that the 19 alignable sequences belong to three distinct and closely related ITS groups. Group 1 is formed by the two whitish isolates BEh-Aw, group 2 comprises the eight light brown isolates from Lake Belau lakeside (BEl-lb) and group 3 includes the six dark brown isolates from Lake Belau lakeside (BEl-db) and the three isolates from the geographically distant stand Lammer Holz (LH-lb/db).

Sequence similarity within the groups is very high across the whole ITS span. All sequences of group 1 (BEh-Aw), group 2 (BEl-lb) and LH of group 3 are identical over their alignable span. Sequences of the BEl-db isolates within group 3 are also highly similar, except for a few ambiguous nucleotides in some sequences. BEl-db sequences differ by only two bases (ambiguities excluded) within the ITS 2 region from the LH isolates. The distinction of three ITS groups is supported by the high bootstrap values shown in the neighbor-joining based tree in Fig. 1A, where within group 3, BEL-db isolates and the geographically distant LH isolates are displayed in the same cluster.

A FastA search in GenBank based on sequences representative of ITS groups 1, 2 and 3 revealed *Tomentella sublilacina* (U83481) and *Thelephora terrestris* (U83486) to be the next closest relatives. Additional sequences within the Thelephoraceae published by Taylor and Bruns (1997) were found suitable as outgroup sequences i.e. the sequences of *Tomentella cinerascens* (U83483), *Thelephora americana* (U83487), *Thelephora penicillata* (U83484), *Tomentella ramosissima* (U83480) and *Thelephora regularis* (U83485) (Table 1). Phylogenetic relationships between isolates in the present study and with *Thelephora terrestris* and *Tomentella sublilacina* were further analyzed by distance and maximum parsimony-based methods. Distances over the whole aligned ITS length were below 6% among *Tomentella sublilacina*, *Thelephora terrestris* and group 1, 2 and 3 isolates (Table 3). All resulting trees displayed the five outgroup sequences in positions similar to those shown in Fig. 1B, confirming the differentiation of the three ITS groups in the present study, but differing in the positions of *Thelephora terrestris* and *Tomentella sublilacina* relative to the three ITS group sequences (data not shown). The single 50% majority-rule consensus parsimony tree shown in Fig. 1B displays ITS groups 1, 2 and 3 together with *Tomentella sublilacina* and *Thelephora terrestris* as a polytomous group. Within this group, clearly defined clades of group 1 and 2, as well as group 3 are indicated by high bootstrap values but relationships to *Tomentella sublilacina* and *Thelephora terrestris* are not further resolved. This uncertainty of the relationships between these closely related sequences can be explained by the different similarities among sequences found within the two spacer regions (Table 3). The ITS 1 region of all isolates of group 1, 2 and 3 is more similar to *Tomentella sublilacina* than to *Thelephora terrestris*. Distances in the ITS2 spacer, in contrast, place group 1 and 2 closer to *Thelephora terrestris* than to *Tomentella sublilacina*, while LH isolates of group 3 display slightly higher similarity to *Tomentella sublilacina* than to *Thelephora terrestris*, and BEl-db isolates have almost identical dis-

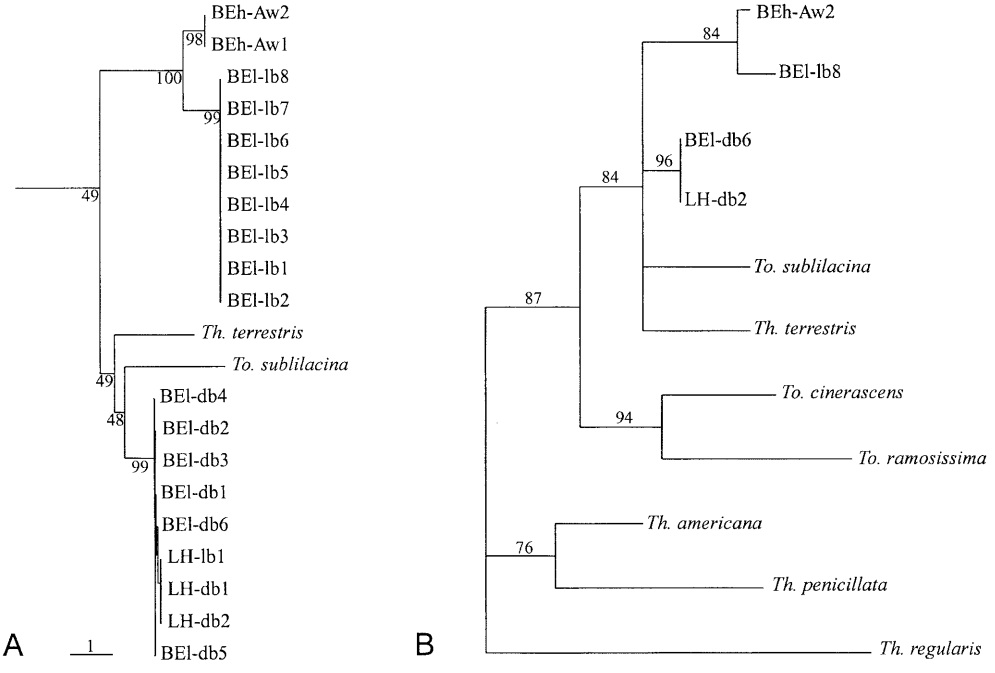

Fig. 1A,B Relationships between isolates of the present study and members of Thelephoraceae published by Taylor and Bruns (1997) (Table 1). **A** Neighbor-Joining tree constructed from a 1000 replicate bootstrap analysis and based on alignment 1 (Table 1). **B** 50% majority rule parsimony tree from a 1000-replicate bootstrap analysis under the branch and bound option based on alignment 2 (Table 1). Branch lengths (horizontal dimension only) are proportional to phylogenetic distances and are given in substitutions per 100 bases (scale bar valid for both trees). The length of vertical lines has no meaning and was adjusted for ease in labeling termini (*Th. Thelephora*, *To. Tomentella*)

Table 3 Jukes-Cantor distances within the ITS region covered by the spans of alignment 1 (Table 1) between sequences of BEh-Aw (ITS group1), BEl-lb (ITS group 2), BEl-db and the geographically distant isolates (LH) (the latter two in ITS group 3), *Thelephora terrestris* (U83486) and *Tomentella sublilcina* (U83481). Ambiguities were considered for distance calculations. Part 1: Distances within the ITS region (above diagonal) and within the 5.8S rDNA region (below diagonal). Part 2: Distances within the partial ITS1 region (above diagonal) and within the ITS2 region (below diagonal) (*To. sub. Tomentella sublilcina, Th. ter. Thelephora terrestris*)

ITS

5.8S	BEh-Aw	BEl-lb	BEl-db	LH	To. sub.	Th. ter.
BEh-Aw		1.5	3.9–4.2	3.9	5.9	5.6
BEl-lb	0.0		4.6–4.9	4.7	6.0	5.8
BEl-db	0.6	0.6		0.3–0.4	3.9–4.1	4.2–4.3
LH	0.6	0.6	0.0		4.1	4.1
To. sub.	0.6	0.6	0.0	0.0		5.8
Th. ter.	0.6	0.6	0.0	0.0	0.0	

ITS1

ITS2	BEh-Aw	BEl-lb	BEl-db	LH	To. sub.	Th. ter.
BEh-Aw		2.3	5.9	5.9	8.4	10.2
BEl-lb	1.9		5.9	5.9	7.8	8.3
BEl-db	4.6–5.4	6.6–7.4		0.0	5.9	7.7
LH	4.8	6.8	0.7–1.0		5.9	7.7
To. sub.	7.8	8.8	5.1–5.8	5.7		10.9
Th. ter.	5.8	7.8	4.5–4.9	4.3	6.2	

tances to *Thelephora terrestris* and to *Tomentella sublilacina* (Table 3).

Group 1 and 2 isolates share the same single base substitution within the 5.8S gene differentiating them from *Tomentella sublilacina*, *Thelephora terrestris* and group 3 isolates, which have identical sequences throughout the 5.8S region (Table 3). The sequence of the whitish isolate BEh-Uw1 showed 98% similarity in the 5.8S rDNA sequence to sequences of the ITS groups 1, 2 and 3 isolates, but exhibited high sequence dissimilarities within the ITS1 and ITS2 spacer regions and a sequence deviation of 40% over the whole ITS sequence length. A FastA search found 87.8% identity in a 452-bp overlap of BEh-Uw1 to the ITS sequence of an unidentified basidiomycete (U65607), the DNA of which was isolated from the phylloplane of bamboo leaves (Zhang et al. 1997) (Table 1). Additional database searches with the sequence of BEh-Uw1, which were kindly performed in private databases by F. Martin (INRA Nancy, France), K. Egger (UNBC, Canada) and M. Weiss (University of Tübingen, Germany) did not yield further homologous fungal ITS sequences.

Discussion

Differentiation by morphological features

Isolates obtained from mycorrhizal roots of *Alnirhiza cystidiobrunnea* first characterized by their culture morphology, were further analyzed for ITS sequence polymorphism. Colony morphology of the mycorrhizal isolates was not satisfactory for differentiation of all isolates. The difficulties in differentiating fungal isolates by morphological criteria in this study are well known for vegetative mycelia of many mycorrhizal fungi (Zak and Marx 1964) and have been demonstrated clearly by complementary studies on RFLP patterns and culture morphology types for *Cenococcum* (LoBuglio et al. 1991).

The same difficulty exists with mycorrhizal tips, although slight color variation of the tips may be recognizable with experience (Horton and Bruns 1998). However, a shift from lighter to darker brown may also be observed from younger to older parts of single tips of this type and, therefore, color remains a feature very difficult to define unambiguously. As shown by the results of sequence comparisons discussed below, *"Alnirhiza cystidiobrunnea"* may not comprise a single species but more likely circumscribes at least two closely related but distinct clades.

Analysis of a partial sequence within the rDNA ITS

The high homology within the 5.8S rDNA of all fungal isolates of the present study is consistent with the known high conservation of this gene (Hershkovitz and Lewis 1996). The ITS1 and ITS2 spacer regions could both be properly aligned and displayed variable regions appropriate for establishing relationships on a deeper taxonomic level among the investigated strains, as also found for the ITS region in other studies (Hershkovitz and Lewis 1996; Martin et al. 1998). In contrast to ITS sequences of *Armillaria* (Chillali et al. 1998), the ITS2

region of our isolates showed no higher variability than the ITS1 region. Comparison of ITS1 and ITS2 regions of our three sequence groups with *Tomentella sublilacina* and *Thelephora terrestris* revealed that both spacer regions varied in different ways, leading to different clusters when analyzed separately. Data from other fungal taxa also showed that variation is not located in either the ITS1 or the ITS2 spacer but is rather dependent on the fungal taxa (Waalwijk et al. 1996). In addition to the ITS region, other DNA regions of phylogenetic diagnostic value for phylogenetic analysis should be analyzed, especially when results are ambiguous.

ITS sequence analysis placed our ITS groups 1, 2 and 3 to *Tomentella sublilacina* and *Thelephora terrestris* but without a clear attribution to either species. Taylor and Bruns (1997) published a phylogenetic tree with *Thelephora terrestris* on the same branch as *Tomentella sublilacina* and a general phylogenetic position closer to *Tomentella* spp. than to other *Thelephora* spp.. A tree based on a 350-bp region of the mitochondrial large subunit rDNA also displays *Thelephora terrestris* in the same clade as *Tomentella sublilacina* (Gardes and Bruns 1996). The sequences of the three ITS groups obtained in our study with a range of similarity to both *Tomentella sublilacina* and *Thelephora terrestris*, apparently link these two species closely together. Although only North American specimens of *Tomentella sublilacina* and *Thelephora terrestris* were considered, ITS sequences of *Tomentella sublilacina* and *Thelephora terrestris* of European origin show high similarities within ITS groups 1, 2 and 3 (U. Köljalg, personal communication). Further clarity on relationships between these species will be achieved by comparison of a range of sequences from geographically distant specimens of fruiting bodies of *Thelephora terrestris* and *Tomentella sublilacina*.

The degree of ITS sequence deviation of 1.5–6.0% between *Tomentella sublilacina, Thelephora terrestris* from North American fruiting bodies and the three closely related ITS-groups in our study is within the range of ITS variability at the species level (1–2 %) or interspecific level (3–5 %) for *Laccaria* (Gardes et al. 1991) and of species within *Suillus* (<10%) (Kretzer et al. 1996). It is considerably beyond the variation found in species considered to comprise species complexes, such as *Pisolithus* (Martin et al. 1998) or *Cenococcum* (Hseu et al. 1996). The similarity of 98.5% of group 1 (BEh-Aw) and group 2 (BEl-lb) isolates and a common base substitution in the highly conserved 5.8S gene in both groups suggest that they belong to the same species. Taking into account the morphological differences between these two isolate groups and the fact that there are no unequivocal clues for setting species limits in fungi (Bruns et al. 1991), further research is required to clarify their relationship. In addition to ITS, other DNA regions of different phylogenetic diagnostic value such as the highly variable IGS and the more conserved nuclear and mitochondrial ribosomal genes should be examined.

Isolates BEh-Aw (ITS group 1), BEl-lb (ITS group 2), BEl-db (ITS group 3) and LH (ITS group 3) had identical sequences or differed only by ambiguities. These groups with identical sequences originating from the same sampling areas may represent genets as the area occupied by a genet can vary considerably (Anderson et al. 1998; Bonello et al. 1998). Since ITS sequence polymorphism may not be sufficient to discriminate at the intraspecific level (Hseu et al. 1996), more highly resolving PCR-based methods such as analysis of random amplified polymorphic DNA or microsatellite primed PCR fingerprints (Hseu et al. 1996; Anderson et al. 1998; Bonello et al. 1998) should be applied to elucidate intraspecific variability of the investigated isolates.

The identity and also the taxonomic position of isolate BEh-Uw1 remains unresolved due to the limited availability of ITS sequences. Other DNA regions suitable for differentiation at the genus or family level, such as more conserved parts of ribosomal nuclear and mitochondrial genes (Bruns et al. 1991), may be suitable for further attempts to identify this mycobiont.

Acknowledgements We gratefully acknowledge the help of U. Köljalg, F. Martin, K. Egger and M. Weiss, who checked our ITS sequence data against their databases. Thanks to B. Mogge and H.-J. Bach for helpful discussion and correction of the manuscript. The research program was funded by the German Research Community (Deutsche Forschungsgemeinschaft, grant No. Mu-831/6–1).

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