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Lactarius ectomycorrhizae on *Abies alba*: morphological description, molecular characterization, and taxonomic remarks

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Abstract: To date, the ectomycorrhizae formed by silver fir (*Abies alba*), an ecologically valuable and indigenous tree species in many European mountain forests, have been poorly investigated. We characterized the mycorrhizae formed by three *Lactarius* species (*Lac. subsericatus, Lac. intermedius, Lac. salmonicolor*) on silver fir, on the basis of material originating from central Italy. The identification of the fungal symbiont was achieved by means of morphoanatomical observations of mycorrhizae, and by comparison of ITS sequences obtained from mycorrhizae and sporocarps of putative fungal partners. Sequences also were obtained from specimens of the same species but from different geographic origin or from closely related *Lactarius* species. A maximum likelihood analysis of the data was performed. On the whole, the resultant tree is in good agreement with sporocarp and mycorrhiza morphology. RFLP patterns were calculated from sequence data. A discussion on the main morphoanatomical characters distinguishing the *Lactarius* ectomycorrhizae reported in this study from those already described belonging to related species, is also included. The accuracy of different methods to identify mycorrhizae formed by closely related *Lactarius* species on silver fir, are discussed.

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INTRODUCTION

Though silver fir (*Abies alba* Miller) is an ecologically valuable and indigenous tree species in many European mountain forests, very little is known about its ectomycorrhizae (see Comandini et al 1998, and references therein). In the present study, we describe the ectomycorrhizae of three *Lactarius* species on *A. alba* from central Italy, by standard morphological and anatomical parameters (Agerer 1991): *Lactarius subsericatus* (Kühner & Romagn.) ex Bon, *Lac. intermedius* (Krombh.) Berk. & Broome, and *Lac. salmonicolor* R. Heim & Leclair. Mycorrhizae of the latter species were compared with an existing description (Pillukat 1996). Identification of the fungal symbionts was achieved by means of sequence comparison between mycorrhizae and sporocarps of the expected fungal partner species, in addition to morphoanatomical analysis of mycorrhizae.

Lactarius subsericatus (syn. *Lac. ichoratus* ss. Romagnesi) is a representative of the subgenus *Russularia* (Fr.) Kauffman sect. *Russularia* Fr. The species is closely related to *Lac. fulvissimus* Romagn. from which it differs by the spore ornamentation composed of mostly isolated warts (more reticulate in *Lac. fulvissimus*). In the field, *Lac. subsericatus* is recognized from the latter species by its more greasy pileus surface, the milk which stains yellow on white tissues, the darker lamellae and an odor resembling that of *Lac. subdulcis* (Pers.: Fr.) Gray (rubber-like, in *Lac. fulvissimus* much like the odor of *Lepiota cristata* (Alb. & Schw.: Fr.) Kummer). For further differences between these two taxa see Schwöbel (1979). The insufficiently known species *Lac. brittanicus* Reid also seems very closely related, if not conspecific. *Lac. subsericatus* is associated with a broad range of both coniferous and deciduous trees on calcareous soil in western Europe, but collections of broadleaved hosts are distinguished by some authors as *Lac. subsericatus* f. *pseudofulvissimus* Bon (e.g., Bon 1980).

Lactarius intermedius [non *Agaricus intermedius* Fr., = *Lac. scrobiculatus* (Scop.: Fr.) Fr.] fits in the subgenus *Piperites* (Fr.) Kauffman sect. *Zonarii* Quél. subsect. *Scrobiculati* Hesler & A.H. Sm. It is closely relat-

TABLE I. Material used for DNA sequencing

Species	Host	Origin	GenBank
Lac. subsericatus S[a]1	*Abies alba*	Italy	AF140254
Lac. subsericatus ECM[b]	*A. alba*	Italy	AF140255
Lac. intermedius S	*A. alba*	Italy	AF140256
Lac. intermedius ECM	*A. alba*	Italy	AF140257
Lac. salmonicolor S1	*A. alba*	Italy	AF140258
Lac. salmonicolor ECM	*A. alba*	Italy	AF140259
Lac. subsericatus S2	*Picea abies*	Germany	AF140260
Lac. subsericatus S3	*Fagus sylvatica*	Germany	AF140261
Lac. cf. *fulvissimus* S	broadleaves	France	AF204678
Lac. fulvissimus S	broadleaves	Belgium	AF204679
Lac. scrobiculatus S1	*P. abies*	Germany	AF140262
Lac. scrobiculatus S2	*P. abies*	Germany	AF140263
Lac. salmonicolor S2	*A. alba*	Germany	AF140264
Lac. salmonicolor S3	*P. abies, F. sylvatica, Pinus sylvestris* (*A. alba*)	Germany	AF140265
Lac. deterrimus S1	*P. abies*	Germany	AF140266
Lac. deterrimus S2	*P. abies*	Germany	AF140267
Lac. semisanguifluus S	*P. sylvestris* (*P. abies*)	Germany	AF140268
Lac. quieticolor S	*P. sylvestris*	Germany	AF140269

[a] S = Sporocarp.
[b] ECM = Ectomycorrhizae.

ed to *Lac. scrobiculatus* from which it differs by the paler, azonate pileus, the scarcely pubescent margin soon becoming smooth in mature specimens, the larger spores which are ornamented with finer and less connected warts and ridges, and its exclusive association with *Abies*, while *Lac. scrobiculatus* is known to grow with *Picea* (Heilmann-Clausen et al 1998). *Lactarius intermedius* is only known from calcareous soils within the natural distribution of its host tree in Europe. A detailed description is given by Marchand (1980).

Lactarius salmonicolor [syn. *L. laeticolor* (S. Imai) Imazeki] belongs to the subgenus *Piperites* sect. *Dapetes* Fr. It is distinguished from the other European members of this group by its exclusive association with *Abies* species and the uniform salmon to orange color without greenish or reddish tinges. For a detailed account on this group, we refer to Heilmann-Clausen et al (1998). *Lactarius salmonicolor* seems widely distributed in the northern hemisphere and is reported from western and central Europe (Heilmann-Clausen et al 1998), Turkey (Sesli and Baydar 1995), Japan (e.g., Hongo 1960 as *Lac. laeticolor*), Mexico (Heim 1953); and from *Abies*-plantations outside the natural distributional area.

As the three *Lactarius* species considered in this study all possess closely related species that may occur in the same area, associated with the same or different tree species, we wanted to find out about the levels of resolution of different methods for identifying mycorrhizae, i.e., identification by their morphoanatomical features, by ITS sequences, and by ITS PCR-RFLP, dealing with these closely related species.

MATERIALS AND METHODS

Sampling and characterization.—*Lactarius* ectomycorrhizae and sporocarps were collected during the growing seasons 1995–1998 from two silver fir stands in the Gran Sasso-Laga National Park, central Italy: "Fonte Gelata" (artificial stand, planted in early 1950s; 1000 m asl) and "Colle Pelato" (natural stand; 1100 m asl) (TABLE I). Details of the two study areas, including mycocoenological, phytosociological and pedological aspects, are reported in Comandini (1997).

Soil cores were taken from beneath the sporocarps. Subsequently, the samples were carefully washed in water, and ectomycorrhizal roots were separated under a dissecting microscope (Leica Wild M 10) for macroscopical characterization, as described by Agerer (1991). Particular care was taken to isolate mycorrhizal roots of *A. alba* from those of other host trees in the sampling sites (mainly *Fagus sylvatica* L.). Methods to characterize ectomycorrhizae have been comprehensively explained by Agerer (1986, 1987–1998, 1991), and a glossary of all terms used has been published by the same author (Agerer 1987–1998). Munsell (1975) has been used as reference for the description of colors of ectomycorrhizae.

Mantle preparations of fresh ectomycorrhizae were fixed on slides with polyvinyl lactophenol for microscopic investigations. For light microscopy, observations were made with a Leitz Laborlux S microscope and photographs were taken with Ilford Panf Plus 50 films. Cross and longitudinal sections (3–5 μm thick) were cut on a cryotome (Frigocut

2800, Reichert-Jung). Voucher specimens of both *Lactarius* sporocarps and mycorrhizae collected in Italy were deposited in AQUI (Herbarium Mycologicum Aquilanum) as fixed material (4% glutaraldehyde), together with slides. Reference specimens of the German material are kept in Tübingen, Institute of Botany, Spezielle Botanik, Mykologie.

The ectomycorrhizae were identified by macroscopic and microscopic analysis, evaluation of consistent features, in particular those in common with sporocarps of the corresponding fungal symbionts, such as the latex color, by matching of field-collected sporocarps with associated ectomycorrhizae, and by matching ITS sequences (see below).

Molecular identification.—Sequences of the internal transcribed spacer (ITS) of the nuclear ribosomal genes were obtained from sporocarps and mycorrhizae listed in TABLE I. For identifying the mycorrhizae of *Lac. subsericatus, Lac. intermedius,* and *Lac. salmonicolor,* the sequences of one sporocarp and of one sample of mycorrhizae (a couple of mycorrhizal tips originating from the same system) of each species were analyzed and compared. If available, sequences obtained from specimens of the same species but from different geographic origin or of closely related species also were considered. Additional material of *Lac. subsericatus* from southwestern Germany (near Tübingen) and central Germany (near Kassel) and of *Lac. salmonicolor* from southwestern Germany (near Balingen or Villingen, respectively), was included. *Lactarius deterrimus* Gröger, *Lac. quieticolor* Romagn., and *Lac. semisanguifluus* R. Heim & Leclair, also were analyzed since they are close relatives of *Lac. salmonicolor.* Preexisting molecular sequence data for *Lac. deliciosus* (L.: Fr.) Gray, which also belongs to the *Dapetes* group, were extracted from GenBank and used for comparative purposes. *Lactarius scrobiculatus* (Scop.: Fr.) Fr. also was considered as a closely related species of *Lac. intermedius,* and *Lac. fulvissimus* Romagn. as a closely related species of *Lac. subsericatus.*

Prior to DNA extraction, ectomycorrhizae were stored in 50% ethanol at room temperature. Sporocarps were dried. DNA isolation from sporocarps was carried out as described in Eberhardt et al (1999). DNA was extracted from mycorrhizae using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturers.

The ITS region within the nuclear ribosomal genes was amplified by the polymerase chain reaction (PCR) using the primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al 1990). Details of PCR reactions are given in Eberhardt et al (1999). The PCR product obtained was purified using the QIAquick protocol (Qiagen, Hilden, Germany). Direct sequencing of PCR products was performed with the PCR primers as sequencing primers. Cycle sequencing was conducted using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, Great Britain) followed by electrophoresis on an automated sequencer (ABI 373A Stretch, Applied Biosystems, Foster City, California). Raw data were processed by the 373A Data Collection- and Data Analysis software (versions 1.2.1, Applied Biosystems, Foster City, California). Sequencing was carried out according to the protocols supplied by the manufacturer, except the cycle sequencing reaction volumes were reduced by half and the cycle sequencing reaction mix diluted 1:1 with bidistilled water. Sequence editing and sequence alignment of sporocarps and their putative mycorrhizae for identification purposes was performed using Sequence Navigator (version 1.0, Applied Biosystems, Foster City, California).

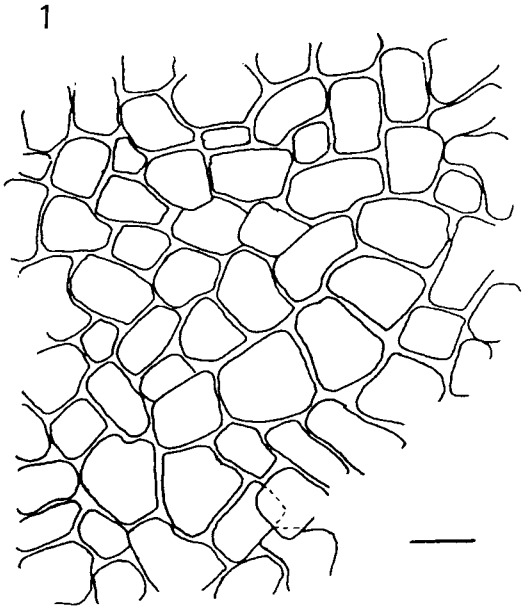
Sequences were aligned using the Clustal method of Megalign (version Power Macintosh 3.12e, DNASTAR, Madison, Wisconsin). Adjustments were inserted manually. Phylogenetic analyses were carried out by PAUP*, version 4.0b4a (Swofford 2000). Maximum likelihood analysis was performed under the Hasegawa-Kishino-Yano model as heuristic search with 100 replicates, and random input order of sequences. Bootstrap values were calculated with the "fast stepwise addition" option and 100 replicates.

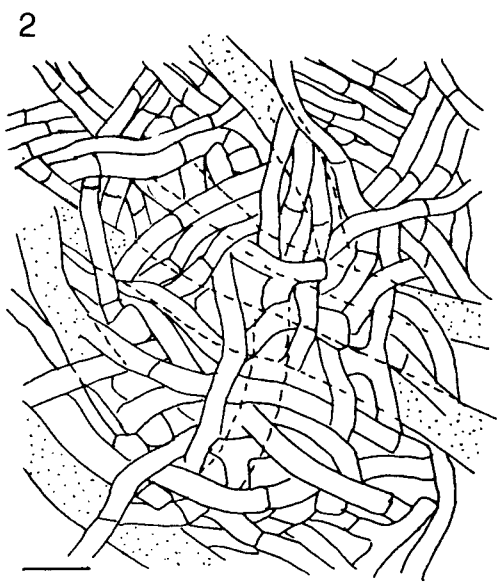
To calculate restriction fragment length patterns (RFLP), the sequences obtained were cut or extended to represent the PCR product of the primer pair ITS1 and ITS4 (White et al 1990). The primers are known to specifically amplify fungal DNA from mycorrhizae of *A. alba, F. sylvatica, Picea abies* Karst., *Pinus sylvestris* L. and some other tree species (Eberhardt unpubl, Jonsson et al 1999, Kårén et al 1997). Seven of the sequences were complete or only the sequences of the primers had to be completed. The sequences of *Lac. semisanguifluus, Lac. deterrimus* (S1), and *Lac. deliciosus* were missing about 35–50 bp at one or at both ends. This was not considered to interfere decisively with the results, because the added sequence stretches were part of coding regions and identical in the remaining sequences. Restriction sites of eight endonucleases (*Alu*I, *Cfo*I, *Eco*RI, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I and *Taq*I) were determined using MapDraw (version 3.08, DNASTAR, Madison, Wisconsin). Restriction patterns were considered as distinct when at least one of the fragments was more than about 10 bp different from the respective band in an otherwise matching pattern or when an additional band of at least 70–80 bp was present in a RFLP pattern.

RESULTS

Descriptions of ectomycorrhizae.—Lactarius subsericatus mycorrhizae are simple or monopodial-pinnate, more infrequently irregularly pinnate. Mycorrhizal systems range from 5.5 to 8.4 mm long and from 0.43 to 0.78 mm diam. Unramified ends are straight to bent, measuring from 1.3 to 3.2 mm long and from 0.4 to 0.7 mm diam. The surfaces of unramified ends are smooth, yellowish-red (5YR 5/8), turning reddish-brown with age. Emanating hyphae were not observed. The mantle secretes a white-yellowish milk when injured. Rhizomorphs are absent.

In surface view, the outer mantle layer is pseudoparenchymatous with polygonal cells, their shapes ranging from subglobose to more elongated (FIGS. 1, 8). Hyphal cell walls are 0.5–0.7 μm. The middle and inner layers are plectenchymatous. In the middle mantle layer, hyphae are mixed with a coarse net of rather straight or dichotomously branched lactifers





FIGS. 1, 2. *Lactarius subsericatus* ectomycorrhizae. 1. Pseudoparenchymatous outer mantle layer with polygonal hyphal cells. 2. Plectenchymatous inner mantle layer with branched lactifers visible through the middle layer. Bars = 10 μm.

with white-pale yellow latex. The inner mantle layer sometimes lacks a distinctive hyphal pattern, but in other places has a net-like arrangement of hyphal bundles (FIG. 2). Hyphae of the inner layer are branched, with numerous septa and lacking clamp connections. Anastomoses were not observed. In the very tip, the outer part of the mantle is pseudoparenchymatous, with the same structure as in the subapical region. Hyphal cells are densely packed and difficult to measure because of small dimensions. The inner mantle layer of the very tip is plectenchymatous. Lactifers are scarce in the inner layer, rather abundant in the middle layer.

From longitudinal sections, a mantle 30–60 μm thick, with 2–3 distinguishable layers can be observed. The outermost layer is pseudoparenchymatous and formed by polygonal cells, lactifers always absent. The rest of the mantle is plectenchymatous. Most of the lactifers are present in the middle layer, whereas scarcely observed in the inner one. The very tips show the same structure as the subapical region, but hyphal cells are more densely packed. The Hartig net surrounds 2–3 rows of cortical cells, forming palmetti-like lobes of 2–7 μm diam in plan view, and forming 1 (2) rows of irregular cells, 2–5 μm thick, in sections.

Lactarius intermedius mycorrhizae are simple or monopodial-pinnate. Mycorrhizal systems range from 4.4 to 5.8 mm long and from 0.6 to 0.7 mm diam. Unramified ends are generally bent, more infrequently straight or tortuous, from 1.5 to 2.5–3 mm long and from 0.4 to 0.5 mm diam. The surfaces of unramified ends are usually smooth, yellowish-brown (10 YR 5/8), slightly grainy in young specimens. Older mycorrhizae become dark yellowish brown. The mantle secretes a yellowish milk when injured. Emanating hyphae were not found. Rhizomorphs are quite rare, and generally connected with the mantle in restricted points; rhizomorphs mostly straight, rarely ramified, and with smooth margins. The color of rhizomorphs is as in the rest of the mycorrhiza.

The outer surface of the mantle is completely covered by a uniform layer of pale yellow/brown crystalloid particles, generally of small dimensions. These resemble little scales in shape, but bigger ones, mainly rhomboidal or elliptic in shape and measuring 3–4 μm long, can also be observed (FIG. 3). Such crystalloid particles were present on fresh as well as on glutaraldehyde-preserved mycorrhizae. Because their presence prevented microscopical observations, we attempted to dissolve them by soaking mycorrhizae in various solvents. Of these, H_2SO_4 50% in water (v/v) for 30 s or more, proved to be most efficient. Afterwards the mycorrhizae were rinsed thoroughly with water.

In treated mycorrhizae, observations were as follows. The outer mantle has a pseudoparenchymatous structure with quadrangular or more elongated hyphal cells, oriented in a parallel way in some parts (FIG. 4). Hyphal cells are not densely packed, cell walls inconspicuous. Lactifers are absent. The middle layer is plectenchymatous, hyphae are mixed with a coarse net of straight or branched lactifers, with a yellowish content and inconspicuous cell walls (FIG.

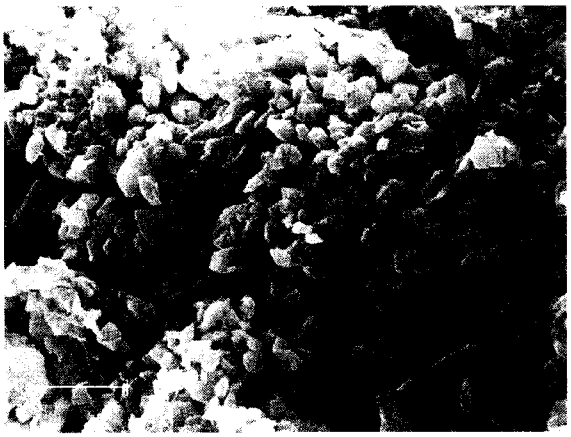


FIG. 3. SEM photomicrograph of crystal-like particles on the surface of untreated *Lac. intermedius* ectomycorrhizae. Bar = 5 μm.

5). The inner mantle layer is plectenchymatous with parallel or irregularly arranged hyphae. Anastomoses are sometimes present. Hyphae are frequently septate with inconspicuous cell walls, clamp connections never observed (FIG. 5). At the very tip, the outer surface of the mantle is pseudoparenchymatous with the same structure as in the subapical region. Hyphal cells are densely packed and difficult to measure as in the inner part of the mantle. Lactifers are very abundant, especially in the middle layer, showing the same features and dimensions as in the subapical region. Rhizomorphs are undifferentiated with rather smooth margins, from 30 to 80 μm diam, and densely covered by crystalloid particles. Within the rhizomorphs, hyphae are thin walled, uniform in diam (3–4 μm), mostly compactly arranged but in some places only loosely woven (FIG. 6). Hyphae are often filled with homogeneous light yellow content (like lactifers). In the distal part of the rhizomorphs, abundantly connected and agglutinated hyphae can sometimes be observed (FIG. 6). Clamp connections are absent.

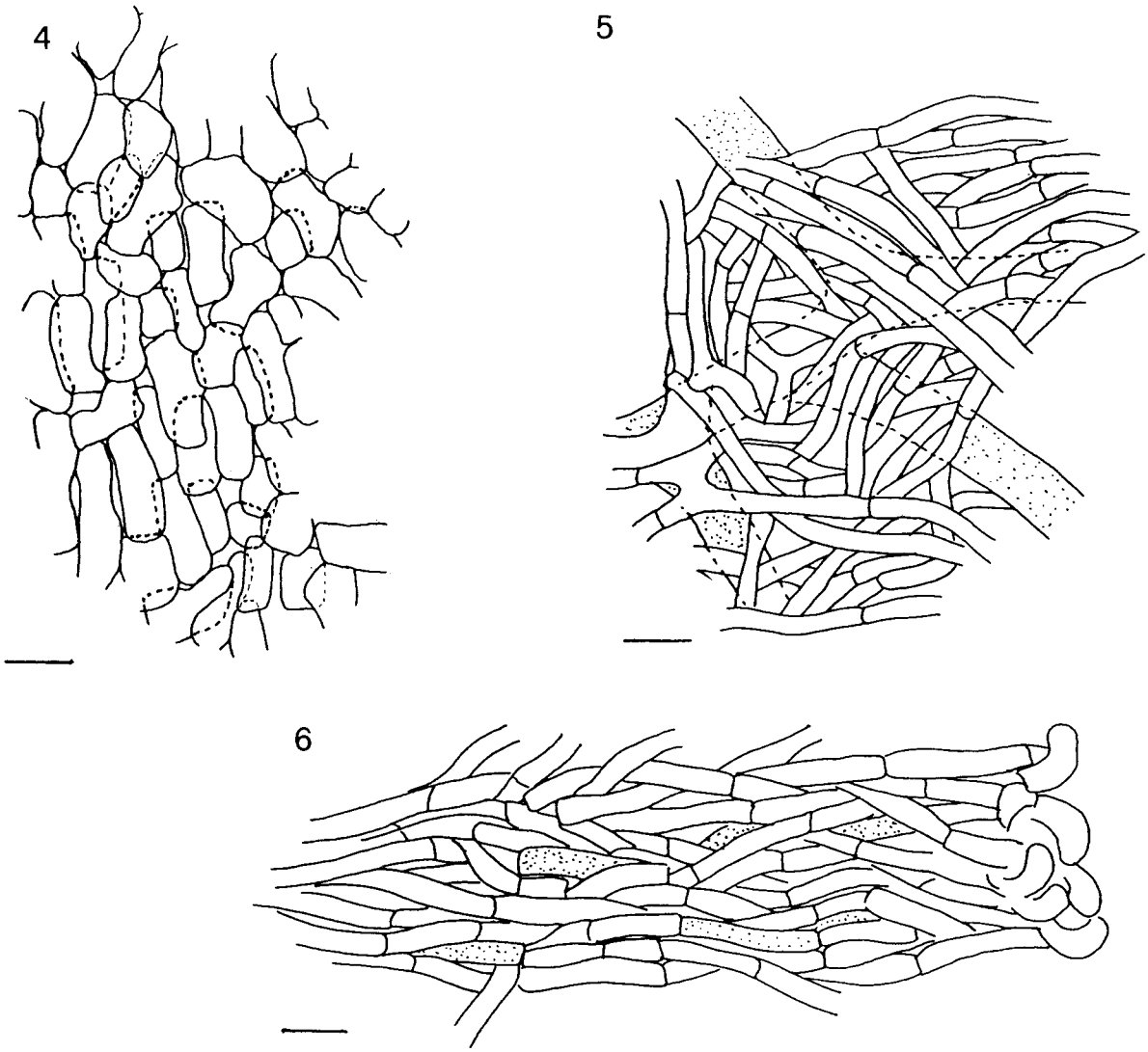
From longitudinal sections, a mantle 30–50 μm thick can be observed, its surface overlaid by a 5 μm thick dense layer of crystalloid particles. The outer mantle layer is composed of tangentially elongated hyphal cells. More elongated hyphal cells, mixed with lactifers, form the middle layer of the mantle. In the inner part of the mantle more elongated and subglobose hyphal cells both are present. The very tip has the same structure as the subapical region, but hyphal cells are more densely packed. Lactifers are mostly present in the middle part of the mantle. Generally three rows of cortical cells are surrounded by the Hartig net, which extends to a depth of 80–100 μm, forming palmetti-like lobes measuring 3–4 μm in width.

Lactarius salmonicolor ectomycorrhizae have already been fully described by Pillukat (1996) on the basis of samples originating from Germany. The outer mantle is plectenchymatous, hyphal cells are rather straight and form characteristic short, often bent protrusions. The inner layer of the sheath is plectenchymatous. The interested reader is directed to Pillukat's paper for more details (Pillukat 1996). Comparing the published features with those of the samples we have collected and studied in Italy, it generally appears that no significant differences exist either in the morphological characters of the ectomycorrhizae or in the anatomical structures of the different mantle layers. Only minor details were observed differentiating our material from the German samples. In our samples, lactifers, located in the middle or inner layers, were of more homogeneous diameter ranging from 5 to 9 μm [versus 4.5–7 (13) μm], and lactifers' cell walls were rather thin and not up to 1.8 μm thick as in the German samples. Moreover, the interhyphal matrix material reported in the outer and in the inner layers of the mantle for German samples has never been observed in Italian ectomycorrhizae. The mantle thickness differs conspicuously between Italian and German material, ranging from 30 to 40 μm in the Italian ectomycorrhizae (FIG. 7), versus 40–70 μm for the German samples in the subapical region, and from 45 to 50 μm versus 80 μm at the extreme tip.

DNA analysis.—For all of the three species, ITS sequences of a sporocarp and of mycorrhizae from underneath the sporocarp could be obtained (TABLE I). The length of the sequences from sporocarps and their putative mycorrhizae that were compared were 738 bp for *Lac. subsericatus,* 731 bp for *Lac. salmonicolor,* and 675 bp for *Lac. intermedius.* The less conserved spacer regions ITS1 and ITS2 are completely included in the compared sequences, apart from the ITS2 of *Lac. intermedius,* the last 5 bp of which are missing from the sequence of the mycorrhizae. No sequence differences could be detected within any of the species, thus confirming that the mycorrhizae described above were formed by the respective fungal species.

Sequence similarities. ITS sequences of two sporocarps of *Lac. subsericatus* and *Lac. salmonicolor* from Germany were obtained and compared to the sequences of the respective species from Italy and show little or no differences (TABLES I, II).

Lactarius fulvissimus belongs to the same, yet unresolved, species complex as *Lac. subsericatus.* Two sporocarps, morphologically unambiguously assigned to *Lac. fulvissimus,* were considered (TABLE I). The ITS sequence of one isolate (named *Lac.* cf. *fulvissi-*

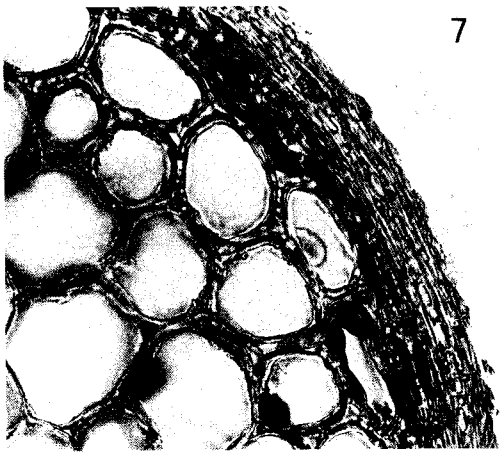


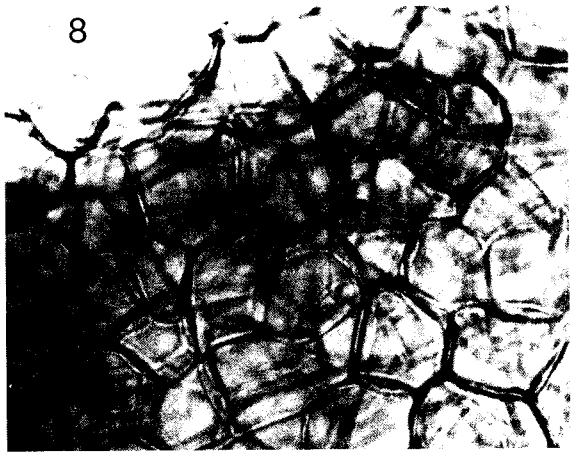
FIGS. 4–6. Treated *Lactarius intermedius* ectomycorrhizae. 4. Pseudoparenchymatous outer mantle layer with quadrangular or more elongated hyphal cells. 5. Plectenchymatous inner mantle layer with parallel or irregularly arranged hyphae and with branched lactifers visible through the middle layer. 6. Undifferentiated rhizomorphs with isodiametric hyphae, compactly arranged or rather loosely woven, often filled with homogeneous content. Abundantly connected and agglutinated hyphae in the distal part of the rhizomorphs. Bars = 10 μm.

mus in TABLES I, II) was identical to that of the *Lac. subsericatus* isolates, the other sequence differed in ten single base mutations (TABLE II). This is more than the variation found among *Lactarius* isolates unambiguously assigned to one species. The interspecific variation among the isolates of other species tested is higher. Thus, the results do not provide clear indications as to the taxonomic status of the two species.

The ITS of three other species of the section *Dapetes* were sequenced, *Lac. deterrimus,* associated with spruce, as well as *Lac. quieticolor* and *Lac. semisanguifluus* associated with pine (TABLE I). In addition, the ITS sequence of *Lac. deliciosus,* associated with pine, is available from GenBank as U80999. The ITS sequences of two sporocarps of *Lac. deterrimus,* from different locations in Germany (ca 300 km apart) differed only slightly (TABLE II). *Lactarius salmonicolor, Lac. deterrimus* and *Lac. semisanguifluus,* each associated with a different host tree, are the most similar in their ITS sequences. The differences among the pine-associated species *Lac. semisanguifluus, Lac. quieticolor* and *Lac. deliciosus* are more pronounced. Multiple insertions or deletions (up to 20 bp) occur in several positions of the alignment (TABLE II).

Lactarius scrobiculatus is a close relative of *Lac. intermedius,* and is associated with spruce (Heilmann-Clausen et al 1998). Two sporocarps of *Lac. scrobi-*





FIGS. 7, 8. Anatomical characters of *Lactarius* ectomycorrhizae. 7. Cross section of *Lac. salmonicolor* ectomycorrhiza with a rather thin mantle. ×400. 8. Pseudoparenchymatous outer mantle layer of *Lac. subsericatus* ectomycorrhiza. Noteworthy, the surface of the mantle lacks an hyphal reticulum. ×1000.

culatus, collected ca 40 km apart in southwestern Germany (TABLE I), showed again little difference. The difference between *Lac. intermedius* and *Lac. scrobiculatus* sequences amounted to about 2% (TABLE II). Taking into account the host specificity of these species and the intraspecific homogeneity encountered to date, the 2% interspecific variation should be sufficient (a) to add corroborative evidence that *Lac. scrobiculatus* and *Lac. intermedius* are two separate species and (b) to make an identification of the mycorrhizae of each species possible.

Variation occurs almost exclusively in the spacer regions, coding regions are mostly identical for all sequences. Namely the 5.8S rRNA sequence does not vary between the *Lactarius* species considered.

Maximum likelihood analysis. The aim of this analysis is to show how well morphologically based taxa are confirmed by molecular data. No outgroup species was included, because the data set is too limited to address phylogenetic questions. The alignment is available from TreeBASE (S479, M702). Maximum likelihood analysis resulted in one optimal tree,

TABLE II. Differences between isolates of the same or related species

Isolates compared	Differences	Differences (%)
Lac. subsericatus		
S1–S2	0 bp of 719 bp	0.0
S1–S3	2 bp[a] of 745 bp	0.3
Lac. subsericatus S1–*Lac.* cf. *fulvissimus*	0 bp of 686 bp	0.0
Lac. fulvissimus–*Lac.* cf. *fulvissimus*	10 bp of 705 bp	1.4
Lac. salmonicolor		
S1–S2	0 bp of 747 bp	0.0
S1–S2	0 bp of 722 bp	0.0
Lac. deterrimus S1–S2	4 bp of 694 bp	0.6
section *Dapetes*	alignment 668 bp	
Lac. deliciosus–*Lac. deterrimus* S2	41 bp	6.1
Lac. deliciosus–*Lac. quieticolor*	52 bp	7.8
Lac. deliciosus–*Lac. salmonicolor*	40 bp	6.0
Lac. deliciosus–*Lac. semisanguifluus*	45 bp	6.7
Lac. deterrimus S2–*Lac. quieticolor*	51 bp	7.6
Lac. deterrimus S2–*Lac. salmonicolor*	25 bp	3.7
Lac. deterrimus S2–*Lac. semisanguifluus*	25 bp	3.7
Lac. quieticolor–*Lac. salmonicolor*	53 bp	7.9
Lac. quieticolor–*Lac. semisanguifluus*	60 bp	9.0
Lac. salmonicolor–*Lac. semisanguifluus*	24 bp	3.6
Lac. scrobiculatus S1–S2	1 bp of 736 bp	0.1
Lac. intermedius–*Lac. scrobiculatus* S2	16 bp of 764 bp	2.1

[a] Ambiguous sequence readings.

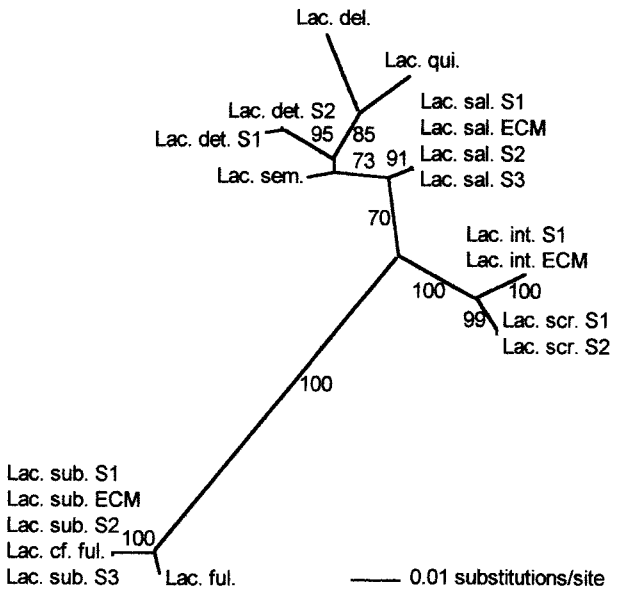


FIG. 9. Maximum likelihood analysis with bootstrap analysis (100 replicates). Lac. *Lactarius*; del. *deliciosus*; det. *deterrimus*; ful. *fulvissimus*; int. *intermedius*; qui. *quieticolor*; sal. *salmonicolor*; scr. *scrobiculatus*; sem. *semisanguifluus*; S sporocarp (for numbering see TABLE I); ECM ectomycorrhizae. Isolates not specified are from sporocarps.

shown in FIG. 9. Branch lengths within subgenera are shorter than the branch separating isolates of the subgenera *Piperites* and *Russularia*. Moreover, this branch is confirmed by a bootstrap value of 100%. The section *Dapetes* is backed by a bootstrap value of 70%, *Lac. intermedius* and *Lac. scrobiculatus* of the section *Zonarii* form a cluster with 100% bootstrap support. Branches housing conspecific isolates are always backed by bootstrap values of at least 95% and mostly of close to zero branch lengths. This implies that isolates from mycorrhizae always cluster with the isolates of sporocarps of the same species. As expected from sequence similarity, the sequence of *Lac.* cf. *fulvissimus* clusters with the isolates of *Lac. subsericatus*.

Restriction fragment patterns. Not all species can be expected to be separable based on the length of their ITS PCR products (TABLE III). Putative restriction fragment lengths of eight endonucleases are given in TABLE III. Closely related species have many restriction sites in common, so differentiation will be dependent on the realization of the few predicted distinguishing restriction sites.

Lactarius subsericatus and *Lac. fulvissimus* are least closely related to the other species, producing restriction patterns distinct from those of the species of the subgenus *Piperites*. The divergent isolate of *Lac. fulvissimus* differs from the other *Lac.* cf. *fulvissimus* isolate and from *Lac. subsericatus* by one restriction site (TABLE III). In spite of the small sequence variation between the *Lac. deterrimus* isolates, differences are sufficient to produce a different banding pattern with *Cfo*I. The differences in RFLP patterns of eight enzymes with 22 cleavage sites between one of the *Lac. deterrimus* isolates and *Lac. semisanguifluus* are slight (TABLE III). Two fragments in the RFLP of *Hinf*I differ in about 20 bp from the respective fragments of the other species. The restriction patterns of *Lac. intermedius* and *Lac. scrobiculatus* vary in two restriction sites of *Mbo*I (TABLE III), one of which can be expected to produce a distinctive banding pattern. Among the fungal partners of pine, *Lac. deliciosus* and *Lac. semisanguifluus* can only be distinguished by three restriction sites of two different enzymes, six enzymes producing similar patterns. *Lactarius quieticolor* is set apart by the length of its ITS PCR product. In addition, indels change the position of restriction sites such that the resulting fragments vary notably in size. *Lactarius intermedius* and *Lac. salmonicolor*, associates of fir, can be expected to produce distinctly different restriction patterns for three of the enzymes. More enzymes distinguish the partners of spruce, *Lac. deterrimus* and *Lac. scrobiculatus* (TABLE III).

DISCUSSION

The mycorrhizae of *Lac. subsericatus* previously have not been described, though descriptions of a number of different ectomycorrhizae of *Lactarius* species sect. *Russularia* are available. Their features are discussed by Agerer (1995) and Palfner and Agerer (1996). Although the host trees associated with these different mycorrhizal types belong to several genera both of conifers and broadleaves, several common features within this group can be pointed out, such as the orange–brown color of the ectomycorrhizae, the lack of macroscopically visible lactifers on the surface, and a pseudoparenchymatous structure of the outer mantle layer which is generally covered by thin hyphae arranged in a net-like to ramified star-like array. The pseudoparenchymatous structure of the outer mantle layer is generally formed by angular cells. Rhizomorphs are quite rare, with differentiated or undifferentiated structures (Palfner and Agerer 1996). Cystidia have been observed only in few cases, e.g., in *Lac. mitissimus* (Fr.:Fr.) Fr. (Weiss 1991) and in *Lac. rubrocinctus* Fr. (Brand 1991). In *Lac. subdulcis* and *Lac. rubrocinctus* ectomycorrhizae, intracellular infections caused by a second fungus, *Leucoscypha leucotricha* (A. & S. ex Fr.) Bound (*Pezizales*) have been observed (Brand 1991, Brand and Agerer 1986). Most of the common features of the ectomycorrhizae of *Lactarius* sect. *Russularia* also can be ob-

TABLE III. Presumed restriction fragment lengths of the ITS1/ITS4 PCR products calculated from sequence data

Species[a]	*Alu*I	*Cfo*I	*Eco*RI	*Hae*III	*Hinf*I	*Mbo*I	*Msp*I	*Taq*I
Lac. subsericatus	487	368	406	458	389	263	378	278
AF140254	161	227	322	245	263	206	263	274
	80	133		25	68	131	87	74
					8	106		59
						22		31
								12
Lac. fulvissimus	487	368	406	458	389	263	641	278
AF204679	161	227	322	245	263	206	87	274
	80	133		25	68	131		74
					8	106		59
						22		31
								12
Lac. deterrimus	505	372	403	471	306	269	639	197
AF140266	232	267	334	266	173	197	98	168
		98			170	143		119
					80	97		117
					8	22		72
						9		59
								5
Lac. deterrimus	503	267	403	469	306	269	639	197
AF140267	232	215	332	266	173	197	96	166
		155			168	141		119
		98			80	97		117
					8	22		72
						9		59
								5
Lac. semisanguifluus	500	365	403	466	288	269	645	197
AF140268	232	273	329	266	177	197	87	159
		94			161	134		121
					98	101		119
					8	22		72
						9		59
								5
Lac. deliciosus	501	358	396	475	347	261	635	261
U80999	233	270	338	259	298	198	99	169
		106			81	144		125
					8	100		120
						22		59
						9		
Lac. quieticolor	486	358	396	459	331	262	621	278
AF140269	232	206	322	259	213	237	97	262
		154			86	197		73
					80	22		59
					8			46
Lac. salmonicolor	504	365	403	470	386	269	642	289
AF140258	232	270	333	266	342	197	94	197
		51			8	139		191
		50				100		59
						22		
						9		
Lac. intermedius	506	375	410	473	393	267	572	293
AF140256	241	372	337	274	346	206	175	195
					8	149		117
						103		83
						22		59

TABLE III. Continued

Species[a]	*Alu*I	*Cfo*I	*Eco*RI	*Hae*III	*Hinf*I	*Mbo*I	*Msp*I	*Taq*I
Lac. scrobiculatus	505	374	409	472	392	267	571	292
AF140262	240	371	336	273	345	146	174	195
					8	139		116
						103		83
						59		59
						22		
						9		

[a] The sequences employed are identified by their GenBank accession numbers.

served in *Lac. subsericatus* mycorrhizae on *A. alba*, with the exception of the hyphal reticulum over the pseudoparenchymatous outer mantle layer, which has never been found in our *Lac. subsericatus* samples (FIG. 8). Rhizomorphs were never observed in our samples.

The mycorrhizae of *Lac. intermedius* are described here for the first time. *Lactarius scrobiculatus* ectomycorrhizae, associated with *P. abies* and *Tsuga heterophylla* (Raf.) Sarg., have been comprehensively described by Amiet and Egli (1991) and Kernaghan and Berch (1997), respectively. From a comparison of the ectomycorrhizal types formed by *Lac. scrobiculatus* and *Lac. intermedius*, only few differences can be noted. The presence of a dense layer of crystalloid particles on the surface of *Lac. intermedius* mycorrhizae is particularly intriguing. It is a consistent feature of all mycorrhizae so far examined. This layer was never observed on *Lac. scrobiculatus* mycorrhizae (Amiet and Egli 1991, Kernaghan and Berch 1997, Egli, Kernaghan pers comm). A closer inspection of these particles through a SEM microscopy apparatus equipped with an EDS X-ray microanalysis instrument, revealed a general appearance of densely packed scales and an elemental composition rich in Si and O (data not shown). Thus, it is possible that these crystalloid particles are indeed soil elements that stick tenaciously to the surface of the mantle, since they are not removed by rinsing the mycorrhiza with water nor by brushing. Further work is under way to shed light on this point. Interestingly, crystalloid particles have recently been observed on the ectomycorrhiza formed by another *Lactarius* species, namely *Lac. controversus* Pers., on *Populus alba* L. (Jakucs et al 2000).

Most of the other features distinguishing *Lac. intermedius* and *Lac. scrobiculatus* mycorrhizae are largely due to the different host species (Pillukat and Agerer 1992), such as the shape of mycorrhizal systems (monopodial irregularly pinnate to coralloid in *Lac. scrobiculatus*/spruce mycorrhizae, generally simple or monopodial-pinnate in *Lac. intermedius*/fir) and the depth of Hartig net (in *Lac. scrobiculatus*/spruce mycorrhizae it surrounds all cortical cells up to the endodermis while in *Lac. intermedius*/fir it generally surrounds only 3 rows of cortical cells). The outer mantle layer of *Lac. intermedius* mycorrhizae, though essentially similar in structure (apart from some differences concerning dimensions of hyphal cells), does not possess fusiform cystidia as described by Amiet and Egli (1991) for *Lac. scrobiculatus*. The intracellular infection of an unidentified fungus reported by Amiet and Egli (1991) in *Lac. scrobiculatus* ectomycorrhizae, has never been observed in our samples, nor the lacunae present in the outer mantle layers of *Lac. scrobiculatus* as described by Kernaghan and Berch (1997).

A comprehensive discussion of the main diagnostic characters of the described *Lactarius* ectomycorrhizae belonging to sect. *Dapetes*, was carried out by Pillukat (1996). Subsequently, the description of the mycorrhizae formed by *Lac. rubrilacteus* Hesler & A. H. Sm. on *Pseudotsuga menziesii* (Mirb.) Franco appeared (Eberhart and Luoma 1997), as well as a short account of *Lac. deliciosus* var. *aerolatus* Smith mycorrhizae on *Abies lasiocarpa* (Hook.) Nutt. (Kernaghan et al 1997). These mycorrhizae also are typical for the section *Dapetes* in their plectenchymatous mantle structures and the presence of lactifers concolorous with the milk in sheath and rhizomorphs. Unlike other descriptions of mycorrhizae of this section, neither agglutination of mantle hyphae nor the presence of matrix material was observed in our samples. Since matrix material was described from the mantles of *Lac. salmonicolor* mycorrhizae by Pillukat (1996) but was not observed in our specimens, the presence of matrix material, or at least its presence in distinguishable quantities, may not be a constant feature even within the same species.

A comparison of our Italian samples with *Lac. salmonicolor* mycorrhizae described from Germany (Pillukat 1996) revealed that mantle thickness is another distinguishing anatomical feature. Indeed, as reported above, Italian samples possess mantles only about half as thick as those measured by Pillukat (1996), both in the subapical region and in the very tip. Of the numerous samples of *Lac. salmonicolor* mycorrhi-

zae collected in a period spanning four years and in various localities in Italy, all showed a comparable thickness of the mantle. Considerable variation in the mantle thickness within a single mycorrhizal type (i.e., same mycobiont species on the same host tree species), has already been reported in the literature, and differences in mantle thickness have also been observed when comparing natural with synthesized mycorrhizae (Agerer and Weiss 1989, Egli et al 1993, Miller et al 1991, Treu 1990, Weiss 1991). However, available data do not allow drawing definitive conclusions as to the influence of specific parameters or conditions (environmental factors, growth medium composition, developmental stage of the mycorrhizae, etc.) on the change of mantle thickness. Given the important functional role played by the fungal mantle in the mycorrhiza nutrient input and storage processes, it is conceivable that a marked alteration in the mantle thickness under different growth conditions and habitats might prove to have an adaptive significance.

The ITS of the nuclear ribosomal genes has become one of the most widely used genomic regions for the identification of ectomycorrhizae (e.g. Erland et al 1994, Goodman et al 1996–1998, Jonsson et al 1999, Kårén et al 1997, Kraigher et al 1995, Pritsch et al 1997). In most of these studies, identification was obtained by restriction patterns (RFLP) of the ITS PCR product with a given set of endonucleases. For *Lac. subsericatus, Lac. intermedius,* and *Lac. salmonicolor* we showed that the entire ITS sequences were identical for conspecific mycorrhizae and sporocarps. While sequence identity between isolates of the same origin might be expected, we could also show that isolates of *Lac. subsericatus* and *Lac. salmonicolor* from Germany and Italy, or *Lac. subsericatus* and *Lac. deterrimus* from distinct locations in Germany, revealed the same or almost identical sequences. Moreover, even association with different tree species, such as fir, spruce and beech, did not lead to divergent ITS sequences for *Lac. subsericatus.*

Molecular phylogenetic studies (Bruns et al 1998, Moncalvo et al 2000) result in a common terminal cluster of *Russula* and *Lactarius* species, thus confirming the common origin of the agaricoid *Russulaceae.* In recent molecular studies on the phylogeny of the *Russulales* (Miller et al unpubl, Henkel et al unpubl), the monophylly of the genus *Lactarius* (including species of the same sections or subgenera treated in this study) is questioned. Irrespective of the supposed paraphyly of *Lactarius,* the result of the maximum likelihood analysis presented in this study confirms morphological data. Similarity of sporocarp morphology is reflected by terminal clusters, bootstrap support, and branch lengths separating the respective taxa, regardless of the taxonomic level, species, section, or subgenus. Moreover, species known or presumed to form morphologically similar mycorrhizae appear in the same clusters. The only exception to the agreement of sporocarp morphology and sequence similarity is *Lac.* cf. *fulvissimus.*

Owing to the morphological differences between *Lac. fulvissimus* and *Lac. subsericatus,* we prefer to treat them as separate species. Others, i.e. Heilmann-Clausen et al (1998), do not consider them distinct species. The molecular verdict is ambiguous. The sequence identity of *Lac.* cf. *fulvissimus* (morphologically unambiguously belonging to *Lac. fulvissimus)* and the *Lac. subsericatus* isolates argues for merging both species, following the general opinion that ITS sequences are species specific (Baura et al 1992, Chen et al 1992, Gardes and Bruns 1993, Gardes et al 1991, Lee and Taylor 1992, etc.). Nevertheless, levels of variation within species and among closely related species differ considerably between different taxa. Compared to the intraspecific variation of ITS sequences detected in other ectomycorrhizal fungi (Anderson et al 1998, Baura et al 1992, Eberhardt et al 1999, Mankel et al 1998, Shinohara et al 1999), the intraspecific variability of the ITS in the *Lactarius* species studied here, is rather low, as is the interspecific variation between most species of the same subgenus. Even an intraspecific variation of 1.4%, the level of variation between *Lac. fulvissimus* and *Lac.* cf. *fulvissimus* or *Lac. subsericatus,* can be considered low. On the other hand, *Lac. scrobiculatus* and *Lac. intermedius* are only separated by a few additional single base mutations. So levels of inter- and intraspecific variability of the ITS may not always be separable in *Lactarius.* As a consequence, species-specificity of ITS sequences cannot be taken for granted in this genus. The molecular evidence is not considered sufficient to deviate from the view that *Lac. subsericatus* and *Lac. fulvissimus* are two morphologically distinct species. The observed sequence variation between the *Lac. fulvissimus*-like isolates may reflect a high level of intraspecific variability within this species, possibly due to only recent speciation.

Given the widespread application of ITS PCR-RFLP to the identification of ectomycorrhizae in community studies, fragment lengths were calculated from sequence data. Our putative RFLP results show that for identifying *Lactarius* ectomycorrhizae, the resolution of ITS PCR-RFLP is not great enough to ensure identification of species of the same section or even subgenus when applying a standard set of three to five endonucleases. Until the presence or absence of certain restriction sites will be known to be of diagnostic value for the identification of certain spe-

cies, additional means of identification of mycorrhizae should not be disregarded.

The enzymes arbitrarily selected have been previously applied to the identification of ectomycorrhizae by Horton and Bruns (1998), Kårén et al (1997), and Pritsch et al (1997). In praxi, occasionally additional (faint) bands may occur in RFLP patterns resulting from incomplete restriction or single base mutations in a minority of the PCR products. The fragment size of these bands can again be explained by sequence data, omitting single restriction sites. On the whole, restriction patterns and fragment lengths calculated from sequence data agree well (Eberhardt unpubl, Gandeboeuf et al 1997).

Mycorrhizal morphology and anatomy can be employed to distinguish mycorrhizae formed by *Lactarius* species of the different sections from one another. Within sections, morphological differences tend to be slight, though judging from the above comparisons, the resolution provided by morphological characters is sometimes greater than that of RFLP analysis. The presence of rhizomorphs and cystidia in mycorrhizae of *Lactarius* species is variable, and therefore the mere absence of such structures is not sufficient for differentiating between mycorrhizal types. Additional characters must be taken into account. Considering the host specificity of *Lac. deterrimus* and *Lac. semisanguifluus,* root morphology might help to separate mycorrhizae, while mycorrhizal anatomy can be expected to be fairly similar. The mycorrhizae of *Lac. semisanguifluus* and *Lac. quieticolor* have not been described so far, but with the exception of *Lac. paradoxus* Beardslee & Burl. (Danielson 1984) mycorrhizal anatomy is fairly uniform within the section (see Pillukat 1996). For distinguishing the mycorrhizae of *Lac. semisanguifluus, Lac. quieticolor* and *Lac. deliciosus,* all associated with pine, environmental conditions may give important hints towards mycorrhizal identity. While *Lac. deliciosus* typically occurs on neutral, calcareous, often sandy soils with *P. sylvestris* and *Juniperus communis* L., *Lac. semisanguifluus* is found in grass rich forests on calcareous soils, and *Lac. quieticolor* prefers acid, often sandy soils (Marchand 1980, Michael et al 1983, Heilmann-Clausen et al 1998). Site-by-site comparisons of sequences of species of the subgenus *Piperites* lead to the impression that adaptation to different hosts in *Lac. scrobiculatus* and *Lac. intermedius* and in the group *Lac. semisanguifluus/Lac. salmonicolor/Lac. deterrimus* was accompanied by slighter sequence divergence as ecological adaptations among the pine-associated species of the *Dapetes* group.

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