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Ectomycorrhizae of *Lactarius lignyotus* on Norway spruce, characterized by anatomical and molecular tools

Abstract The ectomycorrhizae of *Lactarius lignyotus* on Norway spruce are comprehensively described by morphological and anatomical characteristics. Identification of ectomycorrhizae was performed by tracing mycelia to the fruitbodies and also by molecular tools, using polymerase chain reaction (PCR) amplification of the fungal DNA. The newly described ectomycorrhiza is compared to ectomycorrhiza of the related *Lactarius picinus*. The amplified DNA products of the two fungi and their ectomycorrhizae could be distinguished by characteristic fragments after digestion with *Hin*f1.

Key words Ectomycorrhizae · Characterization and identification · *Lactarius lignyotus* · Polymerase chain reaction (PCR) · Restriction digest

Introduction

Ectomycorrhizae of eight species of the genus *Lactarius* occurring on Norway spruce are well described (Agerer 1987–1993). In our study we describe the ectomycorrhizae of *Lactarius lignyotus* Fr. on Norway spruce, *Picea abies* (L.) Karst., by morphological and anatomical parameters (Agerer 1991). The identification was possible by tracing mycelia from the fruitbodies to mycorrhizae. The descriptions are compared to the ectomycorrhizae of a closely related species, *Lactarius picinus* Fr. (Agerer 1986). Furthermore, we employed molecular tools for identification of ectomycorrhizae as suggested in the review by Bruns et al. (1991). For molecular identification, we analysed fungal variability of the internal transcribed spacer (ITS) region in the nuclear ribosomal units using two taxon-specific primers which have been shown to amplify the basidiomycete component in ectomycorrhizae (Gardes and Bruns 1993). The comparison of digested fragments of amplified DNA from the two fungi and mycorrhizae of *L. lignyotus* was shown to be fungus specific, fast and reproducible.

Considered as part LV of the series "Studies on ectomycorrhizae" of the Institute for Systematic Botany, Munich; part LIV: Agerer et al. (1994)

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Materials and methods

Isolation and characterisation of ectomycorrhizae

Natural mycorrhizae and attached rhizomorphs were isolated from soil (O_H/A_H layer) in mixed pro-naturally managed Norway spruce stands in north Slovenia (Mislinja, close to Velenje, belonging to the Pohorje mountain range, at 850 m above sea level). Methods for isolation and characterisation of ectomycorrhizae (morphological and anatomical features) and the terms necessary for exact descriptions are according to Agerer (1991) and Agerer (1987–1993). For extraction of DNA, polymerase chain reaction (PCR) and digestion of the amplified products, the methodology of Gardes and Bruns (1993) was slightly adapted.

DNA extraction

DNA from frozen tissues (10–300 mg of sporophore tissue or ectomycorrhizae) was thawed in 600 µl of 2×CTAB lysis buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM Na_2-EDTA, 2% CTAB, 0.2% mercaptoethanol) and incubated at 68° C. After chloroform-isoamyl alcohol (24:1) extraction, DNA was precipitated in cold isopropanol for 30–60 min. The pellet was washed with 70% ice-cold ethanol, dried and resuspended in 150 µl TE buffer (1 mM Tris-HCl, 0.1 M Na_2-EDTA pH 8.0). Prior to amplification, the quantity of DNA was measured fluorometrically and diluted 10-, 100- and 500-fold in sterile double-distilled water.

PCR amplification

For PCR amplification, aliquots of 12.5 or 25 µl of the diluted DNA were combined with an equal volume of PCR mix containing: buffer (Boehringer-Mannheim, with $MgCl_2$ adjusted to 2.5 mM), nucleotide triphosphates (200 µM each), primers (ITS1-F and ITS4-B (1 µM), generously supplied by Dr. Tom Bruns, Berkeley, Calif.) and *Taq* polymerase (Boehringer-Mannheim, at 0.6 units per 25 µl final volume or 1.2 units per 50 µl final volume). Each reaction was overlaid with a drop of mineral oil (Sigma). Temperature cycling was carried out using a programmable heat block (Perkin-Elmer) as follows: denaturation at 94° C for 85 s, followed by 13 cycles of denaturation at 95° C for 35 s, annealing at 55° C for 55 s, extension at 72° C for 45 s. For the following 13 cycles, the extension steps were continuously lengthened from 60 s to 120 s and for the last 9 cycles from 120 s to 180 s. After the 35 cycles were completed, the samples were incubated for 10 minutes at 72° C and soaked at 4° C until storage or analysis. A negative control (no DNA template) was used in every experiment to test for the presence of DNA contamination of reaction mixtures.

Restriction digests

Restriction digests of the amplified DNA were performed using restriction enzymes *Alu*1 or *Hinf*1 (MBI Fermentas) by diluting 8 µl of PCR product with 8 µl of the mix of twofold concentrated enzyme buffer and 3 units of restriction enzyme. The reactions were then incubated for 1 h at 37° C.

Electrophoresis

The PCR products and their restriction digests were electrophoresed for 3.5–4 h on 1.4 or 1.8% agarose (SeaKem) gel in Tris-borate-EDTA buffer (TBE) pH 8.0, at 70 mV (bands containing ethidium bromide). Two DNA molecular-weight markers (Boehringer-Mannheim) were applied to the gels: marker IV (13 bands ranging from 19329 to 421 bp) and marker VI (11 bands ranging from 2176 to 154 bp). The gels were photographed under ultraviolet light on polaroid film.

Results

Morphological characters of *L. lignyotus* Fr.

Mycorrhizal system irregularly monopodially-pyramidal, in loose clusters, ramified systems up to 12 mm long, unramified ends up to 1.5 mm long and 0.5–0.7 mm in diameter, axes 0.4–0.6 mm in diameter; unramified ends straight or slightly bent, sometimes slightly beaded and tapering to distal ends; mantle surface smooth, white, silvery due to air entrapped between hyphae, older parts with larger dark grey patches (air between hyphae displaced by water); very tips grey or slightly ochre; soil particles glued to mantle (predominantly at points of ramification); rhizomorphs not found; emanating hyphae infrequent (Fig. 1).

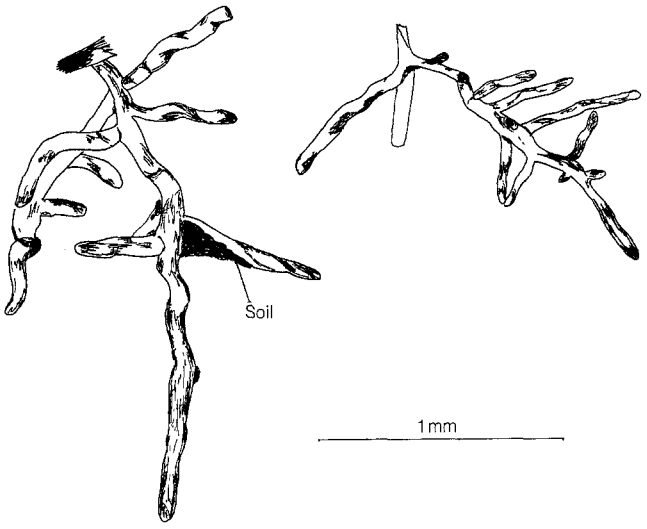

Fig. 1 *Lactarius lignyotus* × *Picea abies:* habit (from SLO 00428)

Anatomical characteristics of surface

Inner surface of mantle (Fig. 2a) hyphae more densely arranged, laticifers (4) 5–7 µm in diameter, hyphae 2–3 (3.5) µm in diameter, cells (6) 12–16 (27) µm long. *Surface of tip* cystidia-like hyphal ends 9–19 (26) × 2–5 µm, hyphae 2–4 µm thick, hyphal cells mostly 12–18 µm long, laticifers 3.5–5.5 µm in diameter. *Middle layers of mantle* (Fig. 2b–d, g) loosely plectenchymatous, hyphae net-like arranged, with gelatinous matrix, laticifers frequent, with H-shaped ramifications, hyphae (1.5) 2–3 µm in diameter, cells (4) 8–18 (21) µm long, septa infrequently with central globular thickenings; laticifers 4.5–7 µm in diameter, walls approximately 0.5 µm thick, septa infrequent. *Outer surface of mantle* (Fig. 2e, f) consisting of short, cylindrical or clavate, sometimes irregularly bent hyphal end-cells, containing droplets, 9–12 (17.5) × (2) 2.5–4.5 (5.5) µm (mantle type I: Agerer 1987–1993, 1991), soil particles adhering to the mantle, there these hyphal ends infrequent, hyphae of mantle growing towards soil debris. *Rhizomorphs* not found. *Emanating hyphae* 2.5–3 µm in diameter, irregularly bent, walls approximately 0.5 µm thick. *Cystidia-like hyphal ends* (Fig. 2e, f) see outer mantle surface.

Anatomical characteristics, cross section

Mantle (Fig. 3a) plectenchymatous throughout, (20) 30–35 (49) µm thick, three different layers discernable, layers often variable in thickness, hyphae thin-walled; outer layer composed of thick, stout, irregularly bent hyphal ends, reminiscent of cystidia, this layer 8–12 µm thick, but sometimes lacking; middle layer consisting of looser arranged hyphae, running mostly in parallel to root surface and oriented rather perpendicular to longitudinal axis of root, hyphae radially 2–3.5 µm thick, laticifers 5–8 µm, hyphae tangentially (3) 10–15 (30) µm, laticifers found even up to 70 µm; inner layer with hyphae mostly shown in cross sections, radially 2–3 µm thick, tangentially 2–10 µm; calyptra cells as irregularly shaped, strongly light-reflecting lines very close to root

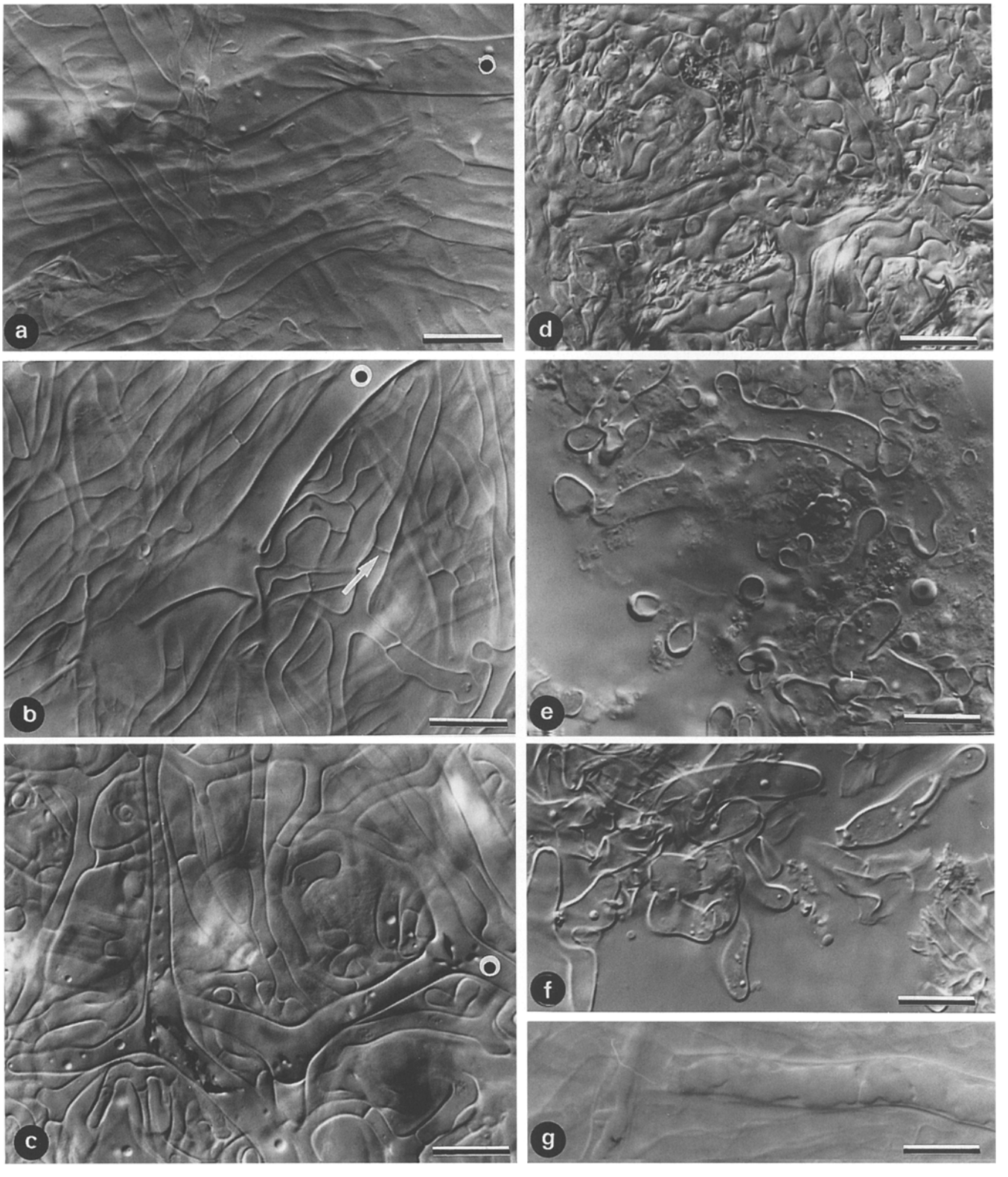

Fig. 2a–g *L. lignyotus* × *Picea abies:* plan views of mantle at different levels of focus. **a** Inner surface of mantle, with densely arranged hyphae and with one laticifer. **b** Middle layer of mantle close to inner surface, less densely arranged hyphae with gelatinous matrix in between and a laticifer; central globular thickenings in the septa (*arrow*). **c** Middle layer of mantle, close to outer side, hyphae net-like arranged, with some laticifers, note latex in the laticifers. **d** Outer layer of mantle just beneath the cystidia-like protruding hyphal ends. **e** Cystidia-like hyphal ends of mantle surface with soil particles. **f** Cystidia-like hyphal ends, irregularly shaped and with latex droplets. **g** Laticifer with coagulated latex. Microscopy with Nomarski's interference contrast. **a–f** In lactic acid; **g** lactic acid just added. *Points* laticifers. All figures from SLO 00428; *bar* = 10 μm

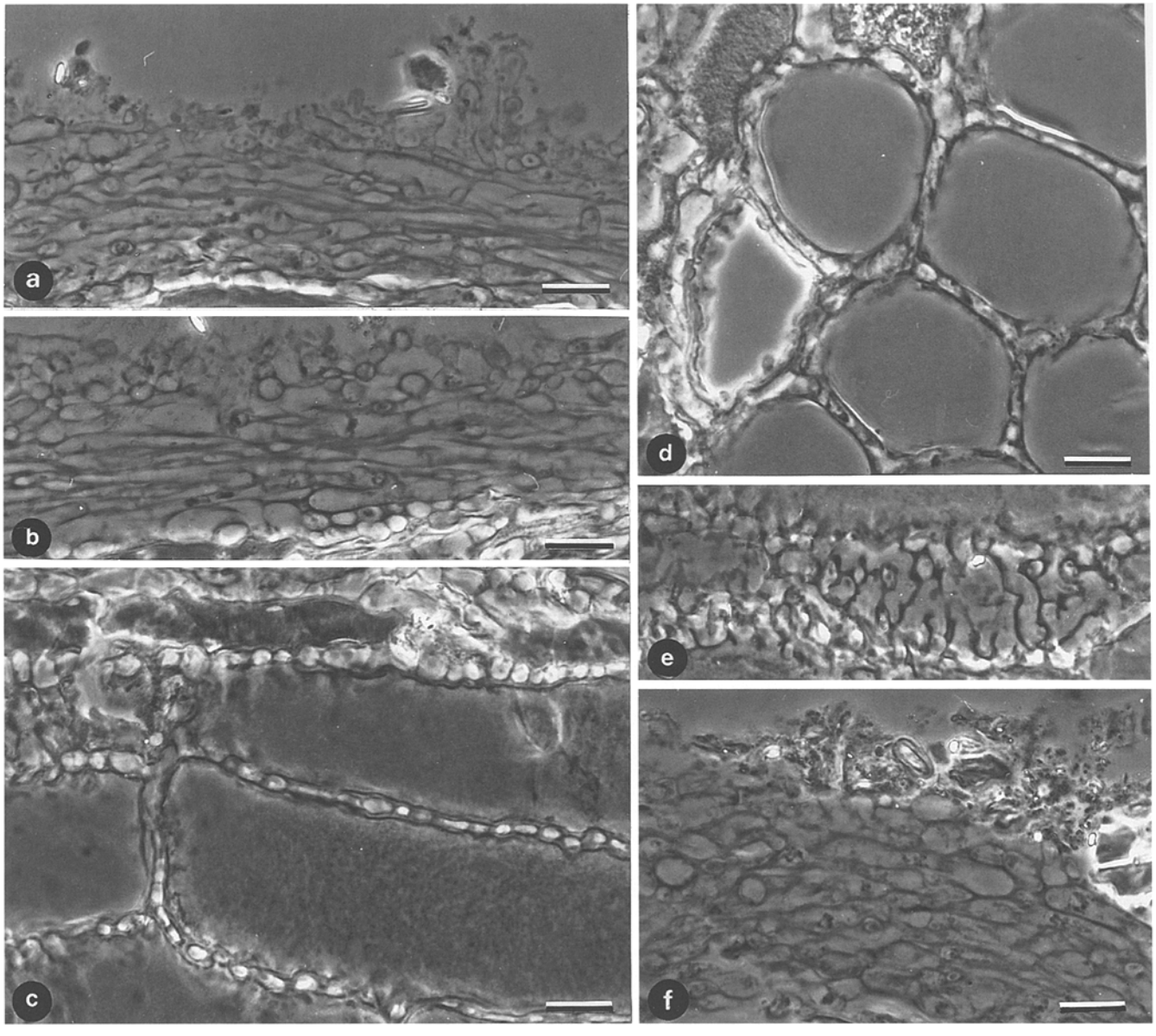

Fig. 3a–f *L. lignyotus* × *Picea abies:* sections of ectomycorrhizae. **a** Cross section, mantle with portions of tannin cells. **b** Longitudinal section, mantle with portions of tannin cells. **c** Longitudinal section, tannin and cortical cells with Hartig net. **d** Cross section, tannin and cortical cells with Hartig net. **e** Longitudinal section, plan view of Hartig net. **f** Longitudinal section through very tip, mantle. Microscopy with phase contrast. All figures from SLO 00428; *bar* = 10 μm

surface. *Tannin cells* (Fig. 3d) tangentially oval to cylindric, 1–2 rows, tangentially (15) 23–55 (70) μm , radially 5–25 (30) μm , TCt (average tangential length of tannin cells) = 37.8 μm , TCq (average ratios between tangential length and radial width of tannin cells) = 3.8; Hartig net 3–5 (10) μm thick, composed of 1 (2) rows of hyphal cells, hyphal cells in section round or radially short cylindric. *Cortical cells* (Fig. 3d) radially to mostly tangentially oval, tangentially (15) 20–37 (43) μm, radially (13) 17–35 (50) μm, CCt = 26.9 μm , CCq = 1.2; Hartig net (2) 3 cortex cell layers deep (tannin cells not included), mostly reaching endodermis; Hartig net 2–3 μm thick, composed of one row of hyphal cells; Hartig net hyphae short to long cylindrical, walls between cells distinct.

Anatomical characteristics, longitudinal section

Mantle (Fig. 3b, f) corresponding to cross section, but layers not very distinct, especially middle layer not pronounced; outer layer as in cross section; hyphae of middle layer radially 2–5 μm thick, with laticifers even up to 7 μm, tangentially 3–15 (30) μm; hyphae of inner layer radially 2–3 μm thick, tangentially (2) 3–10 μm; residues of calyptra cells as strongly light reflecting as in cross-section, irregularly shaped lines very close to root surface. *Mantle of mycorrhizal tip* (Fig. 3f) slightly gelatinous, 30–35 (40) μm thick, laticifers even up to

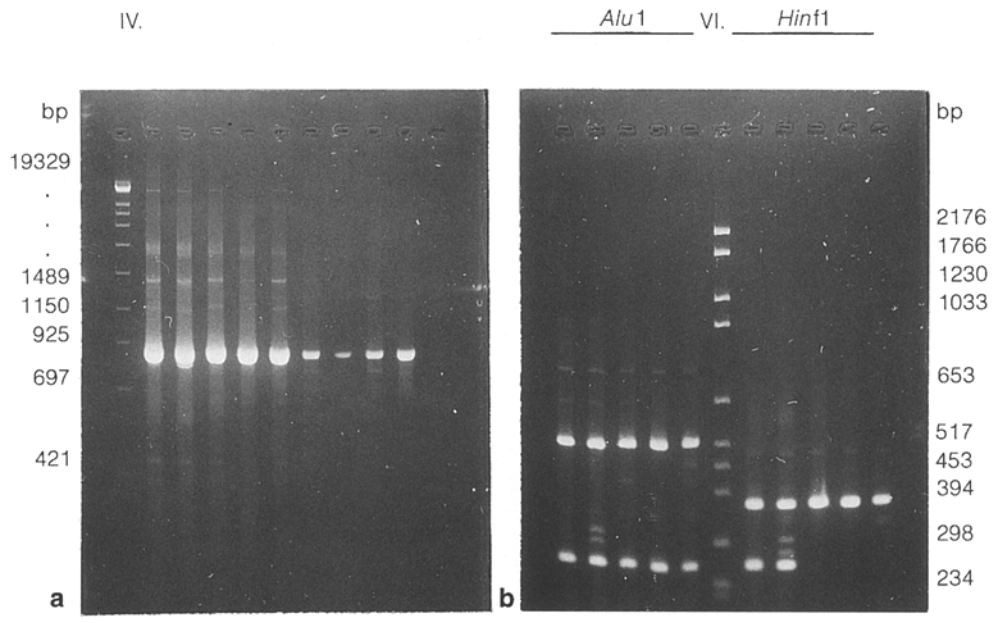

Fig. 4a, b PCR amplification of fungal ITS region in the ribosomal repeats using ITS1-F and ITS4-B primers. **a** Preferential amplification of fungal DNA from fruitbodies (lanes 2, 3 *L. picinus,* lanes 4–6, 10 *L. lignyotus*) and ectomycorrhizae (lanes 7–9 *L. lignyotus* x *Picea abies*); lane 1: molecular-weight marker IV; lane 11: negative control. Dilutions of DNA prior to amplification: lane 10 10-fold, lanes 2–5, 8 100-fold, lanes 6, 7, 9, 11 500-fold. **b** *Alu*1 (lanes 1–5) and *Hinf*1 (lanes 7–11) restriction digests of PCR-amplified fungal DNA from fruitbodies (lanes 1, 2, 7, 8 from *L. picinus,* lanes 3, 4, 9, 10 *L. lignyotus*) and ectomycorrhizae (lane 11 *L. lignyotus* × *Picea abies*); lane 6: molecular-weight marker VI. Dilutions of DNA prior to amplification for fruitbodies 100-fold, for mycorrhizae 500-fold. *L. picinus* from LJU FU2 029, *L. lignyotus* from LJU FU2 028, *L. lignyotus* × *Picea abies* from SLO 00428

7 μm, tangentially 2.5–10 (15) μm, laticifers up to 30 μm long. *Tannin cells* (Fig. 3c) long elliptic to oval, approximately parallel to root surface, tangentially (25) 40–110 μm, radially (4) 8–22 (27) μm, TCt = 75.2 μm, TCq = 6.1. *Cortical cells* (Fig. 3c) elliptic to oval to irregularly cylindric, slightly obliquely to root surface or often parallel, tangentially (29) 55–110 μm , radially (12) 15–36 μm, CCt = 83.8 μm, CCq = 3.6. *Hartig net in plan view* (Fig. 3e) of a weakly ramified palmetti-type, lobes (2) 2.5–4 (5) μm broad, with hyphal septa.

Colour reactions in different reagents

Acid fuchsin: slightly pink; brilliant – cresyl – blue: blue; 70% ethanol: slightly ochre tint destains slightly; iron sulphate: ochre; 40% formol: no reaction; guaiac: blue; 10% KOH: ochre; lactic acid: slightly ochre tint destains slightly; Melzer's reagent: ochre, improves contrast; phenol: darkens slightly; sulpho-vanillin: greyish-pink, well-distinguishable laticifers, latex greyish olive-green.

Autofluorescence

Whole mycorrhiza: UV, 254 nm: no reaction; UV, 366 nm: no reaction. Mantle in cross section: UV filter 340–380 nm: bluish, laticifers more intensive; blue filter 450–490 nm: greenish, laticifers more intensive; green filter 530–560 nm: slightly reddish, laticifers more intensive.

Staining of nuclei and test for siderophily with aceto-carmine

Mostly pairs of nuclei, 1.5 μm in diameter, often very close together; siderophilous granula often present in laticifers, in the cystidia-like hyphal ends and in normal hyphae of the middle mantle-layers, sometimes in small patches very densely aggregated; individual particles up to 0.5 μm long and up to 0.2 μm thick, if very densely aggregated, dimensions unclear.

PCR amplification and restriction digest

Amplified ITS region from *L. picinus* (sporocarp), *L. lignyotus* (sporocarp) and *L. lignyotus* x *Picea abies* (ectomycorrhiza) DNA using ITS1-F and ITS4-B primers all show a distinct fragment between the marker bands of 925 and 697 bp, at approximately 850 bp (Fig. 4a). These fragments from both fungi can be digested into two distinct bands with the *Alu*1 enzyme at 530 bp and 250 bp (Fig. 4b, left). The pattern of the bands using this enzyme could not be used to differentiate between the two fungus species. However, *Hinf*1 produced two characteristic bands at 360 bp and 250 bp for *L. picinus,* but only one band at 360 bp for the fungus and the mycorrhizae of *L. lignyotus* (Fig. 4b, right). Therefore, the restriction digestion using the *Hinf*1 enzyme for digestion of the amplified ITS region provided a differential and characteristic pattern of bands for the two different fungal species and their mycorrhizae.

Material studied and method of identification

Reference specimen: Slovenia, Pohorje, Mislinjski jarek, Forest Research Plot of the Forestry Institute of Slovenia, 850 m above sea level, exposition SW, inclination 40°, shallow distric kambisol, pH $CaCl_2$ 3.66 (sieved sample), *Luzulae albidae – Fagetum* illyricum, predominant tree species Norway spruce (*Picea abies* (L.) Karst), with some larch (*Larix decidua*), in understorey beech (*Fagus sylvatica*) and hazel (*Corylus avellana*); found under spruce, 21 October 1993, fruitbody determined by A. Piltaver (Ljubljana), fruitbodies LJU FU2 028, ectomycorrhiza SLO 00428 in Herbarium Ljubljana and in Herb. R. Agerer (M). Additional material: Slovenija, Pohorje, Mislinjski jarek, Forest Research Plot of the Forestry Institute of Slovenia, 18 August 1993 (fruitbody LJU FU2 019; mycorrhiza SLO 00275), 16 September 1993 (mycorrhiza SLO 00319), 21 October 1993 (mycorrhiza SLO 00383, SLO 00405).

Discussion

L. lignyotus belongs to *Lactarius* sect. *Plinthogali* (Singer 1986). Only one further species associated with spruce is characterized with respect to its ectomycorrhizae in detail out of this section: *L. picinus* (Agerer 1986). *L. lignyotus* ectomycorrhizae are identical to the ectomycorrhizae of all species of this section so far known: *L. acris* (Brand 1991), *L. fuliginosus* (Brand 1991), and *L. picinus* (Agerer 1986). All ectomycorrhizae form the unique mantle type I, with characteristic, often irregularly shaped, protruding hyphal ends, containing densely arranged droplets, often stainable by sulfo-vanillin (Agerer 1994).

The ectomycorrhizae of *L. lignyotus* and *L. picinus* can not easily be distinguished. The laticifers of *L. picinus* seem to be slightly thicker (up to 9 μm) and possess thicker walls (1 μm), the cystidia-like hyphal ends on the surface are slightly thinner in *L. picinus* (3–4 μm). *L. picinus* possesses rhizomorphs but in *L. lignyotus* ectomycorrhizae they could not be found. However, as the rhizomorphs are often very scanty in *L. picinus* this difference should not be overemphasized. One difference could be found with respect to colour change in guiaiac. Only *L. lignyotus* showed a blue-green reaction; but again this feature must be treated with caution, as often this change is dependent upon the freshness of the material to be tested.

The ectomycorrhizae of both species can be clearly distinguished by the variability of their sequences in the ITS region of ribosomal repeats. Using the enzyme *Hinf*1, the restriction digest pattern of the PCR-amplified DNA of the two fungi was different (Fig. 4b). We found the method applied to be fast and reproducible as already observed by Gardes and Bruns (1993). Thus this approach complements the classical anatomical characterization. It is relatively simple in comparison to anatomical determination which requires extensive experience. Furthermore, classical determinations of ectomycorrhizae in soil cores can offer 'group' type determinations: for example, if an ectomycorrhiza is found to show all anatomical characteristics for *L. badiosanguineus*, it can still only be classified as 'belonging to the *L. badiosanguineus* group', since there might exist some as yet unidentified types possessing the same characteristics. In contrast to these 'group' identifications, the applied PCR technique offers clear species determination, providing restriction digest patterns are known.

We suggest that PCR methods should be used and further developed for use with every anatomical description of ectomycorrhizae in the future. The method itself must accordingly be further developed in respect of new probes, such as mitochondrial rRNA-oriented species-specific oligonucleotide probes, as tested by Bruns and Gardes (1993). Molecular biology approaches can not replace classical taxonomical procedures but they may simplify and add to the objectivity of these and thus allow researchers to deal with large numbers of ectomycorrhizae. One such example would be the screening for potential bioindications of forest site pollution by the estimation of abundance of ectomycorrhizal types known to be diagnostic for polluted sites.

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