Mycologia, 102(6), 2010, pp. 1263–1273. DOI: 10.3852/09-311

Description and identification of *Alnus acuminata* ectomycorrhizae from Argentinean alder stands

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Abstract: The objective of this study was to describe the morphological and anatomical features of five unidentified ectomycorrhizal types of *Alnus acuminata* and to complement their identification based on ITS-rDNA sequence analysis. The combined approach of morphotyping and sequence analysis based on ITS sequence comparison with sequences contained in GenBank and the UNITE database let us assign three of the five field-collected ectomycorrhiza morphotypes to the tomentella-thelephora lineage that closely matched European and North American species. The sequencing results within *Tomentella* point toward alder specific clades within *T. sublilacina*, *T. ellisii* and *T. stuposa* sensu lato. The two other EcM morphotypes matched *Lactarius omphaliiformis* and a *Russula* sp. Better focused, concomitant fruit body surveys are needed for accurate identification of South American ectomycorrhizal fungi because of the evidence of cryptic speciation in both agaricoid and resupinate mycobionts.

Key words: Andean alder, ectomycorrhizas, ITS sequence analysis, morphological anatomical characterization, morphotyping, *Tomentella*

Submitted 13 Dec 2009; accepted for publication 21 Apr 2010.
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INTRODUCTION

Genus *Alnus* associates with a few dozens of ectomycorrhizal (EcM) fungal species that are highly host specific (Miller et al. 1991, 1992; Molina 1979). Examples include *Alpova diplophloeus* (Zeller and Dodge) Trappe & Smith and several species of genera *Cortinarius*, *Lactarius*, *Alnicola* and *Russula* (for a detailed list see Becerra et al. 2005c). In addition several host promiscuous species of EcM fungi, such as *Cenococcum geophilum* Fr. (Trappe 1964) and *Tomentella sublilacina* (Ellis and Holw.) Wakef. (Kennedy and Hill 2010, Pritsch et al. 2000), have been reported. Tedersoo et al. (2009) demonstrated the presence of 25 morphotypes comprising 39 species of EcM fungi on root systems of *A. incana* and *A. glutinosa* in Estonia but found that host promiscuity occurred only in ascomycetous mycobionts. Alder-associated fungi differ substantially in biochemical and morphometric characters, suggesting differential functions (Pritsch 1996, Ostonen et al. 2009).

Descriptions of EcM, based on morphological and anatomical features, allow recognition of fungal taxa in field samples and sometimes provide species identification (Agerer 1991). However morphological-anatomical characters usually do not allow differentiation of closely related species. Therefore DNA-based identification methods such as PCR-RFLP and sequence analysis have been established for identification of EcM fungal species (Egger 1995, Horton and Bruns 2001, Kõljalg et al. 2001, Sakakibara et al. 2002).

EcM of Andean alder (*Alnus acuminata* Kunth) were studied during investigations on the ecology of Andean alder stands in northwestern Argentina. Eleven EcM morphotypes of Andean alder have been characterized hitherto by morphological-anatomical studies. Five morphotypes were identified based on matching RFLP patterns of the rDNA internal transcribed spacer (ITS) region from EcM root tips and fruit bodies (Becerra et al. 2002, 2005a, c), while six remained unidentified. In this study we describe five of the six morphotypes and identify these taxa based on ITS sequence comparison with data in public sequence databases such as GenBank and UNITE (Abarenkov et al. 2010, Kõljalg et al. 2005). The remaining morphotype is not described due to consistent failure of molecular identification and exhaustion of material available for DNA extraction.

TABLE I. Identification of *Alnus acuminata* EcM based on sequence comparison to GenBank and UNITE sequences

Voucher	Ectomycorrhiza morphotype	GenBank accession	Best BLAST match	Sequence similarity (percent)
AB06	*Tomentella* cf. *sublilacina*	DQ195590	*Tomentella sublilacina* UDB002972	99.8
AB08	*Tomentella* cf. *stuposa*	DQ195591	*Tomentella stuposa* UDB002428	95.1
AB10	*Tomentella* cf. *ellisii*	DQ195592	*Tomentella ellisii* UDB003326	97.3
AB11	*Lactarius omhaliiformis*	DQ195593	*Lactarius omhaliiformis* UDB002514	100.0
AB12	*Russula* sp.	n.d.[a]	*Russula puellaris* UDB000010	93.8

[a] n.d., not deposited due to >1% ambiguous bases.

MATERIALS AND METHODS

Sampling and sample preparation.—Mycorrhizal roots and EcM fruit bodies were sampled at two sites, Quebrada del Portugués, Tafí del Valle, (Tucumán Province), and Narvaez Range, (Catamarca Province) in northwestern Argentina (NOA), in Feb, Mar, May and Nov (summer, fall, spring) 2000–2002. Details of the two sites, including phytosociological and pedological aspects, are reported in Becerra (2002) and Becerra et al. (2005b). Soil cores (15 × 15 cm) to about 10 cm deep were collected concurrently below fruit bodies. The samples were placed in plastic bags and stored at 4 C until processed.

Morphological descriptions were prepared according to Agerer (1991) from fresh and FEA-fixed (5% formaldehyde, 5% acetic acid, 90% ethanol) EcM. For each morphotype several tips were subjected to comparative anatomical studies. Photographs were taken with a Leica M420 stereo microscope. Several chemical reagents (15% KOH, Melzer's reagent, cotton blue, 70% ethanol, sulpho-vanillin, NH_4OH, and lactic acid) were used to study specific color reactions of mycorrhizae. Description of the EcM follows the terminology of Agerer (1991, 1999) and Miller et al. (1991). Fresh mycorrhizae were prepared for direct microscopy of the hyphal mantle (hand sections or mantle peeled off) according to Agerer (1991). Mycorrhizae were dehydrated previously with a series of 20, 30, 40, 50, 60, 70, 80, 90, 96, 100% ethanol. Mycorrhizae were embedded in Leica Historesin kit and semi-thin tangential, and cross sections were cut with a microtome with steel knives. Peeled off mantles, mantle plan views and sections from resin-embedded mycorrhizae were examined and photographed with a Zeiss Axiophot light microscope at 200–1000× magnification. Characterization of the Hartig net follows Godbout and Fortin (1983). From each morphotype, several tips were stored in CTAB buffer (Doyle and Doyle 1990) at 4 C for up to 1.5 y before DNA extraction. Voucher specimens of FEA-fixed roots were deposited in the Museo Botánico de Córdoba Herbarium (CORD) (Holmgren et al. 1990).

DNA extraction and PCR.—DNA was extracted from single, CTAB-stored EcM root tips with the DNeasy Plant Minikit (QIAGEN, Hilden, Germany). DNA was extracted from at least two replicates from each morphotype. PCR was performed with primers ITS1F and ITS4B (Gardes and Bruns 1993). The 50 µL PCR mix contained 5 µL template DNA in empirically determined dilutions of 1 : 10–1 : 100, 1.5 mM $MgCl_2$, 2 mM dNTPs, 20 pmol each primer, 10% amplification buffer and 1 U Taq polymerase (Invitrogen, Karlsruhe, Germany) added after a 5 min hot start at 95 C. Subsequent cycling conditions were 30 cycles of 95 C, 55 C, 72 C 60 s each and a final extension cycle of 72 C for 10 min. PCR products were checked on an 1.5% agarose gel and purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Sequencing was run on an ABI Prism 3730 capillary sequencer (Applied Biosystems, Foster City, California) by Sequiserve (Vaterstetten, Germany) with primers ITS1F and ITS4B.

Sequence analysis.—Sequences of both strands were edited manually and merged to contigs. The sequences are available in GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers DQ195590–DQ195593. Database search for similar sequences and sequence collection from the respective databases was performed with a MegaBLAST algorithm against GenBank and BLASTN in the UNITE database (Abarenkov et al. 2010). To confirm the sequence comparison-based identification and uncover the phylogenetic affinities of *Tomentella* spp. sequences from *Alnus acuminata* EcM morphotypes and identified fruit body specimens were included in the dataset. Sequences were aligned automatically with Clustal W (http://www.ebi.ac.uk/clustalw/) and corrected manually. Alignments were imported to PAUP* 4.0d81 (Swofford 2002), and a NJ analysis based on raw distances was performed.

RESULTS

Identification of ectomycorrhiza.—The DNA of all five EcM morphotypes was successfully amplified and sequenced. Based on BLAST matches, these taxa belonged to the tomentella-thelephora or russula-lactarius lineages (cf. Tedersoo et al. 2010) that respectively comprised three and two species (TABLE I). The closest relatives of four taxa were well resolved, but that of the *Russula sp.* remained ambiguous in part due to moderate sequence quality. Paucity of extractable material of this morphotype hindered greater replication of DNA analyses. Phylogenetic analyses confirmed BLAST matches and placement of the *Tomentella* species among *Tomentella* and *Thelephora* isolates originating in the northern hemisphere (FIG. 1).

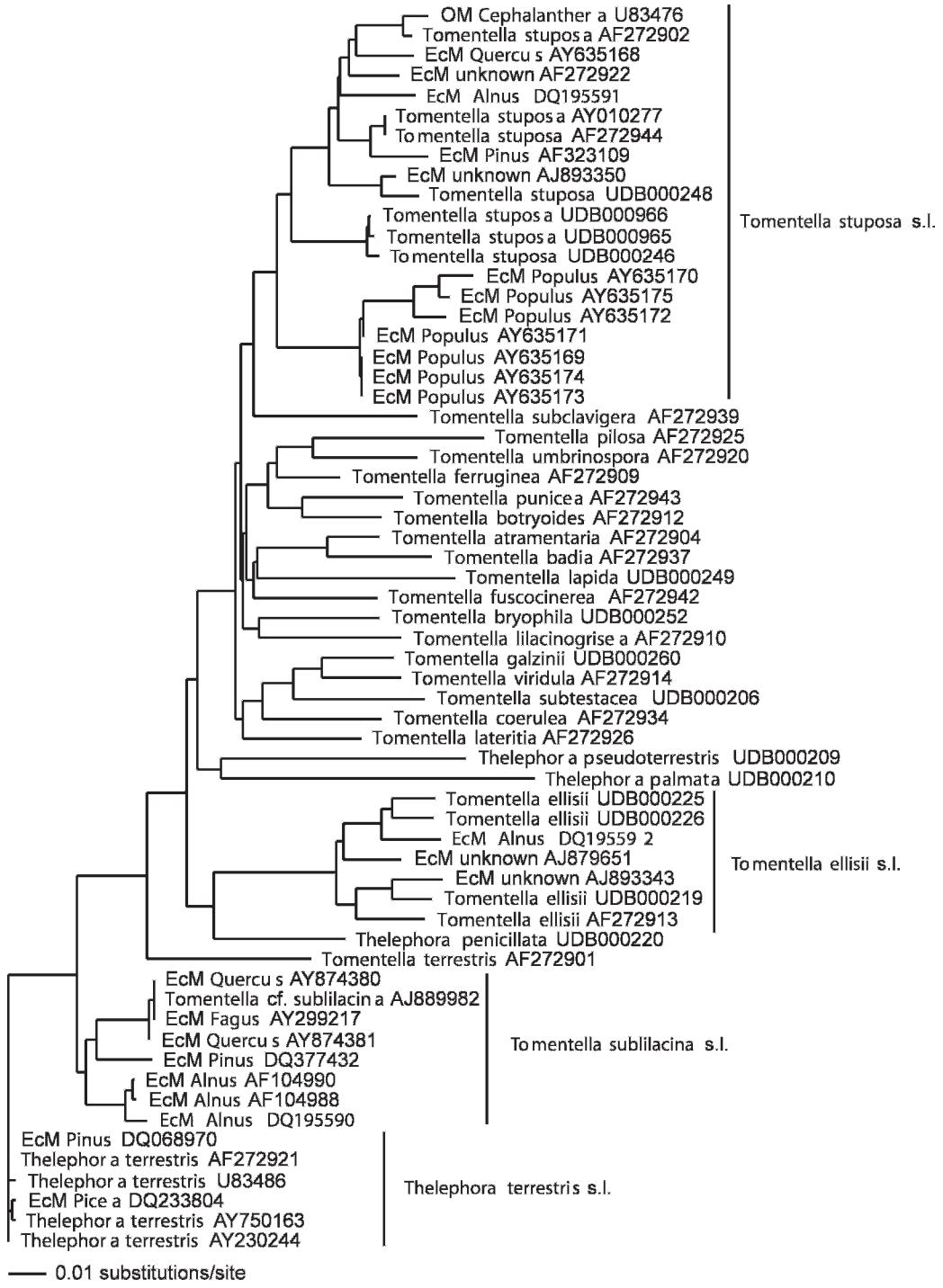
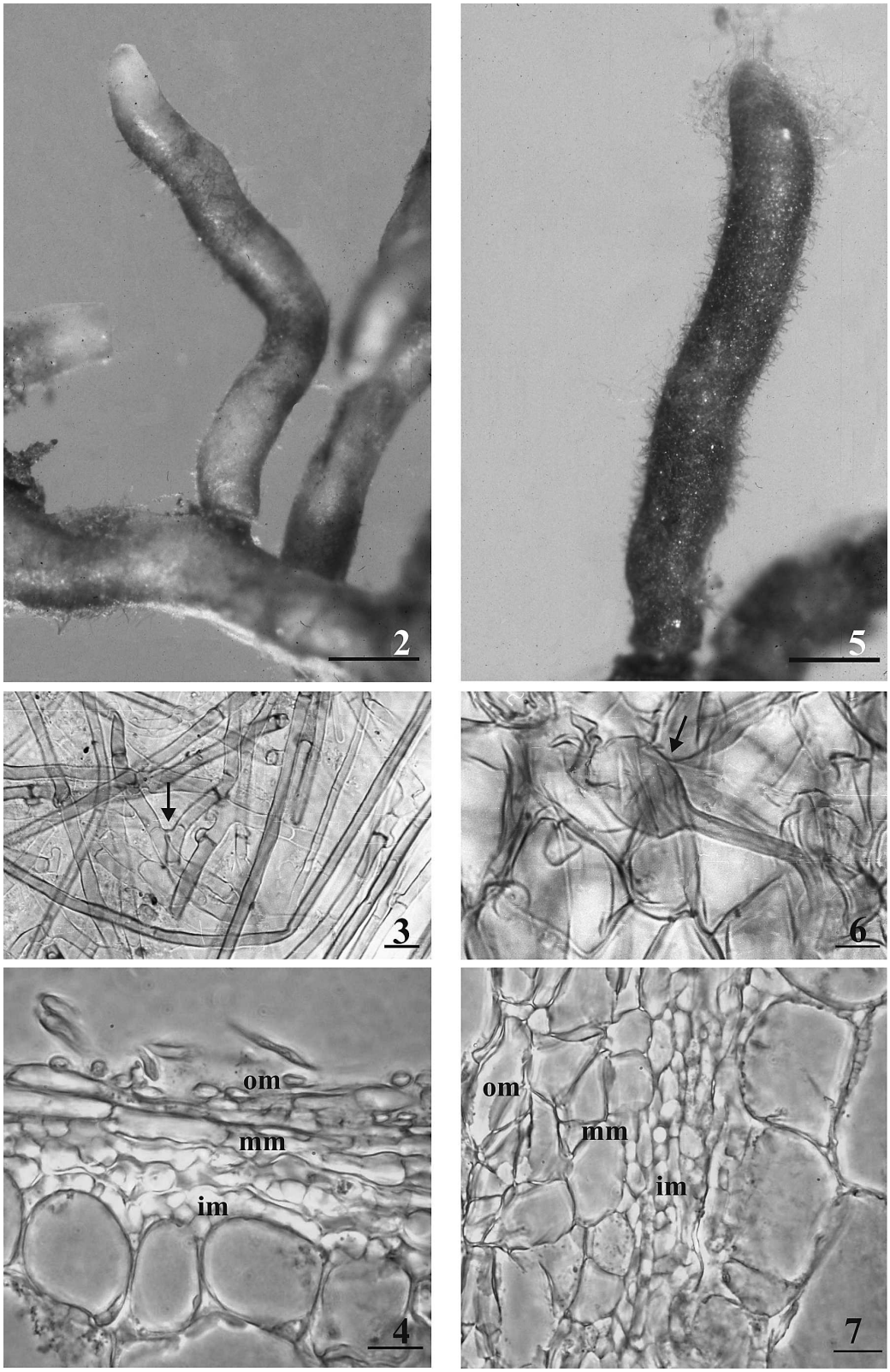


FIG. 1. Neighbor joining phylogram demonstrating the phylogenetic placement of alder-associating fungal taxa (in boldface) among fruit body- and mycorrhiza-derived *Tomentella* species. EcM, ectomycorrhiza; OM, orchid mycorrhiza.

Description of morphotypes.—*Tomentella* cf. *sublilacina* (FIG. 2) (described by Pritsch [1996] as ''*Alnirhiza cystidiobrunnea*'').

Anatomical characters of mantle in plan views. Mantle usually continuous over the root apex. Blue granules and needle-like contents after fixation are lacking. Outer mantle layers plectenchymatous with hyphal net arrangement; hyphae undifferentiated, cylindrical not constricted at septa, cell walls slightly brown, 2.5–4 μm diam, thin-walled (< 0.5 μm); clamps present, anastomoses open (type A, Agerer 1991), septa thinner than walls; cell surface smooth with many soil particles. Middle mantle layers plectenchymatous with parallel hyphal arrangement; hyphal walls slightly brown,



FIGS. 2–4. Light micrographs of *Tomentella* cf. *subliblacina* on *Alnus acuminata*. 2. Ectomycorrhizal system of *T.* cf. *subliblacina*. 3. Emanating hyphae smooth and straight with anastomoses open (→). 4. Longitudinal section of the mantle with three distinct mantle layers; outer mantle layer plectenchymatous (om); middle mantle layer plectenchymatous (mm); inner mantle layer plectenchymatous to pseudoparenchymatous (im). Bars: 2 = 0.5 mm, 3–4 = 10 μm.

4–6 μm diam, walls thin (< 0.5 μm); smooth. Inner mantle layers, plectenchymatous to pseudoparenchymatous, without discernible pattern; cell walls yellowish brown, 4.5–8 μm diam, without clamps.

Anatomical characters of emanating elements. Rhizomorphs up to 32 μm diam, undifferentiated, hyphae loosely woven and of uniform diameter (5–10 agglutinated hyphae), walls slightly brown, with clamps. Emanating hyphae smooth, straight, ramification Y-shaped with one side branch at septum in considerable distance from septum, distance of septa 15–40 μm; clamps present, secondary septa frequent, partially crowded; clamps in dorsal and lateral view thinner than the hypha, in dorsal view oval, in lateral view less than a semicircle; hyphae 2–3.5 μm diam, cell walls slightly thickened (< 1 μm), of uniform thickness; cell walls yellowish brown; elbow-like protrusions lacking. Anastomoses open with a short bridge or bridge almost lacking, anastomosal bridge as thick as hyphae (FIG. 3).

Anatomical characters in longitudinal section. Mantle 23–36 μm thick (FIG. 4), three distinct mantle layers discernible; outer mantle layer plectenchymatous, hyphae tangentially 3–22 μm, radially 1.5–4 μm; middle mantle layer plectenchymatous, hyphae tangentially 6.5–20 μm, radially 3–8 μm; inner mantle layer plectenchymatous to pseudoparenchymatous, hyphae tangentially 3–6.5 μm, radially 3–6.5 μm. Tannin cells lacking. Epidermal cells rectangular, parallel to root axis, tangentially 9–42 μm, radially 8–16 μm. Hartig net paraepidermal, in plan view infrequently lobed; 1–3 μm wide lobes around epidermal cells.

Color reaction with different reagents. Whole ectomycorrhizae bleach with 15% KOH and lactic acid. Emanating hyphae and mantle stain slightly blue with cotton blue. Amyloid reaction (septa and clamps) with Melzer's reagent; none with sulpho-vanillin, 70% ethanol or NH_4OH.

Specimens examined. ARGENTINA. CATAMARCA PROVINCE. Sierra de Narváez. 27°43′S, 65°54′W, 1820 m. Ectomycorrhizae under *A. acuminata*, 7 Nov 2001, *A. Becerra 06* (CORD); TUCUMÁN PROVINCE. Quebrada del Portugués. Tafí del Valle. 26°58′S, 65°45′W, 2187 m.

Tomentella cf. *stuposa*.

Morphological characters. Mycorrhizal systems up to 45 mm long, irregularly monopodial-pinnate, orders of ramification 0–2(3); systems abundant, dense. Unramified ends straight to tortuous, tapering tip, up to 22 mm long and 0.2–0.5 mm diam. Entire mycorrhiza surface ocher to brown when young, dark brown when older (FIG. 5), not carbonizing; surface of unramified ends glistening, short spiny. Root tips blunt and covered by the mantle. Mantle not transparent. Rhizomorphs not observed.

Anatomical characters of mantle in plan views. Mantle continuous over root apex. Blue granules and needle-like contents after fixation are lacking. Outer mantle layers pseudoparenchymatous, with angular and roundish cells, large areas covered with cystidia (FIG. 6); mantle cells 26–38 μm long and 16–25 μm diam, 5–6 cells in a square, 20 × 20 μm; mantle cells walls slightly brown, walls 0.5 μm thick, cell surface smooth. Middle mantle layer pseudoparenchymatous with angular cells; cell walls slightly brown, 18–34 μm long and 11–21 μm wide, cell walls less than 0.5 μm thick, 5–6 cells in a square of 20 × 20 μm. Inner mantle layer plectenchymatous, without pattern, clamps not frequent, cell walls slightly brown, 4–5 μm diam, distance of hyphal septa 8–30 μm, straight ramification infrequent.

Anatomical characters of emanating elements. With clamps, backward-oriented ramifications and reversed clamps present, anastomoses and intrahyphal hyphae not observed. Rhizomorphs not observed. Emanating hyphae straight, ramification Y-shaped, with two side branches at septum in considerable distance from septum, distance of septa 9.5–46 μm, hyphae 5–8 μm thick; cell walls 0.5–1 μm thick, slightly brown, smooth, apical ends simple, walls at tip thinner than remaining walls; secondary septa frequent, evenly distributed, clamps present, without a hole, in lateral view thinner than the hypha, shaped less than a semicircle, in dorsal view oval. Cystidia bottle-shaped with a straight to bent neck (type B, Agerer and Rambold 1998) (FIG. 6), 37–65 μm long, at bases 8–20 μm diam and apically 2–4.5 μm; cell walls slightly brown, uneven thickness, similar to walls of mantle cells; septa lacking, surface of cystidia smooth, without contents.

Anatomical characters in longitudinal section. Mantle 45–65 μm thick (FIG. 7), three distinct mantle layers discernible; outer and middle mantle layer pseudoparenchymatous, cells of outer and middle layer tangentially 12–35 μm, radially 8–27 μm; inner mantle layer plectenchymatous, hyphae tangentially 3–11 μm,

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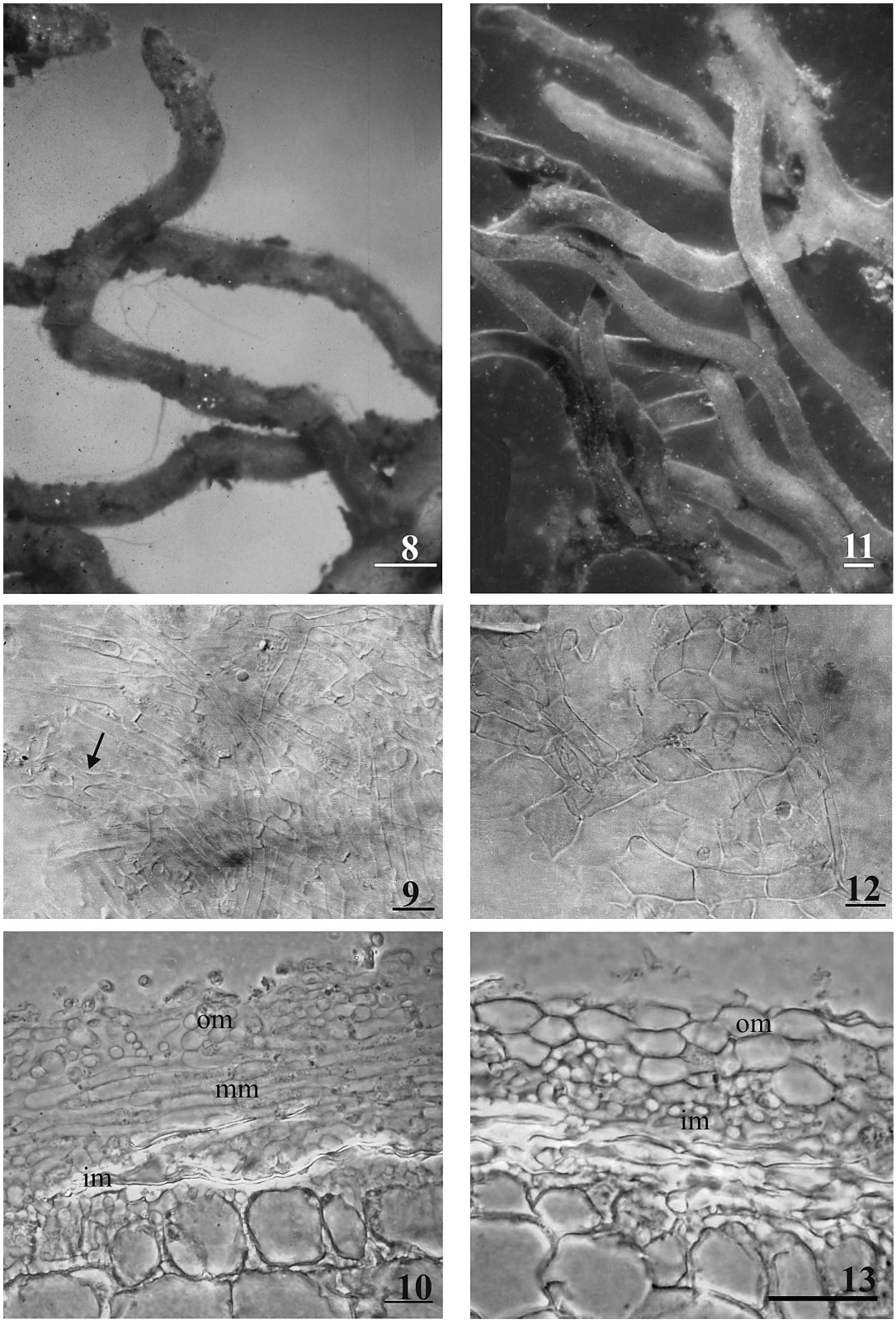
FIGS. 5–7. Light micrographs of *Tomentella* cf. *stuposa* on *Alnus acuminata*. 5. Ectomycorrhizal systems of *T.* cf. *stuposa*. 6. Bottle-shaped cystidia with a straight to bent neck on mantle (→). 7. Longitudinal section of the mantle with three distinct mantle layers; outer (om) and middle mantle (mm) layer pseudoparenchymatous; inner mantle layer plectenchymatous (im). Bars: 5 = 0.5 mm, 6 = 5 μm, 7 = 10 μm.



FIGS. 8–10. Light micrographs of *Tomentella* cf. *ellisii* on *Alnus acuminata*. 8. Ectomycorrhizal systems of *T.* cf. *ellisii*. 9. Emanating hyphae smooth and straight with anastomoses open (→). 10. Longitudinal section of the mantle with three distinct mantle layers; outer mantle (om) and middle mantle (mm) layer plectenchymatous; inner mantle (im) layer pseudoparenchymatous. Bars: 8 = 0.5 mm, 9–10 = 10 µm.

radially 1.5–6.5 μm. Tannin cells lacking. Epidermal cells rectangular to cylindrical, parallel to root axis, tangentially 13–24 μm, radially 8–13 μm. Hartig net paraepidermal to periepidermal, in plan view infrequently lobed; 1.5–4 μm wide lobes around epidermal cells.

Color reaction with reagents. Whole ectomycorrhizae bleach with 15% KOH and lactic acid. Emanating hyphae and whole ectomycorrhizae stain slightly blue with cotton blue. No reaction with Melzer's reagent, sulpho-vanillin, 70% ethanol or NH_4OH.

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Tomentella cf. *ellisii*

Morphological characters. Mycorrhizal systems up to 46 mm long, irregularly monopodial-pinnate, orders of ramification 0–2; systems abundant, dense. Unramified ends straight to bent some tortuous, tapering tip, up to 14 mm long and 0.2–0.4 mm diam, light brown when young, light brown to gray when older (FIG. 8). Surface of unramified ends shiny, loosely stringy. Root tips acute and usually covered by the mantle. Mantle not transparent; mycorrhiza not carbonizing. Rhizomorphs not observed.

Anatomical characters of mantle in plan views. Mantle continuous over root apex. Lacking are blue granules and needle-like contents. Outer mantle layers plectenchymatous with irregularly arranged hyphae, without pattern (type B, Agerer and Rambold 1998), hyphae undifferentiated, cylindrical not constricted at septa, colorless to slightly yellow, 4–5 μm diam, thin-walled (< 0.5 μm), with clamps, septa thicker than walls. Middle mantle layers plectenchymatous, with parallel hyphal arrangement, hyphal walls slightly yellow, 4–5 μm diam, walls thin (< 0.5 μm), smooth. Inner mantle layer pseudoparenchymatous, with inflated cells, 5–11 μm long and 4–7 μm wide, cell walls slightly yellow.

Anatomical characters of emanating elements. With clamps, backward-oriented ramifications and reversed clamps present, septal pores with globular thickenings, anastomoses open, with a long bridge (FIG. 9); cell walls as thick as remaining walls, and anastomosal bridge thinner than hyphae. Rhizomorphs not observed. Emanating hyphae not striking to wavy, ramification approximately Y-shaped, with one side branch at septum considerable distance from septum; hyphal ends tortuous, screw-like; distance of septa 8–52 μm; clamps present, secondary septa frequent, evenly distributed; hyphae 3–4 μm thick, cell walls (< 0.5 μm), evenly thick, cells walls colorless (FIG. 9); surface smooth; clamps without a hole, in lateral view thinner than the hypha, shaped less than a semicircle, in dorsal view oval.

Anatomical characters in longitudinal section. Mantle 70–105 μm thick (FIG. 10), three distinct mantle layers discernible; outer and middle mantle layer plectenchymatous; hyphae of outer and middle layer 4–45 μm tangentially and 2–6 μm radially; inner mantle layer pseudoparenchymatous, hyphae of inner layer 4–7 μm tangentially and 2.5–4 μm radially.

Tannin cells lacking. Epidermal cells rectangular, parallel to root axis, tangentially 24–38 μm, radially 12–15 μm. Hartig net paraepidermal to periepidermal, one row around epidermal cells, in plan view palmetti type; 1.5–6 μm wide lobes around cortical cells.

Color reaction with reagents. Whole ectomycorrhizae bleach with 15% KOH and lactic acid. Sulpho-vanillin: whole ectomycorrhizae bleached, Hartig net stained pink. Emanating hyphae stain slightly blue with cotton blue. Some septa slightly amyloid with Melzer's reagent. No reaction with 70% ethanol or NH_4OH.

Specimens examined. ARGENTINA. CATAMARCA PROVINCE. Sierra de Narváez. 27°43′S, 65°54′W, 1820 m. Ectomycorrhizae under *A. acuminata*, 7 Nov 2001, *A. Becerra 10* (CORD); TUCUMÁN PROVINCE. Quebrada del Portugués. Tafí del Valle. 26°58′S, 65°45′W, 2187 m.

Lactarius omphaliiformis Romagn.

Morphological characters. Mycorrhizal systems up to 18 mm long, irregularly monopodial-pinnate to monopodial pyramidal, orders of ramification 0–2; systems occurring solitary or in few numbers. Unramified ends bent to almost sinuous, preferentially tortuous, tip not inflated, up to 15 mm long and 0.2–0.4 mm diam, entire mycorrhiza surface orange to ocher, slightly yellow when young, brown when older (FIG. 11). Surface of unramified ends smooth covered with soil particles. Root tips blunt and covered by the mantle. Mantle not transparent; mycorrhiza not carbonizing. Rhizomorphs not observed.

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FIGS. 11–13. Light micrographs of *Lactarius omphaliiformis* on *Alnus acuminata*. 11. Ectomycorrhizal systems of *L. omphaliiformis*. 12. Epidermoid cells bearing a hyphal net on the outer mantle layer. 13. Longitudinal section of mantle two distinct mantle layers discernible; outer mantle (om) layer densely pseudoparenchymatous; inner mantle (im) layer plectenchymatous. Bars: 11 = 0.5 mm, 12–13 = 10 μm.

Anatomical characters of mantle in plan views. Mantle continuous over the root apex. Blue granules and a matrix are lacking, but needle-like contents after fixation are present. Outer mantle layers pseudoparenchymatous with epidermoid cells bearing a hyphal net (type Q, Agerer and Rambold 1998) (FIG. 12), mantle cells 7–34 µm long and 6–20 µm wide, walls of hyphal net faint, delicate, inconspicuous, thin, hyphal net not specialized. Walls of mantle cells slightly yellow, thin, 0.3–0.5 µm, hyphae without clamps, septa as thick as walls, surface of cells smooth, more or less gelatinous (sticky); with cells filled with latex that stain in sulpho-vanillin, angular shape, 6–13 µm wide. Inner mantle layer plectenchymatous, without pattern, clamps not observed, cell walls slightly yellow, 2–3 µm diam.

Anatomical characters of emanating elements. Rhizomorphs not observed. Emanating hyphae smooth, wavy, shape not striking, 1.5–3 µm diam, cell walls < 0.5 µm thick, colorless to slightly yellow; elbow-like protrusions lacking; distal ends of hyphae simple; septal distances 4–18 µm, without clamps; with septa as thick as remaining walls.

Anatomical characters in longitudinal section. Mantle 44–58 µm thick (FIG. 13), two distinct mantle layers discernible; outer mantle layer pseudoparenchymatous, hyphae tangentially 6.5–18 µm, radially 3–8 µm; inner mantle layer plectenchymatous, hyphae tangentially 3–10 µm, radially 1.5–6.5 µm. Epidermal cells rectangular, parallel to root axis, tangentially 13–35 µm, radially 9.5–21 µm. Hartig net paraepidermal, in plan view infrequently lobed; 2.4–5 µm wide lobes around cortical cells.

Color reaction with reagents. Whole ectomycorrhizae stain ocher-brown with 15% KOH and bleach with lactic acid. Sulpho-vanillin: walls pink, contents of mantle cells with droplets, laticifers black. Emanating hyphae and laticifers stain slightly blue with cotton blue. Tips of unramified ends and the whole ectomycorrhizae stain black with 70% ethanol. No reaction with Melzer's or NH_4OH;

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Russula sp.

Morphological characters. Mycorrhizal systems up to 10 mm long, simple to irregularly monopodial-pinnate, orders of ramification 0–1; systems solitary or in a few numbers. Unramified ends straight to sinuous, tapering tip, up to 7 mm long and 0.5 mm diam, ocher, yellowish brown when young, brown when older (FIG. 14); surface of unramified ends densely to loosely long, spiny. Root tips acute and usually covered by the mantle. Mantle not transparent; mycorrhiza not carbonizing. Rhizomorphs not observed.

Anatomical characters of mantle in plan views. Mantle continuous over the root apex. Blue granules and needle-like contents are lacking. Outer mantle layers plectenchymatous with irregularly arranged hyphae, without any unique pattern (type B, Agerer and Rambold 1998); hyphae undifferentiated, cylindrical not constricted at septa, walls slightly yellow, 1–2 µm diam, thin-walled (< 0.5 µm), without clamps, septa as thick as walls. Middle mantle layer plectenchymatous, with parallel hyphal arrangement; hyphal walls slightly yellow, 2–6 µm diam, walls thin (< 0.5 µm), smooth, anastomoses without septa. Inner mantle layers plectenchymatous to pseudoparenchymatous, with slightly yellow, inflated cells, 2–7 µm diam, walls slightly yellow.

Anatomical characters of emanating elements. Ramifications backward-oriented clamps and intrahyphal hyphae are lacking. Anastomoses open, with a long bridge, cell walls as thick as remaining walls (FIG. 15). Rhizomorphs not observed. Emanating hyphae straight to wavy, ramification approximately Y-shaped, with one side branch at septum in considerable distance from septum, hyphal ends simple; distance of septa 4–85 µm, clamps lacking, septa evenly distributed; hyphae 1–2 µm thick; cell walls < 0.5 µm, evenly thick, slightly yellow; hyphal surface smooth with soil particles.

Anatomical characters in longitudinal section. Mantle 25–75 µm thick (FIG. 16), three distinct mantle layers discernible; outer and middle mantle layer plectenchymatous, hyphae of outer and middle layer 2.5–40 µm tangentially and 1.5–2 µm radially; inner mantle layer plectenchymatous to pseudoparenchymatous, hyphae of inner layer 3.5–6 µm tangentially and 2.5–5 µm radially. Tannin cells lacking. Epidermal cells rectangular, parallel to root axis, tangentially 24–38 µm, radially 12–15 µm. Hartig net paraepidermal, in plan view palmetti type, lobes 1.5–2.5 µm wide.

Color reaction with reagents. Whole ectomycorrhizae bleaching with 15% KOH and lactic acid. Sulpho-vanillin: whole ectomycorrhizae bleaching, Hartig net staining pink. Emanating hyphae slightly staining blue with cotton blue. Some septa slightly amyloid with Melzer's reagent. No reaction with 70% ethanol or NH_4OH.

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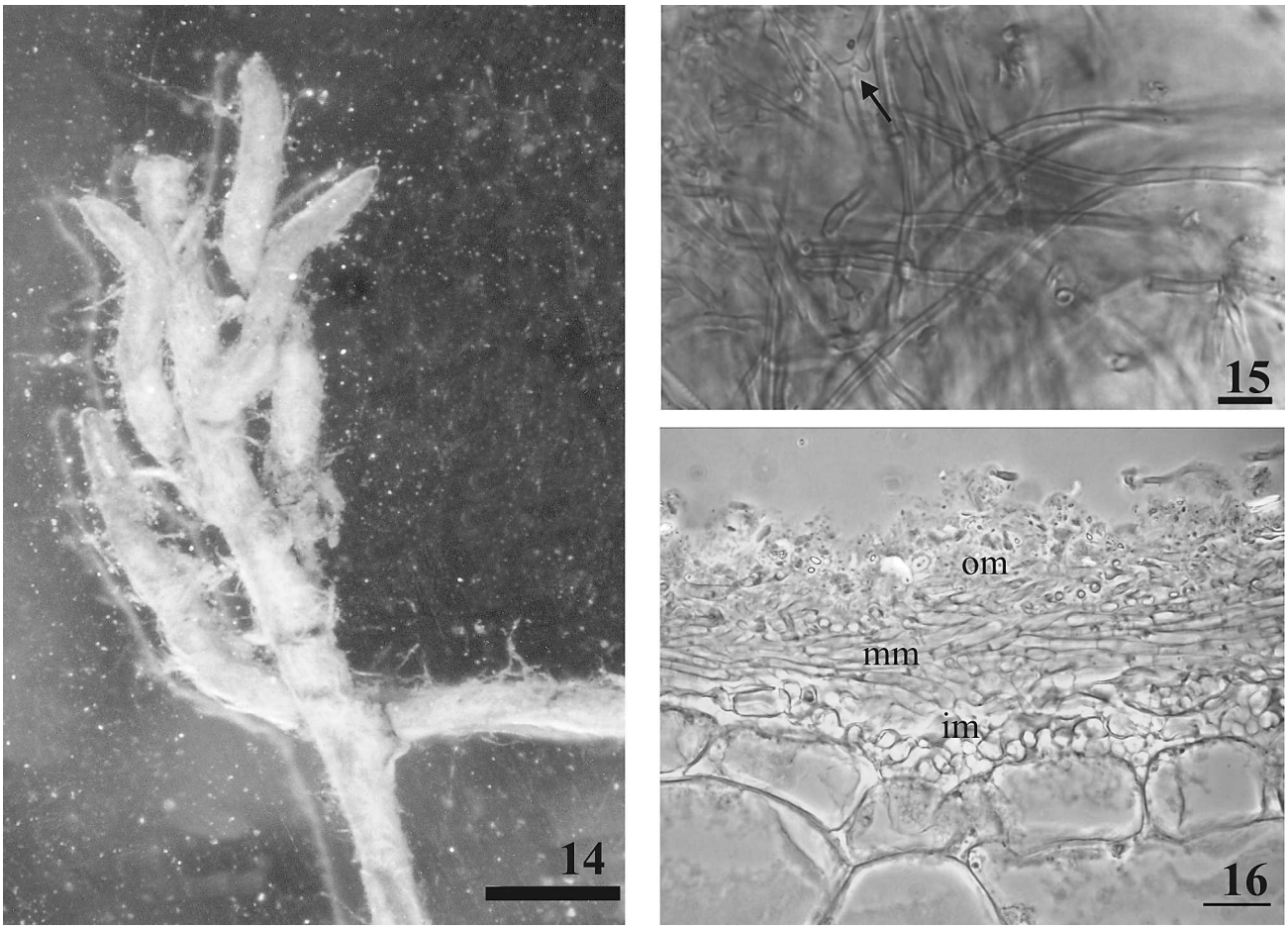
DISCUSSION

In this study the combined approach of anatomotyping and ITS-DNA sequence analysis was performed to



FIGS. 14–16. Light micrographs of *Russula* sp. on *A. acuminata*. 17. Ectomycorrhizal systems of *Russula* sp. 18. Emanating hyphae smooth and straight with anastomoses open (→). 19. Longitudinal section of mantle three distinct mantle layers discernible; outer mantle (om) layer plectenchymatous; middle mantle (mm) layer pseudoparenchymatous; inner mantle (im) layer plectenchymatous. Bars: 17 = 0.5 mm, 18–19 = 10 μm.

identify field-collected EcM of *Alnus acuminata* to species or genus. Three EcM morphotypes were placed in the tomentella-thelephora lineage. *Tomentella* cf. *sublilacina* morphotype clustered with isolates of *Tomentella sublilacina* and shared morphological and anatomical features similar to ''Type I: unknown mycobiont'' on *Alnus rubra* (Miller et al. 1991) and ''*Alnirhiza cystidiobrunnea*'' on *Alnus glutinosa* (Pritsch 1996, Pritsch et al. 1997). Based on the ocher to brown surface and mantle structure (pseudoparenchymatous with angular cells), the *T.* cf. *stuposa* morphotype resembled the ''clavate, dark brown'' and ''type 4'' on *Alnus rubra* described respectively by Neal et al. (1968) and Miller et al. (1991) and *T. stuposa* (Link) Stalpers (Jakucs et al. 2005). However *T.* cf. *stuposa* differs from these mycorrhizas by the bottle-shaped cystidia with a straight to bent neck. The ectomycorrhiza called ''*Piceirhiza nigripunctata*'' and ''*Populirhiza asperula*'' (Agerer et al. 2002, Jakucs et al. 2005) identified as *T. stuposa* (Jakucs et al. 2005) differ considerably in the mantle structure, especially the presence of star-like arranged mantle cells, dense and patchily distributed blue granules and lack of cystidia. The distinct anatomical differences suggest that *T.* cf. *stuposa* belongs to a sister lineage relative to the EcM of *T. stuposa* described by Jakucs et al. (2005). *Tomentella* cf. *ellisii* is characterized by a voluminous envelope of emanating hyphae and the lack of rhizomorphs. The combination of frequent thin-walled hyphae on the mantle surface and a slightly amyloid reaction with Melzer's reagent resemble features of *Tomentella brunneorufa* M.J. Larsen (Agerer and Bougher 2001). Although the phylogenetic analysis suggests an identification of *T.* cf. *ellisii* as the EcM of *T. ellisii*, a description unequivocally attributable to *T. ellisii*, EcM is lacking from the northern hemisphere. Only the *Lactarius omphaliiformis* morphotype was fully identified as species based on 100% match in the ITS region to a European specimen. The currently

described EcM of *L. omphaliiformis* differ by some details from the described EcM of *L.* cf. *omphaliiformis* (Becerra et al. 2005c), indicating that the two descriptions belong to closely related *Lactarius* species. *L. omphaliiformis* has two distinct mantle layers, the outer mantle layer pseudoparenchymatous comprising epidermoid cells that bear a hyphal net (type Q, Agerer and Rambold 1998); whereas *L.* cf. *omphaliiformis* (sensu Becerra et al. 2005c) has three distinct mantle layers, the outer mantle layer pseudoparenchymatous comprising angular to epidermoid cells that bear a hyphal net (type P-Q, Agerer and Rambold 1998). In addition some differences in chemical reactions suggest the presence of closely related species. *L.* cf. *omphaliiformis* EcM had been assigned to this species with RFLP comparison with DNA of *L.* cf. *omphaliiformis* fruit bodies from Argentina, but no sequence data is available. On *Alnus* several species of *Lactarius* have been described in the world, but due to the paucity of morphotype descriptions we cannot evaluate the anatomical differences among species. The morphotypes of both *L. omphaliiformis* EcM and that of the *Russula* sp. generally conform well with descriptions of other species from these genera (Eberhardt 2000, Eberhardt et al. 2000).

Despite strong matches of the ITS sequences generated here to those in public databases, we cannot unambiguously identify the EcM morphotypes of *Alnus acuminata* on a fungal species level. By displaying 100% ITS sequence identity to a European isolate *L. omphaliiformis* may be exceptional in this respect. However Moreau et al. (2006) and Tedersoo et al. (2009) suggested that several taxa of *Alnus*-associated EcM fungi have been subject to cryptic radiation that is difficult to assess with classical taxonomy and specimens collected from restricted geographical area. These closely related molecular-phylogenetic species are difficult to differentiate based on ITS sequence data due to high sequence similarity (Tedersoo et al. 2009). We expect that due to great geographical distance and differences in soil and other ecosystem properties different cryptic species are present in Europe and South America. Therefore sequences from root tips of *Alnus acuminata* should be compared first to fruit bodies collected from the same ecosystems in South America to prevent lumping of species. Collections of fruit bodies in *A. acuminata* forests unfortunately include only agaricoid macromycetes. Based on the results here and in previous studies, a high proportion of EcM fungi belong to taxa with resupinate fruit bodies such as *Tomentella* (Kõljalg et al. 2000; Smith et al. 2007; Tedersoo et al. 2003, 2006). Therefore particular attention should be paid to searching resupinate fruit bodies on the underside of debris to provide voucher material for improved identification of EcM root tips.

ACKNOWLEDGMENTS

This work was partially supported by PROYUNGAS (1999, 2001). AB thanks DAAD, FOMEC and CONICET for the fellowship. AB thanks the people of the Institute of Soil Ecology (Helmholtz Zentrum München, German Research Center for Environmental Health, Germany) and the Division of Organismic Biology: Mycology, Ludwig Maximilian University, München, Germany. KP and RA received financial support from the German Research Foundation (DFG-SFB607). LT and SP received support from FIBIR, ESF grants 6606, 7434 and JD92. U. Kõljalg is thanked for advice on taxon sampling for the *Tomentella* phylogram.

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