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Ectomycorrhizae between *Alnus acuminata* H.B.K. and *Naucoria escharoides* (Fr.:Fr.) Kummer from Argentina

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Abstract Field ectomycorrhizae of *Naucoria escharoides* on *Alnus acuminata* (“andean alder”, “aliso del cerro”) are described in detail for the first time. Naturally occurring ectomycorrhizal roots were sampled beneath sporocarps of *N. escharoides*. The samples were taken from four natural forest plots at two homogeneous *A. acuminata* sites (Tucumán and Catamarca Provinces, Argentina). The ectomycorrhizae were characterized morphologically and compared by means of PCR/RFLP analysis of the internal transcribed spacer region of the nuclear rDNA. The most important morphological features of the ectomycorrhizae are a white to pale yellow mantle, simple to monopodial branches, hyaline emanating hyphae, abundant hyphal bundles emerging more or less perpendicularly from a plectenchymatous mantle, and an acute or rounded apex with or without a mantle. *N. escharoides* fruitbodies have white basal mycelium with emanating hyphae similar to those of andean alder ectomycorrhizae. The RFLP profiles of sporocarps and mycorrhizae were the same.

Keywords Andean alder · Ectomycorrhiza · Characterization and identification · PCR/RFLP

Introduction

Alnus acuminata H.B.K. (“andean alder”, “aliso del cerro”) forests are distributed at 400–3,000 m along the Andes and Central American mountains from southern Mexico to latitude 28°S in northwest Argentina (Grau 1985).

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Alnus acuminata has the ability to colonize ecologically extreme or disturbed habitats. It is tolerant of infertile soils through its ability to form both ectomycorrhizal and actinorrhizal relationships. It grows rapidly and improves soil fertility by increasing soil nitrogen, organic matter, and cation-exchange capacity (Grau 1985). Andean alder is mainly harvested for firewood, pulp, and timber. Thus, it is an important species recommended for management in land reclamation, watershed protection, agroforestry, and erosion control (National Academy of Science 1984).

Alnus ectomycorrhizae in nature and pouch studies have been described for North America (Neal et al. 1968; Froidevaux 1973; Molina 1979; Godbout and Fortin 1983; Massicotte et al. 1986, 1989, 1994, 1999; Miller et al. 1988, 1991, 1992; Molina et al. 1994; Murphy and Miller 1994), Japan (Masui 1926), New Zealand (Mejstrik and Benecke 1969) and Europe (Treu 1990; Agerer et al. 1993; Airaudi et al. 1994; Pritsch et al. 1997a, b), but there are no records for South America.

The morphological and anatomical description of mycorrhizae (Miller et al. 1991) and the identification of their fungal partners (Agerer 1991) are prerequisites for recognizing mycorrhizal diversity in ecosystems and for comparison with other research. An additional approach is to identify mycorrhizae by comparing specific DNA regions of mycorrhizal fungi and sporocarps. Polymerase chain reaction coupled with restriction fragment length polymorphism analyses (PCR/RFLP) have been applied in mycorrhizal research to identify strains of introduced or naturally occurring mycorrhizal fungi (Gardes et al. 1991; Henrion et al. 1992, 1994) and also to differentiate and identify mycorrhizal symbionts unambiguously (Erland et al. 1994; Kraigher et al. 1995; Gardes and Bruns 1996a; Horton and Bruns 2001).

Several members of the genus *Naucoria* have been suspected to form mycorrhizae with alder. *N. escharoides* (Fr.:Fr.) Kummer is probably the most common and widespread species of *Naucoria*. This fungus is found wherever there are suitable damp and boggy areas (Reid 1984). Brunner et al. (1992) mention *N. escharoides* as-

sociated with *A. viridis* and *A. incana* in Switzerland as well as with *A. tenuifolia* and *A. crispa* forests near Fairbanks, Alaska. In Argentina, Raitelhuber (1988) found two species of *Naucoria* [*N. scolecina* Fr. and *N. diplocystis* (Sing.) Raith.] near *A. acuminata*.

Mycorrhizae of *Naucoria* spp. were described by Pritsch et al. (1997a, b) on *A. glutinosa*. These authors mention that *N. escharoides* and *N. subconspersa* Kühn can be distinguished by their sporocarp morphology and produce anatomically identical mycorrhizae. Neither sporocarps nor mycorrhizae from the two species could be differentiated by molecular biological studies (Pritsch et al. 1997a, b).

The aim of this present work was to describe the ectomycorrhizae formed by *N. escharoides* and *A. acuminata* through morphological and anatomical features (Agerer 1991) and to describe the fungus species. We also obtained PCR/RFLP profiles of the internal transcribed spacer (ITS) region of the described morphotype and the fruitbodies.

Materials and methods

Study site

The research area is located in the northwestern region of Argentina. Two sites were sampled: Quebrada del Portugués, Taffí del Valle (Tucumán Province, site A) (elevation 2,187 m; 26° 58'S, 65° 45'W) and the Narvaez Range (Catamarca Province, site B) (elevation 1,820 m; 27° 43'S, 65° 54'W). The vegetation was a nearly homogeneous *A. acuminata* thicket (height 6–15 m, age 20–30 years) with few herbaceous understorey plants. The soils were found to be Entisols with high organic matter content: site A, 1.1–7.4%, and site B, 3–7.5%; pH 4.5–6 (Rojas et al. 1978). Two plots of 30×30 m were sampled at each site.

Sampling and direct identification

Plots were visited from 1997 through 2000, but the sampling was not systematic. In this region, the peak fruiting season is February through May and this period was surveyed intensively for sporocarps.

Eight soil cores of 15×15 cm to a depth of ca. 10 cm were taken directly beneath *N. escharoides* fruitbodies. The soil samples with roots together with sporocarps were placed in plastic bags, leaving the sample as undisturbed as possible, and stored at 4°C during transport to the laboratory.

Samples were examined under a stereomicroscope at ×10–40 magnification for hyphal connections leading from sporocarps to fungal mantles, using the method of Agerer (1991). Ectomycorrhizae were carefully placed in water and cleaned of soil particles. Fresh mycorrhizae were photographed under a Leica M420 stereo microscope. Several reagents (15% KOH; Melzer's reagent; cotton blue; 70% ethanol; sulpho-vanillin; NH₄OH and lactic acid) were used to study specific color changes of mycorrhizae. Afterwards, mycorrhizal roots were fixed in 70% ethanol and stored at 4°C in the dark.

Microscopic analysis

The microscopic description of the ectomycorrhizae follows the terminology of Agerer (1991, 1999) and Miller et al. (1991). Total mantle views were examined and photographed with a Zeiss Axio-phot light microscope at ×200–1,000 magnification.

The characterization of the Hartig net as paraepidermal (penetrating only to the depth of the transverse walls of the epidermal cells) or periepidermal (hyphae entirely encircle the epidermal cells) follows Godbout and Fortin (1983).

The epigeous sporocarps were analyzed macroscopically (size, shape, color, margin, lamellae, etc) and microscopically (basidiospores, basidium, cuticle, cystidia, etc) and identified following the protocols of Reid (1984). The specimens were finally dried and vouchers of mycorrhizae and sporocarps deposited in the "Museo Botánico de Córdoba" Herbarium (CORD) (Holmgren et al. 1990).

DNA extraction and amplification

DNA was extracted from one to three root tips as described by Gardes and Bruns (1993) and from dried lamellae of two sporocarps by the same method.

Species-level characterization of the fungi was based on PCR amplification of the ITS region of the rDNA gene using ITS-1F and ITS-4B (Gardes and Bruns 1993). The primer pairs preferentially amplified specific fragments of basidiomycete DNA from mixtures of plant and fungus DNA. We used the reagents, protocols, and cycling parameters described previously (Gardes and Bruns 1996b).

RFLP analysis

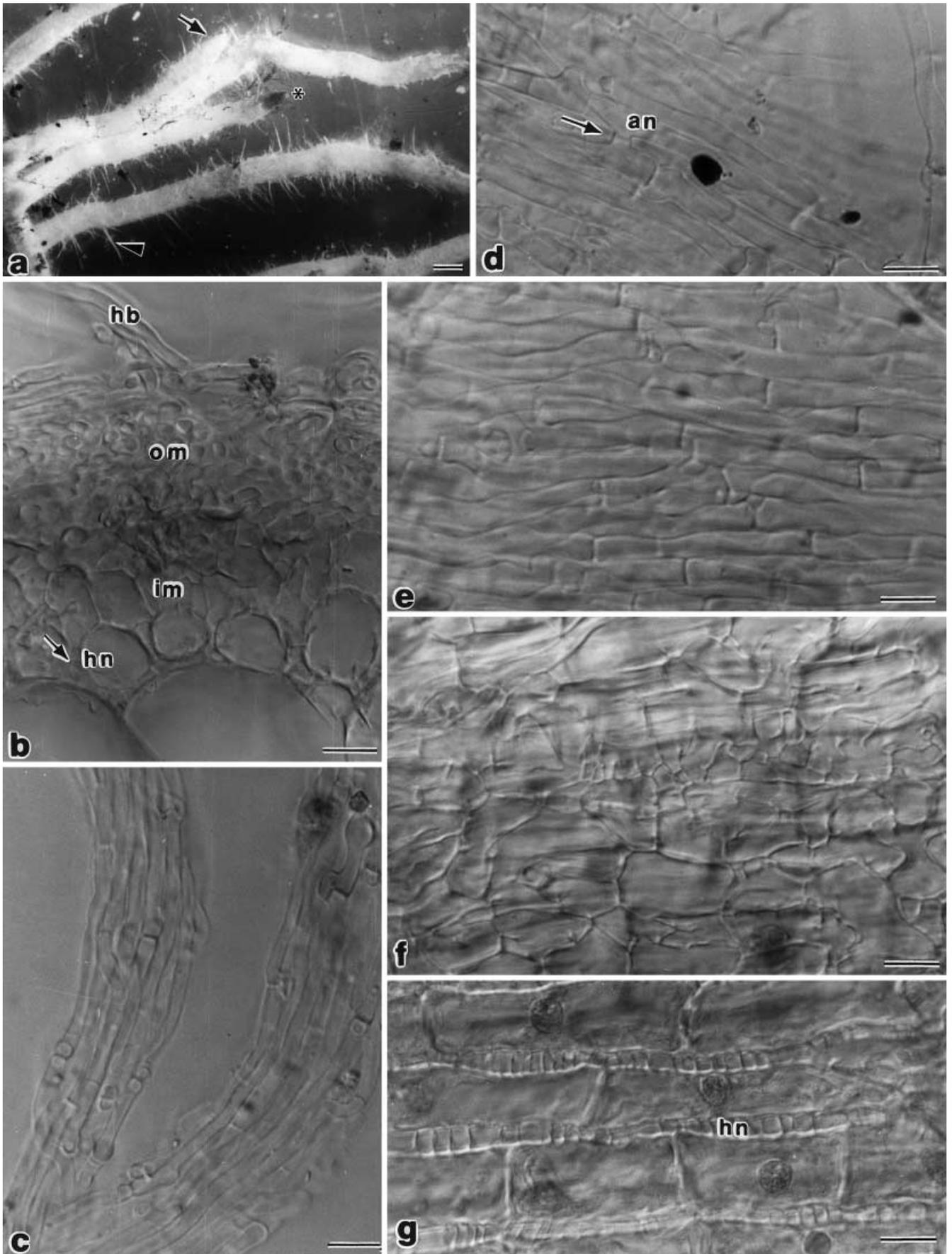
We characterized the ITS region by RFLP analysis, which was used to match mycorrhizae to each other and to sporocarps of voucher collections.

Species-level identification was by RFLP matches after separate digests with *AluI*, *HinfI* and *DpnII*. Aliquots of mycorrhizal and sporocarp DNA, digested by the same enzyme, were loaded side by side for comparison onto a 1% agarose/2% nusieve gel and separated by electrophoresis for 3 h at 100 V in a 1% TBE buffer. A 100-bp DNA ladder (Promega, Madison, Wis.) was used to determine fragment size. The length of the complete ITS was estimated by comparing undigested PCR product run on a 1% agarose/2% nusieve gel with the 100-bp DNA ladder. Gels were stained in ethidium bromide and observed under UV light. Images were analyzed with Scanalytics, Gene Profiler 3.56 software. Band size estimates using agarose gels are approximate, with an error of about 3% (up to 10%).

Results

The direct attempt to identify ectomycorrhizae of *N. escharoides* by tracing hyphal connections between the stipe bases of sporocarps and mycorrhizal root tips was not successful. As there was more than one mycorrhizal morphotype under the sporocarps, an unambiguous attri-

Fig. 1 Light micrographs of ectomycorrhizae of *Alnus acuminata* and *Naucoria escharoides*. **a** Simple to monopodial pinnate morphology of *N. escharoides* ectomycorrhiza collected from the field, mycorrhiza with mantle (arrow), without mantle (*) and hyphal bundles (<); bar 0.5 mm. **b** Mycorrhiza cross section: plectenchymatous outer mantle layer (om) and pseudoparenchymatous inner mantle layer (im), hyphal bundles (hb) and paraepidermal to periepidermal Hartig net (hn, arrow); bar 10 µm. **c** Hyphal bundles of parallel-arranged hyphae, with or without clamps; bar 8 µm. **d** Emanating hyphae from mantle surface, with particles of soil and a simple anastomosis (an, arrow); bar 8 µm. **e** Plectenchymatous outer mantle layer with parallel-arranged hyphae; bar 6.25 µm. **f** Pseudoparenchymatous inner mantle layer irregular shaped; bar 8 µm. **g** Hartig net (hn) surrounding epidermal cells (tangential section); bar 8 µm



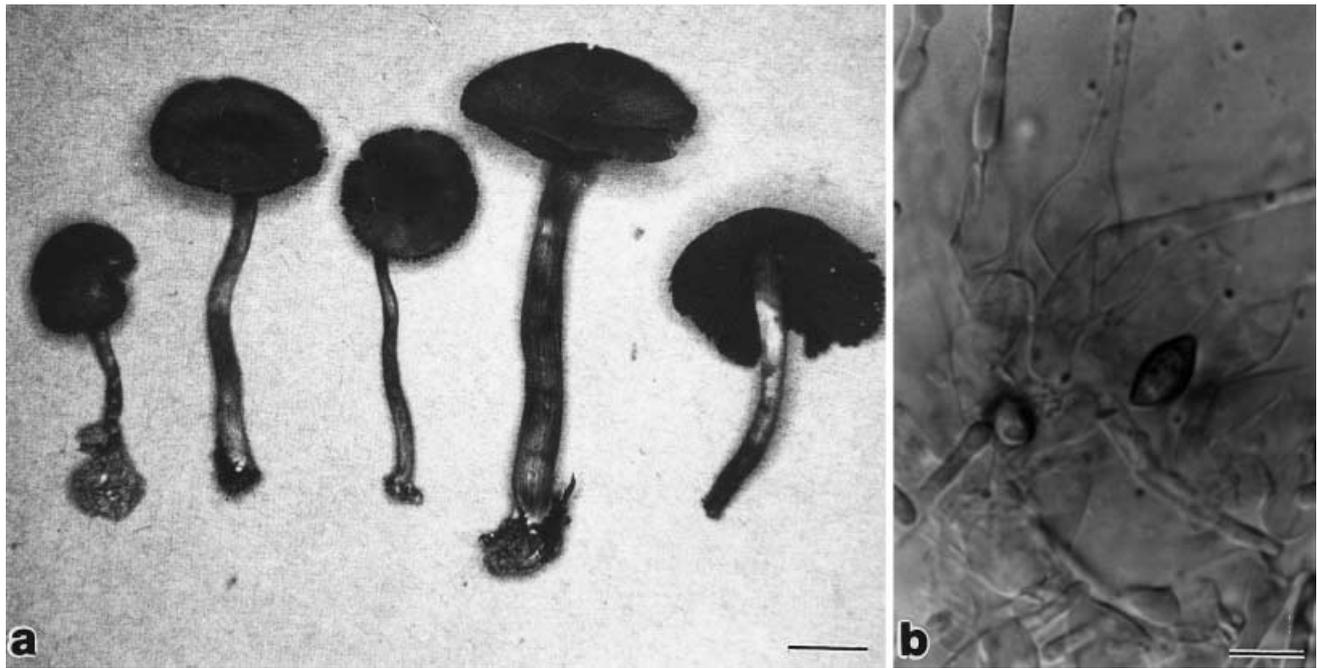


Fig. 2 a Sporocarps of *N. escharoides*; bar 1 cm. b Detail of spores and cystidia from *N. escharoides* gill tissue; bar 10 µm

Table 1 Restriction fragment band size (bp) for *Naucoria escharoides* obtained with *AluI*, *HinfI* and *DpnII* (*ITS* internal transcribed spacer)

Structure	Collection number	Samples (n)	ITS length (bp)	<i>Alu I</i>	<i>Hinf I</i>	<i>Dpn II</i>
Sporocarp	GD160	2	880	255/215/205/85 ^a	385/345/115	525/245 ^a
Mycorrhiza	AB07	1–3	880	265/230/215/90 ^a	425/380/130	565/265 ^a

^a Small fragments probably not detected

bution of ectomycorrhiza to fruitbody was not possible. However, one of the mycorrhiza morphotypes was always present and identical restriction fragment patterns were obtained from PCR/RFLP profiles for this morphotype and sporocarps. The ITS was approximately 880 bp (Table 1).

Description of the morphotype

Naucoria escharoides ectomycorrhizae are simple (unramified) to monopodial pinnate (Fig. 1a), straight to tortuous, 0.6–14 mm long and 0.2–0.4 mm in diameter, white to pale yellow when young, brown when old. Root tips are blunted to acute, mainly with a mantle and some without (Fig. 1a). The surface showed adhering particles of soil; a long stringy mantle was densely covered with emanating hyphae and hyphal bundles in young roots (Fig. 1a); these structures were less abundant in old roots.

Emanating hyphae: hyaline, loosely interwoven emanating hyphae, branched, thin walled, 2–5 µm in diameter, regularly septate with some clamps. Some hyphae with simple anastomoses without septa (Agerer 1991, type A) (Fig. 1d).

Hyphal bundles (Fig. 1a, b, c): arising more or less perpendicularly in connection with the mantle. The bundles are hyaline, straight, tapering, of parallel-arranged hyphae, up to 50 µm wide at the base. Hyphae thin walled, clamped or not.

Mycorrhiza (cross section): the mantle is 39–56 µm thick, differentiated into a plectenchymatous outer layer of parallel-arranged hyphae 2–4 µm in diameter, and a pseudoparenchymatous inner layer of spherical cells, 3–10 µm in diameter (Fig. 1b). Paraepidermal to periepidermal Hartig net, hyphae in one row (occasionally two) between epidermal cells of 1–3 µm in diameter (Fig. 1g). The Hartig net does not penetrate the cortical cells (Fig. 1b). Epidermal cells spherical to elliptical in outline, thick walled, 8–19 µm × 8–11 µm in size.

Mycorrhiza mantle (plan view): mantle usually continuous over the root apex. Outer layer plectenchymatous, hyphae arranged in a parallel manner, hyaline, thin walled, septate with some clamps (Fig. 1e). Inner layer with a pseudoparenchymatous arrangement of rectangular to irregularly shaped 3–11 µm diameter, thin-walled hyphae (Fig. 1f).

Color reactions: whole ectomycorrhizae with 15% KOH slightly reddish and emanating hyphae yellowish;

emanating hyphae and hyphal bundles were blue with cotton blue; yellowish to brown with 70% ethanol; no reactions with Melzer's reagent sulpho-vanillin, NH_4OH or lactic acid. Voucher specimen: ectomycorrhizae under *A. acuminata*, in herb. A. Becerra AB 07 (CORD).

Description of the fruitbodies of *N. escharoides* (Fr.: Fr.) Kummer

Pileus: 0.8–2 cm diameter, campanulate becoming plane, hygrophanous, yellow-ochre, darker at center, nonstriate, somewhat fibrillose in the outer quarter, cracked. Stipe: up to 5 cm high, 1–2 mm wide, pale ochre upward, darker at base fibrillosely striate, with white basal mycelium (Fig. 2a). Gills: adnate, broad, cinnamon, ventricose, whitish edge. Spores: 9.0–11.0 (12.0) \times 4.8–6.4 μm , amygdaliform-limoniform, brown, with minute ornamentation. Basidia: 4-spored, clavate. Cheilocystidia: urtica-hair-like, thin-walled, hyaline, with a swollen base (8.5 μm diameter) and an elongated narrow neck, abruptly terminating in a pointed apex (12 μm long) or tapering gradually from the base into a long narrow neck (1.6 μm apical and 2.4 μm basal diameter) (Fig. 2b). Cap cuticle hymeniform. Habit: solitary to gregarious on soil, associated with *A. acuminata*. Maximal fruiting during summer and fall (February–May). Sporocarp frequency was similar in both study plots. Material examined: Argentina: Quebrada del Portugués, Tafi del Valle (Tucumán Province), elevation 2,187 m; 26° 58'S, 65° 45'W and the Narvaez Range (Catamarca Province), elevation 1,820 m; 27° 43'S, 65° 54'W; 01.05.1997, G Daniele GD 160 (CORD), 06.05.1999, GD 185 (CORD), 12.02.2000, GD 205 (CORD).

Discussion

In this study, we report that field ectomycorrhizae are formed by *N. escharoides* and *A. acuminata* in Argentina.

The majority of ectomycorrhizal fungus species in alder forests are highly specialized at the host genus level (Molina 1979, 1981; Brunner and Horak 1990). Brunner et al. (1992) found *N. escharoides* to be the only ectomycorrhizal species common at two forests sites with *A. tenuifolia* and *A. crispa* in Alaska. Pritsch et al. (1997a) found *N. escharoides* and *N. subconspersa* associated with *A. glutinosa* forests in Lower Saxony, Germany.

Pritsch et al. (1997a, b) carried out detailed studies of the mycorrhizae produced by *N. escharoides* and *N. subconspersa* with *A. glutinosa*. The ectomycorrhizae of *N. escharoides* and *A. acuminata* in our study showed features similar to their findings (color, clamped emanating hyphae, an outer plectenchymatous to inner almost pseudoparenchymatous mantle). Contrary to Pritsch et al. (1997b), we found a stringy mantle with frequent hyphal bundles instead of a woolly, felt-like surface mantle.

Identification of ectomycorrhizal morphotypes in the present study was achieved by comparison of ITS PCR/RFLP profiles of *N. escharoides* fruitbodies and mycorrhizae. We obtained an ITS length of approximately 880 bp, in contrast to the 710-bp ITS obtained by Pritsch et al. (1997a) for species of *Lactarius*, *Russula*, *Naucoria* and unidentified ectomycorrhizae. The larger ITS found in our study is directly a function of using the primers ITS-1F and ITS-4B, which amplify through the priming sites employed by Pritsch et al. (1997a) and amplify a larger fragment. Pritsch et al. (1997a) sampled two species of *Naucoria* (*N. escharoides* and *N. subconspersa*) and could not differentiate these species by ITS RFLP analysis.

Studies on the composition of mycorrhizal populations of "andean alder" are underway to elucidate the diversity of ectomycorrhizae on *A. acuminata* in Argentina.

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