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Edomycorrhizas of *Cortinarius helodes* and *Gyrodon monticola* with *Alnus acuminata* from Argentina

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Abstract Field ectomycorrhizas of *Cortinarius helodes* Moser, Matheny & Daniele (sp. nov) and *Gyrodon monticola* Sing. on *Alnus acuminata* Kunth (Andean alder, aliso del cerro) are described based on morphological and anatomical features. Ectomycorrhizal roots were sampled beneath fruitbodies of C. *helodes* and G. *monticola* from two homogeneous *A.. acuminata* forest sites located in Tucumán and Catamarca Provinces in Argentina. C. *helodes* ectomycorrhizas showed a thick white to beige mantle exuding a milky juice when injured, were bluish toward the apex, and had hyphal strands in the mantle. G. *monticola* ectomycorrhizas showed some conspicuous features like highly differentiated rhizomorphs, inflated brown cells on the mantle surface, and hyaline and brown emanating hyphae with dolipores. Restriction fragment length polymorphism analysis of the nuclear rDNA internal transcribed spacer provided a distinctive profile for each of the collections of fruitbodies and the mycorrhizal morphotypes.

Keywords Andean alder· *Cortinarius helodes* . *Gyrodon monticola* . Morphological characterization . Polymerase chain reaction/restriction fragment length polymorphism

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Introduction

The distribution of *Alnus acuminata* Kunth ranges from the highlands of Mexico to the Andes (Dawson 1990), reaching its southernmost point in the Catamarca province of Argentina. Alders form ectomycorrhizas, and most alder species are used as forage and fuel sources, and quickly increase the forest biomass over previously deforested areas.

Species identification of mycorrhizal morphotypes is often required in biodiversity and ecological studies. The first approach is to characterize mycorrhizas based on morphological and anatomical features (Miller et al. 1991; Agerer 1991); in many cases, as stated by Agerer (1986, 1991, 1995) and Brundrett et al. (1996), hyphal connections can be traced from the fruitbody's stipe base to the mycorrhizas. Additionally, molecular techniques such as polymerase chain reaction coupled with restriction fragment length polymorphism analyses (PCR/RFLP) increase the resolution of identification to the species level (Pritsch and Buscot 1994; Egger 1995; Baldwin and Egger 1996; Eberhardt et al. 1999; Horton and Bruns 2001; Mah et al. 2001; Sakakibara et al. 2002).

Ectomycorrhizal diversity is low in alder forest (Molina 1979, 1981; Gardner and Barrueco 1999). Trappe (1962) and Horak (1963) list a number of fungi within the families Cortinariaceae, Russulaceae, and Boletaceae associated with different *Alnu* s species. Within Cortinariaceae several species have been reported as having a mycorrhizal association with alders (Stangl 1970; Godbout and Fortin 1983, 1985; Brunner et al. 1990; Miller et al. 1991; Moser 2001). Pritsch et al. (1997a, 1997b) described the ectomycorrhizas of *Cortinarius* cf. *helvelloides* (Fr.) Fr. and *Cortinarius* cf. *alneus* (Mos.) Mos. associated with *A.. glutinosa* (L.) Gaertn. Within Boletaceae, four *Gyrodon* species have been reported from North America and Europe associated with *Alnus* spp. (Hayward and Thiers 1984). *Gyrodon monticola* Sing. was reported growing under *A.. acuminata* in Mexico and Argentina (Singer and Morello 1960; Hayward and Thiers 1984; Singer and Gomez 1984) and ectomycorrhizas of

Gyrodon lividus (Bull.) Fr. with *Alnus incana* (L.) Moench were described by Agerer et al. (1993).

The aim of this work was to characterize the ectomycorrhizas formed by *Cortinarius helodes* Moser, Matheny & Daniele and *Gyrodon monticola* with *Alnus acuminata* through morphological and anatomical features, and to confirm their identity with molecular techniques *(PCR/* RFLP).

Materials and methods

Study site

Two forest sites located in the northwestern region of Argentina (NOA) were sampled. Quebrada del Portugués, Taff del Valle (Tucumán Province), elevation 2,187 mm; 26°58'S, 65°45'W, average precipitation ranges between 800 and 1,200 mm, The Narvaez Range (Catamarca Province), elevation 1,820 m; 27"43'S, 65°54'W, average precipitation 1,620 mm, Mean annual temperatures range from 5.8 to 24°C for NOA. The vegetation is a nearly homogeneousA. *acuminata* forest (height 6–15 m, age 20-30 years) with a few herbaceous understory plants such as *Duchesnea* sp. (Rosaceae); *Conyza* sp. (Asteraceae), *Axonopus* sp. (Poaceae), *Selaginella* sp. (Selaginellaceae); *Prunella* sp. (Lamiaceae), etc. (Acefiolaza 1995). Soil types are Entisols with high organic matter content in both locations (Becerra et al. 2002). Two plots of 30x30 m were sampled at each study site.

Sampling and direct identification

Both locations were visited during summer and fall from 1999 through 2001, At every sampling time, soil cores of 15x15 cm to a depth of about 10 cm were concurrently collected below fruitbodies. The samples were placed in plastic bags leaving the sample as undisturbed as possible, and stored at 4°C during transport to the laboratory.

The samples were placed in water and examined for hyphal connections leading from fruitbodies to fungal mantles under a Zeiss stereo microscope at x10 —40 magnification according to the method of Agerer (1991). Alder roots (which are the only structures within these forests that present ectomycorrhizas) and mycorrhizas within soil cores were easy to identify due to their morphological appearance, although roots from herbaceous plants were also present. Within every morphotype, several tips were prepared for DNA extraction, while others were subjected to comparative anatomical studies. Photographs of mycorrhizas 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 for studying specific color changes of mycorrhizas. Afterwards, mycorrhizal roots were fixed in 70% alcohol and stored at 4°C in the dark.

Microscopic analysis

Description of the ectomycorrhizas follows the terminology of Agerer (1991, 1999a) and Miller et al. (1991). Mantle views were examined and photographed with a Zeiss Axiophot light microscope at x200–1,000 magnifications. Characterization of the Hartig net follows Godbout and Fortin's (1983) nomenclature, Fruitbodies were identified following Singer and Digilio (1957, 1960) and Moser (1978) protocols. Voucher specimens and mycorrhizas were deposited in the Museo Botanico de Cordoba Herbarium (CORD) (Holmgren et al. 1990).

DNA extraction and amplification

DNA was extracted from one to three root tips and from dried lamellae from two fruitbodies (Gardes and Bruns 1993) (both extractions were carried out twice). Species characterization of the fungi was based on PCR amplification of the internal transcribed spacer (ITS) region of the rDNA gene by using ITS-1F and ITS-4B primers (Gardes and Bruns 1993). The primer pairs preferentially amplify specific fragments of basidiomycete DNA from mixtures of plant and fungus DNA. We used reagents, protocols, and cycling parameters as described previously (Gardes and Bruns 1996).

RFLP analysis

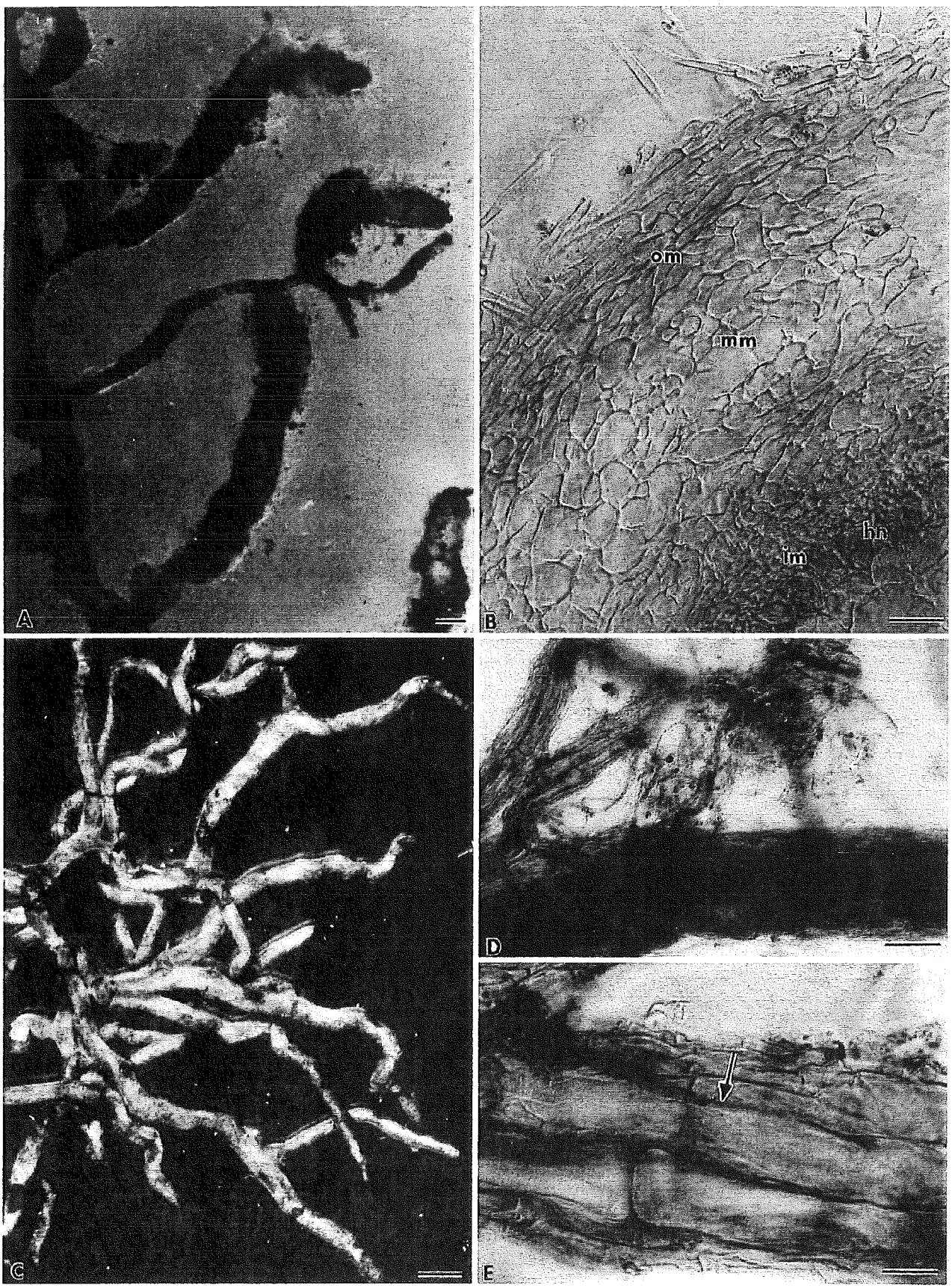
We characterized the ITS region by RFLP analysis, which was used to match mycorrhiza root samples and fruitbodies of voncher collections. Aliquots of DNA from mycorrhizas and fruitbodies of C. *helodes* were digested with *Alu* I, *Hinf* I and *Dpn* II enzymes, whereas G. *monticola* DNA was digested with *Hinf* I, *Dpn* II and *Hae* III enzymes 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-base pair 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-base pair DNA ladder. Gels were stained in ethidium bromide and observed under ultraviolet light. Images were analyzed with Scanalytics, Gene Profiler 4.02 software using default parameters. The ITS base pair lengths are based on scores using a 100-base pair ladder. Scanalytics software calculates the logarithm of all molecular weight standards, then plots the log molecular weight values that intersect each standard data point. To calculate the molecular weight values of the unknown samples, the software substitutes each unknown band migration distance (pixel location) into the linear line equation *(y = mx + b)* for each piece (Hook 2002).

Results

Various fruitbodies and mycorrhizas were collected in the field,, but we present here two fungi (*C. helodes* and G. *monticola)* associated with *A. acuminata* according to the RFLP matches obtained.

The direct attempt to identify ectomycorrhizas of these fungi by tracing hyphal connections between the stipe base and mycorrhizal root was difficult. Within the collected C. *helodes* soil samples only a few mycorrhizas were observed. On the other hand, mycorrhizas of G. *monticola* were abundant under the fruitbodies, Within the molecular analysis, the comparison of PCR/RELP patterns showed a similar band size between ectomycorrhizas and fruitbodies when using three endonuclease

Fig. 1 Light micrographs of *Alnus acuminata* and *Cortinarius helodes* (A, B) collected from the Sierra de Narvaez (Catamarca Province) and *Gyrodon monticola* (C - E) collected from the Quebrada del Portugués (Tucumán Province) and Sierra de Narvaez (Catamarca Province) sites. A Simple (unramified) ectomycorrhizal root tip of C. *helodes; bar* 0.5 mm. B Cross-section showing the plectenchymatous to pseudoparenchymatous outer mantle layer *(om)* (see also Fig. 2 C-D), pseudoparenchymatous middle mantle layer *(mm)* and psendoparenchymatous inner mantle layer *(im);* Hartig net *(hn); bar* 25 µ*s».* C Monopodial to irregularly pinnate ectomycorrhizal root tip of G. *monticola; bar* 0.5 mm. D Rhizomorphs; *bar* 50 µ*m.* E Central core of thick hyphae in the rhizomorph (•-•); *bar* 10 µm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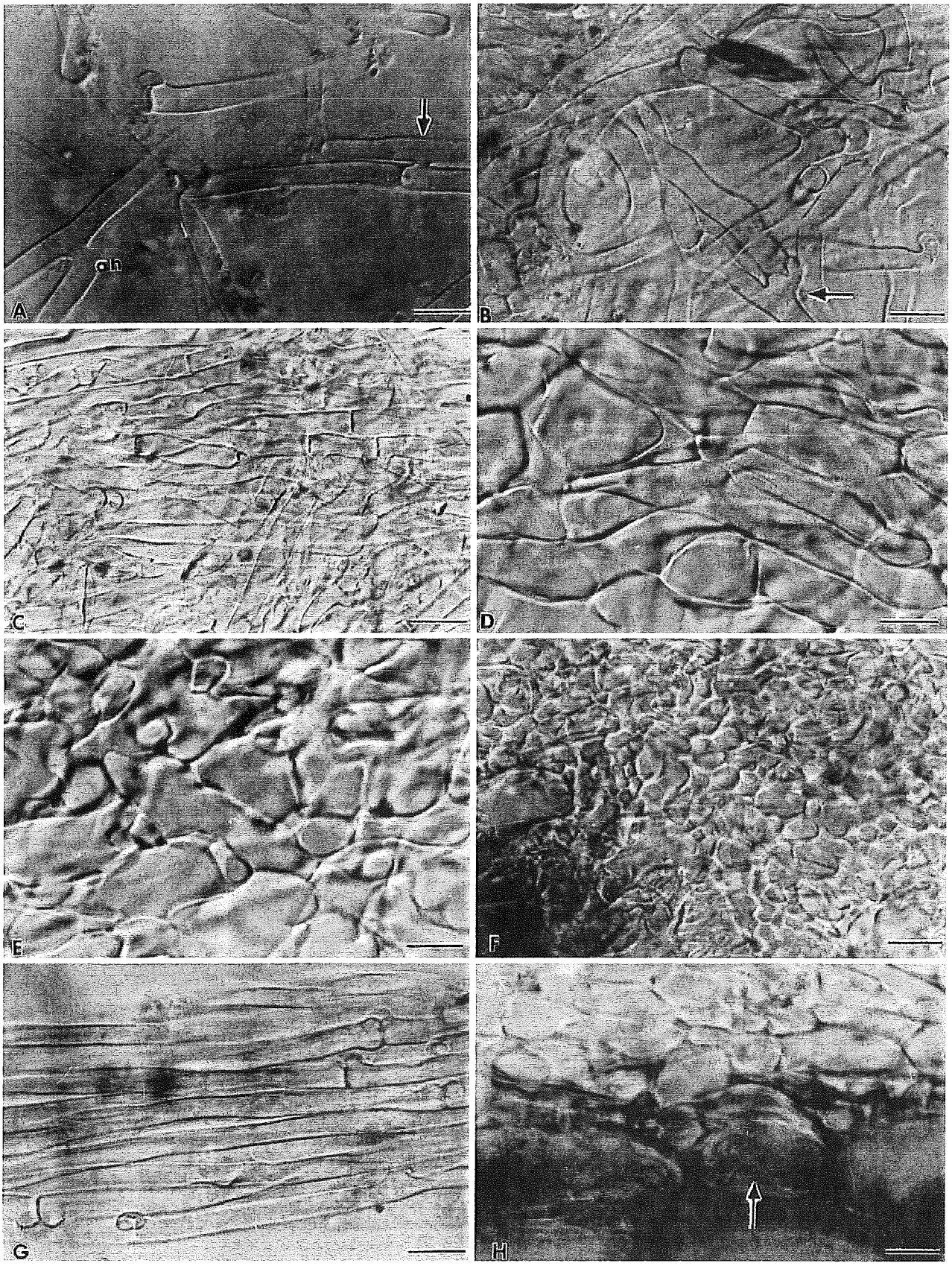

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