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Morphological, molecular and ecological aspects of the South American hypogeous fungus *Alpova austroalnicola* sp. nov.

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Abstract: Field studies in Argentina's Yunga District revealed *Alpova austroalnicola* sp. nov., a hypogeous fungus associated with *Alnus acuminata* ssp. *acuminata*. Morphological and molecular studies based on amplification and sequencing of the nuclear LSU rDNA gene showed its unique identity within *Alpova*. Related genera included in the analyses were *Boletus edulis, Rhizopogon* spp., *Suillus luteus* and *Truncocolumella citrina*. Additional observations of animal diggings around the sites and microscopic examination of fecal pellets of the nine-banded armadillo (*Dasypus novemcinctus novemcinctus*) indicate *A. austroalnicola* is consumed and its spores dispersed by animals.

Key words: *Alnus acuminata,* Boletales, *Dasypus,* molecular systematics, mycophagy, phylogeny

INTRODUCTION

The genus *Alpova* originally was described with a single species, *A. cinnamomeus* C.W. Dodge (1931). Trappe (1975) discovered it had been described earlier as *Rhizopogon diplophloeus* Zeller & C.W. Dodge and recombined it as *A. diplophloeus* (Zeller & C.W. Dodge) Trappe & A.H. Sm. The species is characterized by its hypogeous to sometimes emergent habit; strict association with *Alnus* spp; solid, gelatinous gleba that darkens when exposed to air; smooth, thin-walled, small, ellipsoid to oblong, hyaline to pale brown spores; presence of clamp connections; lack of a hymenial palisade; and a layer of large, inflated cells in the peridium. Trappe (1975) broadened the concept of *Alpova* to include taxa with similar macromorphology that were related to the genus *Rhizopogon*. Beaton et al (1985) broadened it even further by placing several new Australian *Eucalyptus*-associated species in *Alpova*.

Molecular data now have demonstrated that the morphological criteria formerly considered important in defining genera of hypogeous fungi can be inadequate for that purpose. Grubisha et al (2001) have shown that *Alpova diplophloeus* relates to the Boletaceae, whereas the *Rhizopogon*-related species placed by Trappe (1975) in *Alpova* subgen *Alpova* sec *Rhizopogonella* belongs in the ''suilloid radiation,'' in the Rhizopogonaceae. Bougher and Lebel (2002) transferred the Australian species earlier assigned to *Alpova* to their new genus *Amarrendia*. Accordingly, the concept of *Alpova* must revert to the strict sense of its original description. This concept would include two, perhaps four, of the morphologically similar species in Trappe's (1975) *Alpova* subgen. *Alpova* sec. *Alpova*: *A. diplophloeus, A. nauseosus* (Coker and Couch) Trappe, possibly *A. mollis* (Lloyd) Trappe and *A. trappei*. However *A. mollis* is known only from the type collection and *A. trappei* differs strongly from the others in its peridial structure; neither is known to be associated with *Alnus* spp. Grubisha et al (2001) suggested that *Alpova* might not be monophyletic but recognized the need for new studies including more taxa.

A new species of *Alpova* recently was collected in the Yunga District of Argentina in an *Alnus acuminata* Kunth ssp. *acuminata* forest. The distribution of *A. acuminata* ssp. *acuminata* ranges from Venezuela to the Andes in Argentina (Furlow 1979, Cabrera and Willink 1980, Aceñolaza 1995). Molecular phylogenetic analysis can be useful for testing whether species with disjunctive ranges are within the same lineage (Koufopanou et al 1977). This is particularly important when morphological characters are few or apparently have converged enough that it is difficult to separate similar taxa (Rizzo et al 2003). The nucLSU-rDNA gene has been used previously to investigate phylogenetic relationships, particularly in the Boletales, providing suitable resolution for identifying lineages of fungi with good support for terminal branches (Bridge 2002, Grubisha et al 2001, Humpert et al 2001, Moncalvo et al 2000, Wang et al 2002).

We here describe *Alpova austroalnicola* based on

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its unique morphological characters and molecular data obtained from nuc-LSU-rDNA gene analysis. We also obtained data on its use as food and spore dispersal by the Argentine nine-banded armadillo, *Dasypus novemcinctus novemcinctus,* known by the common name of mulita grande or armadillo de nueve bandas. This subspecies inhabits northern Argentina.

METHODS

Sporocarp sampling and morphological description.—Sporocarps were collected in Mar 2001 in a forest dominated by *Alnus acuminata* ssp. *acuminata* in northwestern Argentina. Plots had been established previously near Los Toldos in Salta Province, site M28 (eroded area with isolated groups of *Alnus* trees), elevation 1702 m; 22°14′93″S, 64°40′79″W, and site M42 (*Alnus* dominated forest), elevation 1778 m, 22°16′57″S, 64°43′13″W. The average annual precipitation is 1300 mm. Sites are characterized by the dominant *A. acuminata* spp. *acuminata* ca. 45 y old, plus other relatively abundant species: *Amomyrtella guili* (Speg.) Kausel, *Clethra scabra* Pers., *Ilex argentina* Lillo, *Maytenus cuezzoi* Legname, *Myrica pubescens* var. *glabra* Chev. and *Podocarpus parlatorei* Pilg. Soils are Inseptisols (Haplumbreptes énticos), with high organic matter content (site M28 = 9.77%, site M42 = 9.61%).

Sporocarps were photographed with a Leica M420 stereo microscope. Color reactions and microscopic characters were determined from hand-sectioned mounts in 15% KOH, Melzer's reagent, cotton blue and $FeSO_4$ and photographed with a Zeiss Axiophot light microscope. Voucher specimens were deposited in the Museo Botánico de Córdoba Herbarium (CORD).

Fecal pellet analysis.—Armadillo fecal pellets were collected near animal diggings on the sites where *Alpova austroalnicola* sp nov. was sampled. Twelve samples of collected pellets were analyzed microscopically (25 fields randomly selected per sample at 600×). Fungal elements (spores and hyphae), plant, animal and mineral material were counted following procedures of McIntire and Carey (1989).

Molecular and phylogenetic analysis.—A small amount of sporocarp tissue was ground with a drill-driven plastic pestle in an Eppendorf tube containing 200 μL of 2× CTAB lysis buffer. Additional buffer was added up to 500 μL and mixed; the tubes were frozen and thawed twice, alternating between dry ice and a 65 C water bath. The tubes were incubated in the bath 30–60 min. Chloroform was added to the mixture, which was spun 15 min at 13 000 rpm. The aqueous phase was removed and cleaned with a glass-milk solution (GENECLEAN III®, BIO 101); the extracted DNA then was stored in 30 μL dd H_2O at −20 C.

The nuclear LSU rDNA locus was amplified via polymerase chain reaction (PCR) with LROR and LR3 primers (Vilgalys and Hester 1990). PCR reactions were performed in 50 μL reaction mixtures containing ddH2O, 1 or 2 μL of DNA template, 2 μL of each primer pair (10 μM), 25 μL buffer E (MasterAmp 2 × PCR PreMixes: 100 mM Tris-HCl, 100 mM KCl, 400 uM each dNTP, 5 uM MgCl2, and 4 × MasterAmp Enhancer, Epicentre Technologies, Madison, Wisconsin) and 0.5 μL of 5 U/μL Taq polymerase. The DNA was amplified with a PTC Programmable Thermal Controller and thermal cycling, as follows: 94 C (2 min), [94 C (30 s), 51 C (30 s), 72 C (45 s)] × 30, [94 C (30 s), 53 C (30 s), 72 C (45 s + 5 s per cycle)] × 5, 72 C (5 min), 4 C (15 min). PCR products were viewed on 1% agarose gels (Gibco-BRL ultra PURE, Life Technologies) in a UV light transilluminator (UVP Laboratory products), stained with ethidium bromide and quantified with a low DNA mass ladder (Gibco-BRL Ultra PURE, Life Technologies). The amplified DNA was purified with a PCR purification kit (QIAquick, QIAGEN Inc.). Purified PCR products were sequenced with LROR primer on a 373 DNA Sequencer (Applied Biosystems).

LSU rDNA sequences were assembled with SeqEditor version 1.0.3 (Applied Biosystems) and visually aligned with PAUP* 4.0b10 (Swofford 1999). Two collections of *Alpova austroalnicola* and three collections of *A. diplophloeus* sequences were compared with closely related taxa sequences selected from GenBank: *A. diplophloeus, A. trappei* Fogel., *Boletus edulis* Fr, *Rhizopogon occidentalis* Zeller & C.W. Dodge, *R. truncatus* Linder, *R. villosulus* Zeller, *Suillus luteus* (Fr.) Gray and *Truncocolumella citrina* Zeller. Herbarium, collector and GenBank accession numbers are provided (TABLE I).

Ambiguous insertion/deletions (indels) and gaps were treated as missing data. Phylogenetic analyses were performed in PAUP* 4.0b10. Most parsimonious trees (MPTs) were recovered by the heuristic search option (TBR and MulTrees on) and 1000 replicates of random sequence addition. Support for individual branches was estimated through 1000 bootstrap replicates by the heuristic search option with 100 random sequence additions per replicate, TBR and MulTrees on. Characters were of type "unord" and have equal weight. Of the total number of 646 characters, 586 were uninformative; due to gaps 60 were informative for parsimony.

TAXONOMY

Alpova austroalnicola L.S. Domínguez, sp. nov. FIGS. 1–9

Basidiomata subhypogaea vel hypogaea, globosa vel irregularia, 4–11 × 6–15 μm. Peridium 400–570 μm crassum, pseudoparenchymatum, fibulatum, brunneolum, ube contusum fuscescens. Rhizomorphae concolores, ad peridium adpressae. Gleba solida, lenta vel gelatinosa; loculis 0.4–0.65 mm latis, venis brunneolis separati. Odor nullus. Basidiosporae hyalinae, laeves, ellipsoideae vel oblongae, tunicis parum incrassatus, (5–)6–7(–8.5) × 2–3 μm. Basidia clavata, 25–30 × 4–6 μm, hyalina, octospora. Holotypus hic designatus: Argentina, Salta, Santa Victoria, Los Toldos, inter radices *Alnus acuminatae* ssp. *acuminatae,* L.S. Dominguez 2291.

Etymology.—Latin *austro* (southern) and *alnicola* (dweller with alder), in reference to its association with *Alnus* in the Southern Hemisphere.

TABLE I. Taxa included in the molecular analysis

Taxon	Herbarium and collector number	GenBank
A. austroalnicola	CORD-LSD 2290	AY377574
A. austroalnicola	CORD-LSD 2291	AY377575
A. diplophloeus	OSC 93524 JMT 4745	AY377571
A. diplophloeus	OSC 39950 JMT 5392	AY377572
A. diplophloeus	OSC 34455 JMT 2382	AY377573
A. diplophloeus	OSC 55928, JMT 17685	AF071454
A. trappei Fogel	OSC 56019, JMT 16394	AF071456
Boletus edulis	OSC n/a FAC 1615	AF071457
Rhizopogon villosulus	BPI 841878, JMT 19466	AF071464
Rhizopogon occidentalis	OSC 58923, JMT 17564	AF071453
Rhizopogon truncatus	OSC n/a, LCG212	AF071463
Suillus luteus	isolate JM96/41	AF042622
Truncocolumella citrina	OSC 80861, JMT 19184	AF071465

BPI = U.S. National Fungal Collections-Beltsville, U.S.A.; CORD = Museo Botánico de Córdoba, Argentina; OSC = Oregon State University Herbarium, U.S.A.; LSD = L. S. Domínguez; LCG = L. C. Grubisha; JM = J. Moncalvo isolate; JMT = James M. Trappe; FAC = Francisco A. Camacho; n/a = not available.

Macroscopic characters.—Basidiomata subhypogeous to hypogeous, globose to irregular, 4–11 × 6–15 mm at maturity (FIG. 1). *Peridium* 2-layered, smooth to slightly velvety or felty, 400–570 μm thick in single specimens depending on sporocarp size, at maturity light brown with some darker areas, the depressions paler, when bruised turning dark brown, off-white in cross section, drying dark brown. *Rhizomorphs* concolorous with peridium or slightly darker, up to 80 μm broad, appressed at the basidioma base and scattered on its sides. *Gleba* solid, rubbery to gelatinous, exuding a sticky substance when cut; chambers 0.4–0.65 mm broad, separated by pale brown meandering veins (FIG. 1), maturing from the center of the gleba outward, the content pale brownish yellow, darkening slightly when exposed or aged, drying dark brown to black, hard and waxy when sectioned. *Chemical reactions*: KOH slightly brown on peridium, not reactive on gleba; $FeSO_4$ pale green on peridium, not reactive on gleba. *Columella, stipe* and *basal mycelium* lacking. Odor none.

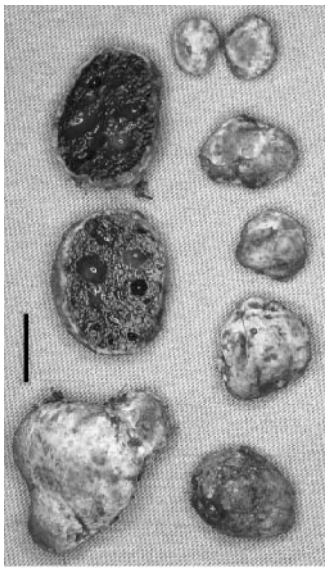
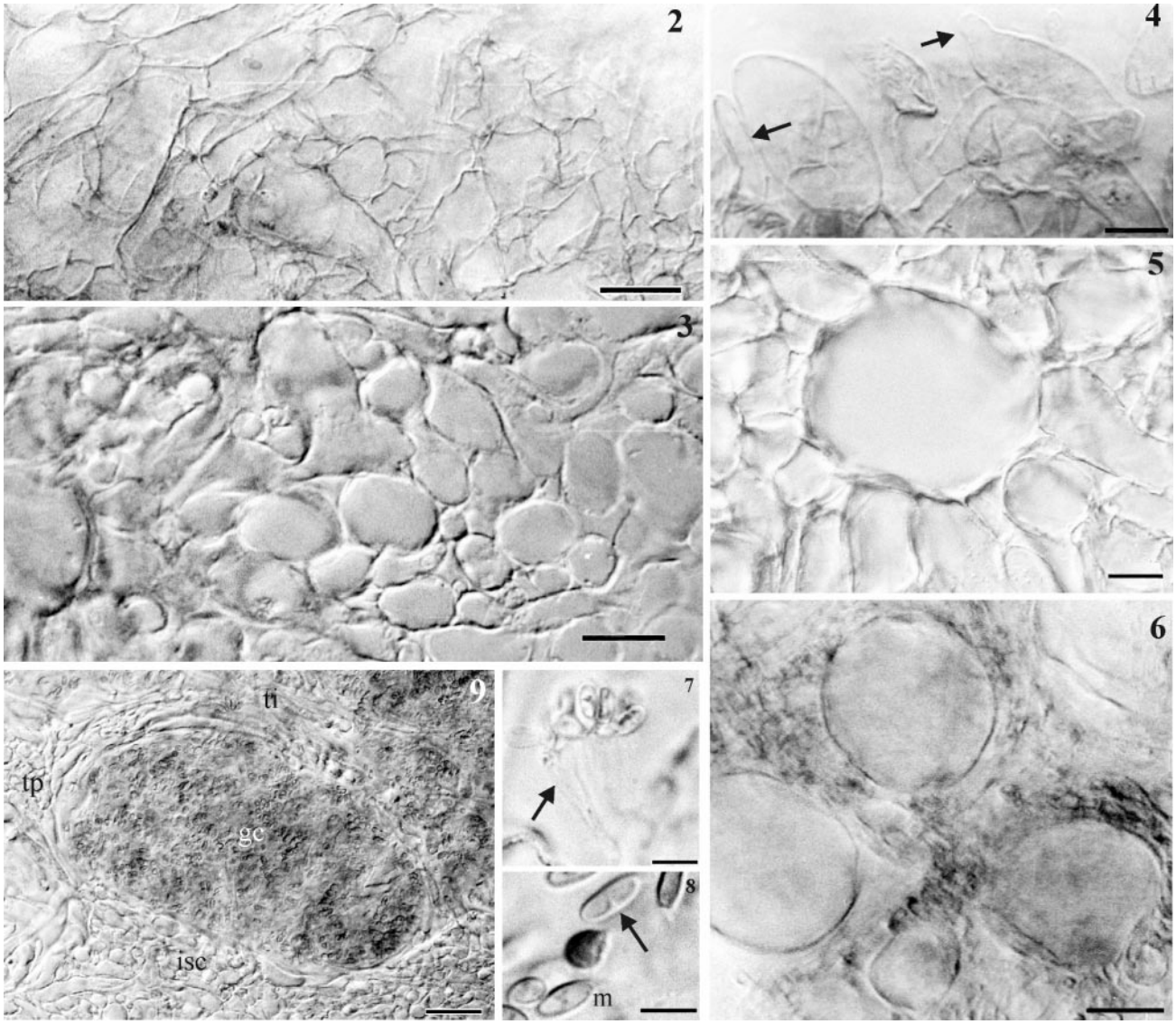


FIG. 1. *Alpova austroalnicola* (LSD 2291).basidiomata. Bar = 5 mm.

Microscopic characters.—Peridiopellis yellow in KOH in cross-section; suprapellis up to 70 μm thick, with some tangled, cylindrical, obtuse hyphae 11–35 × 4.5–8 μm and some projecting and scattered dermatocystidia 4–5.5 μm broad (FIG. 4) on the surface; inward constituted of ± isodiametric cells 8–19 μm broad with walls up to 1 μm thick and pigmented contents (FIG. 2), embedded in a gelatinous matrix at maturity; *subpellis* up to 500 μm, most cells inflated to form a textura angularis/textura epidermoidea gradient, cells in the outer part pale yellow, 12–40 × 5–20 μm mixed with isodiametric cells 8–32 μm broad (FIG. 3), toward the gleba the cells hyaline, 9.5–20 μm broad (FIG. 9) and confluent with the tramal tissue, occasional ''giant'' cells 45–65 μm broad and with walls 1–2 μm thick (FIG. 5) scattered throughout; *conductive hyphae* infrequent, 3–5 μm broad, yellow in Melzer's reagent. *Rhizomorph* hyphae 2–3.5 μm broad, compactly arranged within, on the surface loosely woven, slightly colored and flexuous.

Glebal veins 30–55 μm thick (FIG. 9), of hyaline, parallel to subparallel hyphae 3.2–5 μm broad, with gelatinous-thickened walls in age, at the intersections forming a pseudoparenchyma of cells 5–16 μm broad (FIG. 9). *Locules* in young sporocarps filled by hyaline hyphae 1.5–4 μm broad, basidia and scattered, large, spherical cells with an attachment (FIG. 6) in a gelat-



FIGS. 2–9. Light micrographs of *A. austroalnicola*. 2. Cross section of pellis. 3. Cross section of subpellis of pseudoparenchymatic tissue. 4. Projecting dermatocystidium, note rounded suprapellis cells (→). 5. Isolated ''giant'' cells of subpellis. 6. Spherical cells in gleba of young specimens. 7. Eight-spored mature basidia (→). 8. Spores, note young guttulae spores (→) and mature spores (m). 9. Section through glebal chamber (gc), note tramal plates (tp), tramal intersection (ti) and inner subpellis cells (isc). Bar: 2 = 10 μm, 3 = 16 μm, 4 = 8 μm, 6 = 40 μm, 5 and 9 = 25 μm, 7 and 8 = 5 μm.

inous matrix (FIG. 9), at maturity a few basidia persisting among spores. *Hymenial palisade* lacking; *basidia* abundant, clavate, 25–30 μm long × 2 μm broad at the base and 4–6 μm at the apex, hyaline, thin walled (FIG. 7), autolysing by maturity, 8-spored, the sterigmata less than 0.5 μm long. Mature spores are not attached to basidia although they may be.

Basidiospores hyaline singly, pale yellow in mass, smooth, initially globose, soon becoming ellipsoid to oblong or occasionally allantoid, (5)6–7(8.5) × 2.2–3 μm, the walls initially thin but slightly thickened at maturity, in youth with two guttules giving the appearance of a septum but these usually absent at maturity (FIG. 8), detaching at maturity but often held in the gel in much the same relative position to each other as when attached; spore walls strongly cyanophilic in cotton blue in youth, weak or acyanophilic at maturity, not reactive to Melzer's reagent.

Clamp connections common in all tissues.

Habit, habitat and season.—Subhypogeous to hypogeous among *Alnus acuminata* spp. *acuminata* roots, probably as a mycorrhizal associate, not abundant, most easily found where emergent in banks; Mar.

Specimens examined.—ARGENTINA, SALTA PROVINCE, Santa Victoria, Los Toldos, site M28B, 1702 m, 28 Mar 2001, *L.S.*

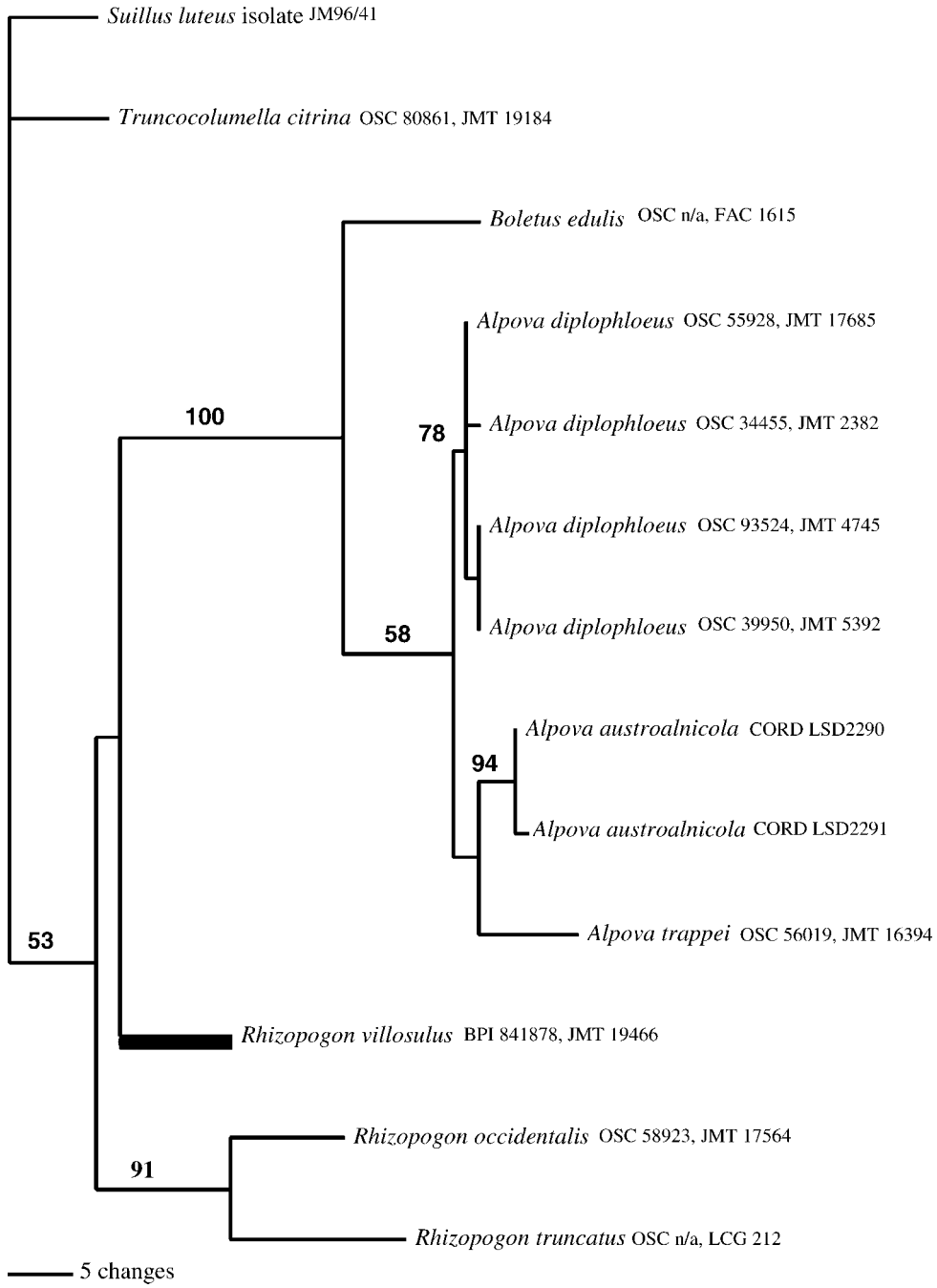


FIG. 10. One out of two most parsimonious trees obtained from the analysis of nuclear LSU rDNA gene. Bootstrap values above 50 are located at the respective internodes. Branches that collapsed in a strict consensus tree are indicated by a thickened line.

Domínguez 2290 (PARATYPE, CORD); site M42, 1778 m, 30 Mar 2001, *L.S. Domínguez 2291* (HOLOTYPE, CORD).

RESULTS

Fecal pellets.—Armadillo fecal pellets contained small amounts of *Alpova austroalnicola* spores. More than 90% of the pellets are represented by plant and mineral material. Mean values of various fungal and other structures observed in the analyzed pellets are provided (TABLE II).

Phylogenetic analysis.—One of the two most parsimonious trees obtained from the analysis of nuclear LSU rDNA gene is shown (FIG. 10). In this topology both *Alpova austroalnicola* collections (CORD LSD

TABLE II. Mean values ($\bar{x}$) of various fungal and other structures observed within the analyzed fecal pellets samples (N=12). Fungal elements are expressed as numbers (*). Plant (leaf fragments and pollen grains) and mineral material (stones) mean values are based on visual estimated percentages (%). (SE) Standard error

Analysis of fecal samples	$\bar{x}$ (SE)
Alpova austroalnicola spores (*)	8.92 (4.4)
Basidiomycete hyphae (*)	30.08 (17.26)
Dark septate spores (*)	21 (10.68)
Dark septate hyphae (*)	10.68 (7.14)
Plant material (%)	43.04 (14.08)
Mineral material (%)	47.04 (13.17)

2290 and CORD LSD 2291) clustered together next to *Alpova diplophloeus* (four collections) and *Alpova trappei,* although the bootstrap figure was low in the *A. austroalnicola-A. trappei* relationship. The grouping of *A. austroalnicola* collections is well supported with a bootstrap value of 94. The relationship of *Alpova* species with *Boletus edulis* is supported by a bootstrap value of 100. The *Rhizopogonaceae* represented by *Rhizopogon occidentalis, R. truncatus* and *R. villosulus* appear as a nonconsistent paraphyletic clade at the base of the tree.

DISCUSSION AND CONCLUSIONS

The combination of morphological characters, molecular data from nuc-LSU rDNA gene analyses and association with *Alnus,* indicate that *A. austroalnicola* is related to *A. diplophloeus,* the type species of the genus. These two species resemble each other closely, but *A. diplophloeus* differs from *A. austroalnicola* in having smaller spores (–5.5[–6] × 3–4.5[–5] μm), larger sporocarps and glebal chambers and a much thicker peridium. *Alpova austroalnicola* is the first hypogeous fungus to be found associated with *Alnus acuminata* spp. *acuminata* in the Southern Hemisphere. *Alpova diplophloeus* associates with *Alnus* species in the Northern Hemisphere (Trappe 1975, Clemençon 1977, Gross 1980, Molina 1981, Godbout and Fortin 1983, Brunner and Horak 1990).

The lack of support for a close relationship of *A. diplophloeus, A. austroalnicola* and *A. trappei* probably reflects the data of Grubisha et al (2001), indicating the nonmonophyletic nature of *Alpova,* although the low number of specimens analyzed, especially *A. trappei* (1), precludes a more accurate interpretation. *Alpova trappei* is associated with North American members of the Pinaceae (Trappe 1975, Fogel 1977), and its peridial structure lacks the peridial layer of inflated cells. The close phylogenetic relationship of *Boletus* (*B. edulis*) and *Alpova spp* is supported strongly, thus representing the boletoid radiation within Boletales (Bruns and Szaro 1992, Bruns et al 1998).

Ours is the first report of mycophagy by the armadillo *Dasypus novemcinctus novemcinctus.* The relatively low number of *A. austroalnicola* spores in the feces suggests the armadillo eats these hypogeous fungi opportunistically. Moreover no other hypogeous fungi have been found in the *A. acuminata* spp. *acuminata* forests, and the number of epigeous mushroom species known in the area is relatively low (Becerra 2002, Nouhra et al 2003), indicating that relatively few, highly specialized, ectomycorrhizal fungi occur in this ecosystem. Similar data have been reported for other *Alnus*-dominated communities (Molina 1979, 1981; Brunner and Horak 1990).

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