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ORIGINAL PAPER

Morphological and molecular identification of the ectomycorrhizal association of *Lactarius fumosibrunneus* and *Fagus grandifolia* var. *mexicana* trees in eastern Mexico

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Abstract A population of *Fagus grandifolia* var. *mexicana* (covering ca. 4.7 ha) is established in a montane cloud forest refuge at Acatlan Volcano in eastern Mexico (Veracruz State), and it represents one of only ten populations of this species known to occur in the country (each stand covers ca. 2–35 ha in extension) and one of the southernmost in the continent. Sporocarps of several ectomycorrhizal macrofungi have been observed in the area, and among them, individuals of the genus *Lactarius* are common in the forest. However, the morphological and molecular characterization of ectomycorrhizae is still in development. Currently, two species of *Lactarius* have been previously documented in the area. Through the phylogenetic analysis of the internal transcribed spacer (ITS) region from basidiomes and ectomycorrhizae, we identified the *Lactarius fumosibrunneus* ectomycorrhiza. The host, *F. grandifolia* var. *mexicana*, was determined comparing the amplified ITS sequence from ectomycorrhizal root tips in the GenBank database with Basic Local Alignment Search Tool. The mycorrhizal system of *L. fumosibrunneus* is monopodial–pyramidal, characterized by its shiny, white to silver and pruinose surface, secreting a white latex when damaged, composed of three plectenchymatous mantle layers, with diverticulated terminal elements at the outer mantle. It lacks emanating hyphae, rhizomorphs, and sclerotia. A detailed morphological and anatomical description, illustrations, and photographs of the ectomycorrhiza are presented. The comparison of *L. fumosibrunneus* and other *Lactarius* belonging to subgenus *Plinthogalus* is presented.

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Introduction

Studies of the characterization of the ectomycorrhizae of native fungi in the neotropics are still limited. In Guatemala and Mexico, some reports have recently been published, describing the ectomycorrhizae of *Lactarius*, one of the most widely represented ectomycorrhizal genus in the region. From this area, the ectomycorrhizae of *Lactarius indigo* (Flores et al. 2005), *Lactarius badiopallescens*, *Lactarius cinereus* (Montoya et al. 2010), and *Lactarius rimosellus* (Comandini et al. 2011) were characterized on a morphological and anatomical basis, and for the three later species, the ectomycorrhizal association with native host trees was determined by means of phylogenetic analyses, displayed on internal transcribed spacer (ITS) sequences. The Mexican montane cloud forest in the Acatlan Volcano (Central Veracruz, eastern Mexico) shelters one of only ten populations of *Fagus grandifolia* var. *mexicana* currently established in the country. Although the stand of *Fagus* trees covers an area of ca 4.7 ha (Williams-Linera et al. 2000, 2003), it represents an interesting natural area for studying the ectomycorrhizal fungal component taking into account its relict condition. As it represents one of the southernmost populations of *Fagus* in North America, its study is relevant to complement current information about biogeographical patterns of ectomycorrhizal fungal species associated with this genus. The vegetation in the crater of the Acatlan Volcano is dominated by *F. grandifolia* var. *mexicana* with some specimens of *Podocarpus matudae* Lundell, *Magnolia schiedeana* Schlecht, and *Quercus* spp.

(cf. also Williams-Linera et al. 2003). Several members of macrofungi belonging to ectomycorrhizal genera have been detected at the site through the presence of above-ground sporocarps; among them, *Lactarius* is well represented. *L. cinereus* Peck and *L. badiopallescens* Hesler & A.H. Sm. were recognized as ectomycorrhizae forming with native trees of *F. grandifolia* var. *mexicana* in the area (Montoya et al. 2010), and *Lactarius fuscomarginatus* Montoya, Bandala, and Haug, a new species recently described (Montoya et al. 2012), was found growing there probably in ectomychorrizal association with this tree species. *Lactarius fumosibrunneus* is a member of subgenus *Plinthogalus* (Burl.) Hesler & A.H. Sm. and has been recorded as one of the most dominant macrofungal species, in terms of basidiomes production (Bandala and Garay-Serrano, personal observation) in the period between June and November, 2006–2010. We provide a morphological description of the *L. fumosibrunneus* ectomycorrhiza, and using sequence data from rDNA ITS region of basidiomes and fungi/host tree from ectomycorrhizal root tips, we demonstrate that *L. fumosibrunneus* forms ectomycorrhizal symbiosis with *F. grandifolia* var. *mexicana* trees.

Materials and methods

Study area

Samples were collected in a stand (ca 4.7 ha) of *F. grandifolia* var. *mexicana* at Acatlán Volcano (19°40′45.7″ N, 96°51′12″ W, 1,950 m altitude), municipality of Acatlán, Veracruz, in eastern Mexico.

Sampling root systems and sporocarps

Basidiomes of *L. fumosibrunneus* and root samples below stipe bases were collected in the studied forest during June–September 2008 and August–October 2009. Fine roots were separated from soil samples under the stereomicroscope. Morphotypes were selected, photographed, and characterized anatomically and morphologically following in part Agerer (1987–2002). Morphotypes were conserved in ethanol at 75 %, and other parts of each sample used for molecular analyses were dried in a hot air dehydrator (±35–45°C). Hand-made sections of root tips were mounted in 3 % KOH and 1 % Congo red (in water) for anatomical characterization of ectomycorrhiza. Macromorphological features of basidiomes were recorded in fresh condition, and microscopical study was developed on dry specimens following Bandala and Montoya (2010); sporocarp collections form part of XAL herbarium (B. Thiers, continuously updated; Index Herbariorum: http://sweetgum.nybg.org/ih/).

DNA extraction, PCR amplification, and sequencing

Ectomycorrhizae with latex and laticifers were selected for molecular analyses. Genomic DNA was extracted from both dried ectomycorrhizal root tips and fruit bodies using the innuPRPE Plant DNA (Analitik Jena AG, Germany) following the manufacturer's recommendations. The ITS region from the ribosomal DNA was amplified for ectomycorrhizal root tips and fruit bodies with primer combinations ITS1F–ITS4 (White et al. 1990; Gardes and Bruns 1993). PCR reactions, purifications of PCR products, and sequencing were performed as described by Montoya et al. (2010). Sequences were edited with the Sequencher software (V 4.1, Gene Codes, Ann Arbor, MI, USA). For the recognition of the plant symbiont, the region ITS1–ITS2 in the rDNA of ectomycorrhizal root tips was also processed for amplification with the ITS1 and ITS4 primers, and fragments were sequenced in the same way as fungi. The Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) at GenBank and UNITE database were used for determining sequence similarities of symbiotic trees; those with high similarities (99 %) were considered as the same taxon.

Phylogenetic analyses of *Lactarius* sequences

Sequences from different *Lactarius* species related to *L. fumosibrunneus* (Hesler and Smith 1979; Bandala and Montoya 2010) were downloaded from the UNITE (http://unite.ut.ee/) and GenBank (http://www.ncbi.nlm.nih.gov/) databases and aligned using POA (Lee et al. 2002). Ambiguously aligned positions from our POA multiple sequence alignment were eliminated using trimAl with the parameter option *automated1* (Capella-Gutiérrez et al. 2009). The data matrix, including 13 *Lactarius* sequences and 645 nucleotide positions, was analyzed by maximum likelihood (ML) as implemented in RAxML version 7.0.3 (Stamatakis 2006). A combined rapid bootstrapping and ML search under the GTRMIX model was computed from 1,000 runs (Stamatakis et al. 2008). The phylogenetic tree was displayed using FigTree V1.3.1 (Rambaut 2009).

Results

A total of 46 collections of *L. fumosibrunneus* (Fig. 2a) as interpreted by Bandala and Montoya (2010) were gathered during 2006–2010 in the studied *Fagus* forest of the Acatlan Volcano. We listed here only the fruit bodies collected in 2008–2009, including those collections used for sequencing (previous collections were recorded by Bandala and Montoya op. cit.). From collections *Garay 3* (EG3) and *Garay 10* (EG10), ITS sequences were obtained and added to the phylogenetic analysis (Fig. 1). *Specimens*

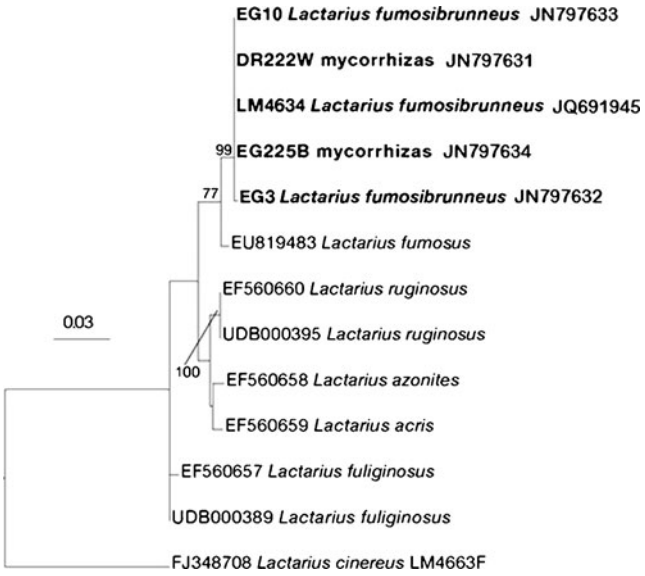

Fig. 1 Phylogenetic relationships of fruit bodies and ectomycorrhizal ITS sequences of *L. fumosibrunneus* obtained in this study. *L. cinereus* was used as the outgroup. Phylogram obtained from a RAxML analysis under the GTRMIX model of DNA evolution. Numbers are maximum likelihood bootstrap values based on 1,000 replicates

examined: MEXICO. Veracruz, Acatlan, Acatlan Volcano, 10 Sep 2008, *Garay 3*; *Garay 10*. 30 Jul 2009, *Montoya 4739*; *Montoya 4740*; *Montoya 4741*; *Montoya 4742*; *Montoya 4743*; *Montoya 4744*; *Montoya 4745*; *Montoya 4746*; *Montoya 4747*; *Montoya 4748*; *Montoya 4749*. 5 Aug 2009, *Garay 70*; *Garay 72*; *Garay 74*; *Garay 76*; *Garay 79*. 12 Aug 2009, *Garay 81*; *Garay 82*; 1 Sep 2009, *Garay 144* (all at XAL).

Nine ectomycorrhizae with latex and laticifers were detected in soil samples, and they were molecularly analyzed but just five were successfully sequenced. Two of these sequences, EG225B and DR222W, cluster together in a phylogenetic analysis with those obtained from *L. fumosibrunneus* basidiomes, with 99 % bootstrap support (Fig. 1). Fruit body sequences were deposited at GenBank under accession numbers JN797632 (EG3), JN797633 (EG10), and ectomycorrhizal sequences under accession numbers JN797631 (DR222W) and JN797634 (EG225B). The other three sequences obtained from ectomycorrhizal root tips (EG10B, EG107CC, and EG238P) (uploaded with accession numbers JQ246011, JQ246012, and JQ246013) shared a high similarity (99 %) with sequences of *L. cinereus* deposited in the GenBank database (accession numbers: FJ348708, FJ348709, FJ348710, FJ348711). For the description of the ectomycorrhizal morphotype of *L. cinereus*, we referred to Montoya et al. (2010).

The host tree was recognized through the comparison of the sequence from the ectomycorrhizal root tip with BLAST in GenBank, which showed a high similarity (99 %) with the sequence AY232929 of *F. grandifolia* var. *mexicana*. The *Fagus* sequence obtained here was uploaded at GenBank under accession number JQ691946

Description of *L. fumosibrunneus* ectomycorrhiza

Morphological characters

Mycorrhizal systems are monopodial–pyramidal (Fig. 2b–d), 5–18 mm length, main axes 0.5–0.8 mm diameter, with 0–2 (–4) levels of ramification, sometimes forming basal clusters; unramified ends straight or slightly bent and cylindrical in distal end, 1–1.7×0.2–0.6 mm; surface slightly rough, pruinose, shiny, white to silver or gray when touched, irregularly tinged with faint pinkish hues, older parts with brown stains; mantle not translucent; secreting white latex when damaged; and tips cylindrical, brownish when old, emanating hyphae, rhizomorphs, and sclerotia absent and turning orange to reddish-orange with KOH 3 %.

Anatomical characters in longitudinal section

Mantle 70–72 μm wide, three mantle layers discernible (Fig. 2e). *Outer mantle* plectenchymatous, hyphae cylindrical, colorless with 2–3 μm in diameter; terminal elements frequent as short, diverticulate (Fig. 2h), laticifers 5–7 μm diameter. *Middle mantle* plectenchymatous, colorless hypha, 3–4 μm diameter, embedded in a gelatinous matrix, laticifers 5–8 μm diameter with yellowish, granulose content. *Inner mantle* in appearance pseudoparenchymatous, compact, composed by tangentially oriented, colorless hyphae 8–17 μm diameter. A tannin layer present, with elliptic or cylindrical cells, oriented in parallel to central axis, 6–11 μm diameter. Hyphae of Hartig net with palmetti structure, penetrating up to the second epidermal cell rows (Fig. 2f), hyphae around epidermal cells appear irregularly subisodiametric in an individual row. Epidermal cells rectangular to cylindrical, some of them oriented obliquely 15–22 μm wide. Clamps absent in all tissues.

Anatomical characters in cross section

Mantle consisting of 10–15 hyphal layers, 50–65 μm wide (Fig. 2g). *Outer mantle* plectenchymatous, hyphae 2–3 μm diameter, thin-walled, terminal elements diverticulate, densely arranged 5–10×7–12 μm. *Middle mantle layer* plectenchymatous, colorless hypha, 3–4 μm diameter, in a gelatinous matrix, laticifers 5–6 μm diameter, with yellowish, granulose content. *Inner mantle* plectenchymatous, with irregularly arranged hyphae, more compactly disposed than those of the middle mantle, hyphae 3–4 μm diameter, other 12–15 μm diameter and shortly septate; laticifers 6–7 μm diameter, with yellowish content. Hyphae of the Hartig net penetrate up to the second epidermal cell rows, hyphae with palmetti structure, hyphae around epidermal cells appear irregularly subisodiametric in an individual row (Fig. 2g). Clamps absent in all tissues.

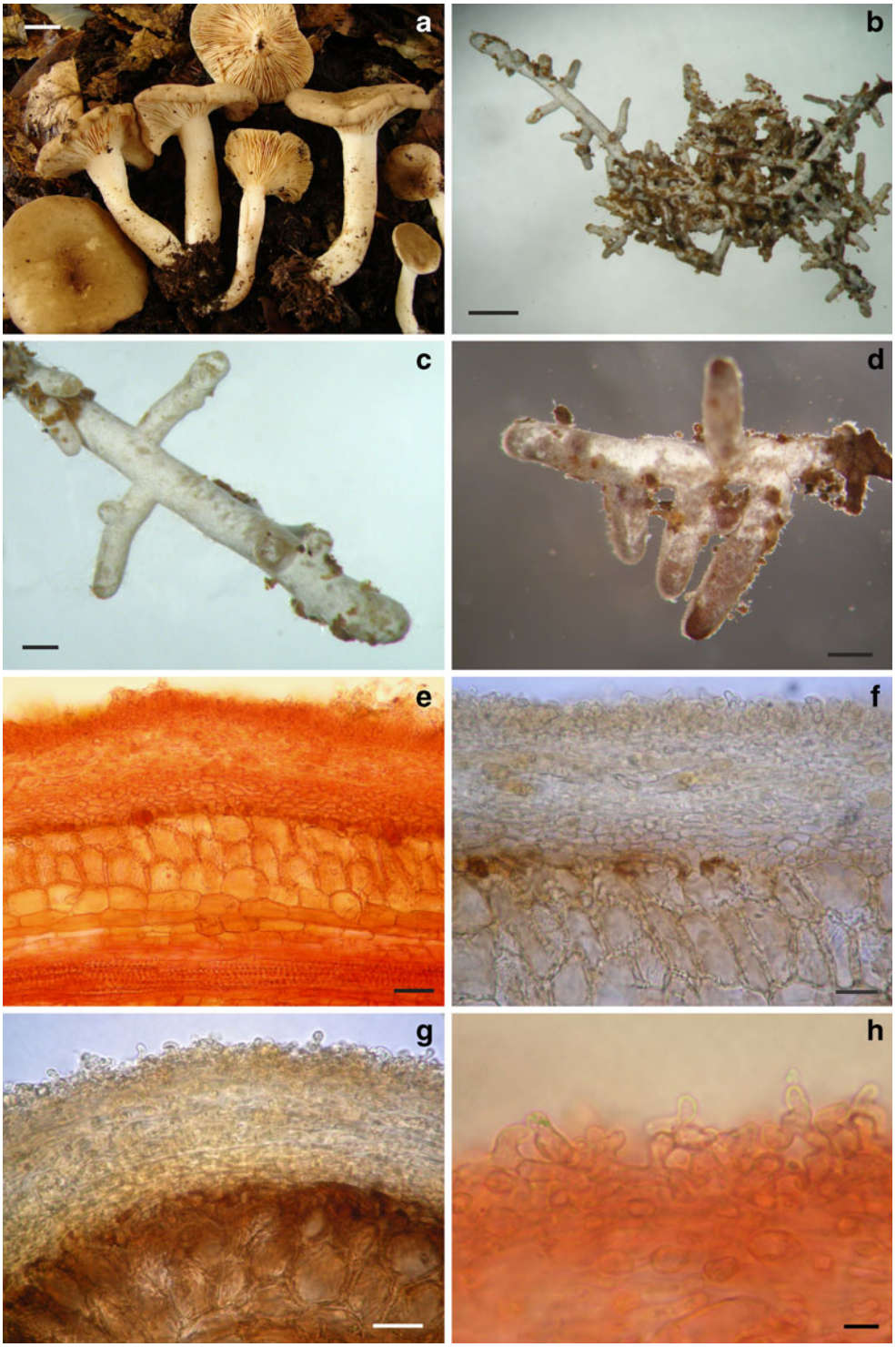

Fig. 2 *Lactarius fumosibrunneus* associated with *F. grandifolia* var. *mexicana*. **a** Fruit bodies (Garay 72); **b–d** ectomycorrhizal (ECM) characteristics under stereomicroscope; **e** ECM in longitudinal section stained with Congo red; **f** details of the outer, middle, and inner mantle and Hartig net in longitudinal section; **g** ECM in cross section, showing the mantle; **h** terminal diverticulated elements on the outer mantle. *Scale bars* **a** 1 cm; **b** 2 mm; **c**, **d** 0.5 mm; **e** 25 μm; **f**, **g** 20 μm; **h** 5 μm

Anatomical characters of mantle in plan views

Outer mantle with rounded to elongated terminal elements (Fig. 3a, b) giving a floccose appearance to the surface, with a plectenchymatous pattern, hyphae 3–4 μm diameter, slightly yellowish, cylindrical, thin-walled (<1 μm), laticifers 5–6 μm diameter, some with granular content. *Middle mantle* layer pseudoparenchymatous with colorless, gelatinized hyphae, 3–8 μm diameter, loosely arranged in several directions, laticifers 5–9 μm diameter, at times with a dense, granular, yellowish content. *Inner mantle* plectenchymatous, with hyphae 4–6 μm diameter, slightly compactly arranged in a more or less net-like disposition (Fig. 3c), laticifers 5–9 μm diameter, with yellowish content. Clamps absent in all tissues.

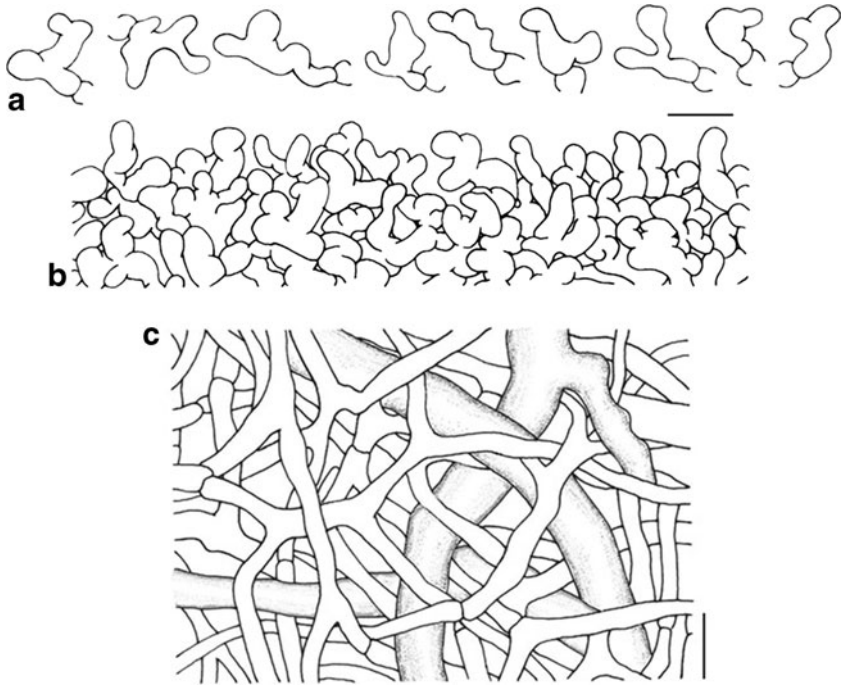

Fig. 3 **a**, **b** Diverticulated elements on the outer mantle of the ectomycorrhiza of *L. fumosibrunneus* (**a** isolated, diverticulated elements; **b** disposition of the elements). **c** Plectenchymatous inner mantle layer: laticifers with yellowish content and hyphae forming a net-like structure. *Scale bar*=10 μm

Discussion

Basidiomes of *Lactarius* species are frequently recorded among the community of ectomycorrhizal fungi observed above ground in the *Fagus* forest on the Acatlan Volcano. *L. badiopallescens*, *L. cinereus*, *L. fumosibrunneus*, and *L. fuscomarginatus* have recently been studied in this area (Bandala and Montoya 2010; Montoya et al. 2010, 2012) and, along with other *Lactarius* species, form part of the above-ground diversity of ectomycorrhizal fungi occurring under *F. grandifolia* var. *mexicana* in the forest studied. The ectomycorrhiza of *L. fumosibrunneus* and the recognition of its association with *F. grandifolia* var. *mexicana* trees were molecularly assessed and reported here for the first time. According to the literature, around 44 ectomycorrhizae of *Lactarius* have been morphologically and anatomically described (Agerer 1987–2002; Agerer and Rambold 2004–2012; Flores et al. 2005; Montoya et al. 2010; Comandini et al. 2011). Among these, ectomycorrhizae of species belonging to subgenus *Plinthogalus* such as *Lactarius acris* (Bolton) Gray (associated with *Fagus sylvatica* L.), *Lactarius lignyotus* Fr. (associated with *Picea* L.), and *Lactarius picinus* Fr. [associated with *Picea abies* (L.) Karst.] (Agerer 1987–2002) have some characteristics in common with *L. fumosibrunneus*. They share ectomycorrhizal root tips with monopodial–pyramidal ramification, surface shiny white or silvery pruinose, terminal elements at the outer mantle (which confers the pruinose surface appearance), and the plectenchymatous pattern of the outer, middle, and inner mantles. Differences between these four species can be observed in that although *L. fumosibrunneus* and *L. acris* share white latex and the presence of a palmetti Hartig net type, they can be distinguished because *L. acris* forms rhizomorphs. As for *L. lignyotus*, the presence of emergent hyphae, and the grayish to olive-green latex distinguish it from *L. fumosibrunneus*. In comparison with *L. picinus*, the *L. fumosibrunneus* ectomycorrhiza lacks both rhizomorphs and a Hartig net protruding toward the endodermis, when observed in cross section. Even when there are differences between the four *Plinthogali* species, the morphological recognition of their mycorrhizae at species level could be difficult. Differences at edaphic habitat are also discernible; members of subgenus *Plinthogalus* in Europe often grow on calcareous soil (Heilmann-Clausen et al. 1998), in contrast with *L. fumosibrunneus*, which grows in Andept soil in Acatlan. In the study area, the ectomycorrhiza of *L. badiopallescens* and *L. cinereus* has been reported to be associated with *F. grandifolia*, but they can be distinguished from that of *L. fumosibrunneus* by differences in color and anatomical characteristics (Montoya et al. 2010). It is known that many *Lactarius* species are specifically related to a certain tree (Heilmann-Clausen et al. 1998), and with regard to *Fagus*, in Europe for example, *L. acris* and *L. pallidus* are considered specifically associated with *F. sylvatica* (Agerer 1987–2002; Agerer and Rambold 2004–2012). In the Acatlan Volcano, *Quercus* trees are also present (Williams-Linera et al. 2000). Further research about ectomycorrhizae associated with *Quercus* could clarify whether the *Lactarius* species studied so far establish ectomycorrhizal associations with a wider range of hosts or if they are obligate associates of *F. grandifolia* var. *mexicana* trees.

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