MORPHOANATOMICAL AND PHYLOGENETIC CHARACTERIZATION OF THE ECTOMYCORRHIZA BETWEEN *LACCARIA SQUARROSA* WITH *PINUS PSEUDOSTROBUS* AND ITS RELEVANCE FOR REFORESTATION PROGRAMS

CARACTERIZACIÓN MORFOANATÓMICA Y FILOGENÉTICA DE LA ECTOMICORRIZA DE *LACCARIA SQUARROSA* CON *PINUS PSEUDOSTROBUS* Y SU RELEVANCIA PARA PROGRAMAS DE REFORESTACIÓN

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Abstract

Background: *Pinus* (Coniferophyta) and *Laccaria* (Basidiomycota) establish ectomycorrhizal symbioses in natural forests. However, their detailed morphoanatomical and phylogenetic characterization have received little attention. Accurate identification of native host symbionts is of paramount relevance to the production of mycorrhizal seedlings for successful reforestation programs.

Questions/Objective: We aimed to determine if *L. squarrosa* is able to establish ectomycorrhizal symbiosis with gymnosperms, thereby widening its host range and highlighting its relevance as a potential inoculant for pine seedlings. Currently, *L. squarrosa* is only known from its type collection associated with the angiosperm *Fagus grandifolia* var. *mexicana*.

Studied species: The fungus *L. squarrosa* and *Pinus pseudostrobus*, a tree endemic to Mexico.

Study site and dates: A *Pinus-Quercus* forest in Piedra Canteada, Nanacamilpa, Tlaxcala; 2018-2020.

Methods: *L. squarrosa* basidiomata were identified and ectomycorrhizal roots were collected and morphoanatomically characterized. For molecular identification, DNA was extracted, PCR was performed targeting the nuclear ribosomal internal transcribed spacer region (nucDNA ITS) for the mycobiont identification and the chloroplastic single-locus *trnL* region for the phytobiont.

Results: In the phylogenetic analyses, our sequences from basidiomata and ectomycorrhizae clustered together with *L. squarrosa* with high values of supporting identity. Meanwhile, *P. pseudostrobus* was molecularly identified as the phytobiont.

Conclusions: This is one of the few worldwide characterizations of *Laccaria* ectomycorrhiza under field conditions and contributes to the understanding of the ecology, distribution, and economic relevance of the symbiotic association. Our data suggest that *L. squarrosa* has potential for use as a native inoculant for *P. pseudostrobus* tree production.

Keywords: Ectomycorrhizal symbiosis, edible wild mushrooms, inoculants, Neotropics, pines.

Resumen

Antecedentes: Las especies de *Pinus* (Coniferophyta) y *Laccaria* (Basidiomycota) establecen simbiosis ectomicorrízicas en bosques naturales. Sin embargo, su caracterización detallada morfológica y molecular ha recibido poca atención, a pesar de que la identificación precisa de fitobiontes nativos es fundamental para la producción exitosa de plantas micorrizadas con fines de reforestación.

Preguntas/Objetivos: Conocer si *Laccaria squarrosa* establece ectomicorrizas con gimnospermas, ampliando su rango de hospederos y relevancia como inoculante ectomicorrízico potencial para la producción de plántulas de pino. Hasta ahora *L. squarrosa* solo se conocía de su colección tipo asociada con *Fagus grandifolia* var. *mexicana*.

Especies estudiadas: *L. squarrosa* y *Pinus pseudostrobus*, árbol endémico de México.

Sitio y años de estudio: Bosque de *Pinus-Quercus*, Piedra-Canteada, Tlaxcala; 2018-2020.

Métodos: Se identificaron basidiomas de *L. squarrosa* y se recolectaron y caracterizaron morfoanatómicamente raíces ectomicorrizadas. Para la identificación molecular, se extrajo ADN, se realizaron PCR de la región espaciadora transcrita interna del ADN ribosomal (nuc-rDNA ITS) para la identificación del micobionte y de la región cloroplástica *trnL* para el fitobionte.

Resultados: En los análisis filogenéticos, las secuencias de basidiomas y ectomicorrizas se agruparon junto con *L. squarrosa* con altos valores de similitud. Mientras tanto, *P. pseudostrobus* se identificó molecularmente como el fitobionte.

Conclusiones: Esta es una de las pocas caracterizaciones mundiales de ectomicorrizas de *Laccaria* en campo; y se contribuye a la comprensión de la ecología y distribución de esta asociación simbiótica económicamente relevante. Se demuestra que *L. squarrosa* podría usarse potencialmente como inoculante nativo para la producción de árboles de *P. pseudostrobus*.

Palabras claves: hongos silvestres comestibles, inoculantes, Neotrópico, pinos, simbiosis ectomicorrízica.

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Mexico is losing an average of 318,000 hectares of forest annually and is one of the countries with the highest deforestation rates, resulting in rapid ecosystem loss and decreasing native biodiversity (including plants and mushrooms) (Pérez-Moreno *et al.* 2020). Mexico is also the center of diversity of *Quercus* and *Pinus* species (Perry 1991, Valencia & Flores-Franco 2006) which are both widely known as ectomycorrhizal (ECM) trees. As Mexico harbors a great diversity of ECM trees, a high number of ECM mushrooms are expected. These ectomycorrhizal associations occur in all forest ecosystems from the tropics to the subpolar zones (Nara 2015). The ECM trees depend on the ECM fungi for obtaining greater access to mineral nutrients and water, while the trees provide a supply of carbohydrates to the symbiotic fungi (Smith & Read 2008).

Laccaria species associate with many ECM trees (Trappe 1962, Mueller 1992, Kropp & Mueller 1999, Wilson *et al.* 2017, DEEMY 2021). It is estimated that 116 species of *Laccaria* are distributed across temperate and tropical regions of the world (Wilson *et al.* 2017). The diversity and taxonomy of the genus *Laccaria* have been well documented (Mueller 1984) including with phylogenetic and molecular studies (Osmundson *et al.* 2005, Vincenot *et al.* 2012, Sheedy *et al.* 2013, Wilson *et al.* 2013, Popa *et al.* 2014, 2016, Montoya *et al.* 2015, Cho *et al.* 2018). More recently, biogeographic (Wilson *et al.* 2017) and genomic studies have used some *Laccaria* taxa (*e.g.*, Martin *et al.* 2008, Kang *et al.* 2020, Li *et al.* 2020) as model species in the understanding of the physiology, ecology, and evolution of ectomycorrhizal symbiosis.

In general, *Laccaria* species are considered early-stage colonizers (Nara *et al.* 2003, Reverchon *et al.* 2012) and therefore play a paramount ecological role in the establishment of ECM host plants (Kropp & Mueller 1999, Nara *et al.* 2003, Ashkannejhad & Horton 2005). They can grow vegetatively and are relatively easy to manipulate under experimental conditions. Several species of the genus, including for example *Laccaria bicolor* (Maire) P.D. Orton, *L. laccata* (Scop.) Cooke, and *L. proxima* (Boud.) Pat., have successfully been used in the production of ECM inoculants for forest management purposes (Molina 1980, Molina & Chamard 1983, Chakravarty & Hwang 1991, Duponnois & Garbaye 1991, Werner & Zadworny 2003, Pérez-Moreno *et al.* 2020). *Laccaria* species such as *Laccaria amethystina* Cooke. and *L. laccata* (Scop.) Cooke also have great cultural, social, and economic value and are harvested for income and as food in different parts of the world (McKnight *et al.* 1998, Pérez-Moreno *et al.* 2008, Kalita *et al.* 2016, Wu *et al.* 2019). *Laccaria squarrosa* Bandala, Montoya & A. Ramos, however, is a recently described species. It is only known from its type locality, where it associates with endangered *Fagus grandifolia* var. *mexicana* (Martínez) E. Murray trees in eastern Mexico (Ramos *et al.* 2017), and from discovery at a market in western Mexico, where it was identified as a rare species by Farfán-Heredia *et al.* (2018) without stating the vegetation type or location from where the specimens were gathered.

Pinus pseudostrobus Lindl. has a wide geographical distribution in Mexico and Central America with a broad altitudinal range between 1,600-3,300 m above sea level (Delgado *et al.* 2007). The species usually establishes pure stands or occurs with some other species of *Pinus*, *Abies*, *Arbutus*, *Juniperus*, or *Quercus* (Perry 1991). In Mexico, *P. pseudostrobus* has been used in reforestation programs especially on degraded soils, and its resins and wood are highly commercialized (Gómez-Romero *et al.* 2013).

During some expeditions to forest located in Piedra Canteada, Nanacamilpa, Tlaxcala, in Central Mexico, to document the diversity of wild edible mushrooms consumed by the local people, we found and collected specimens of *L. squarrosa* (a legitimate species included in the Mycobank database with the number 823034). The study site is in a natural reserve called the “*Santuario de las Luciernagas*” (The Sanctuary of the Fireflies) famous for the displays of fireflies that inhabit its *Pinus-Quercus* forest. The local people make an income from this activity during the summer, motivating them to conserve the forest and manage its resources. Basidiomata of *L. squarrosa* are also harvested as a source of food by members of the Nahua ethnic groups in this area.

In this study, we aim to determine for the first time whether *Laccaria squarrosa* establishes ectomycorrhizal symbiosis with the gymnosperm *Pinus pseudostrobus* in natural conditions, confirming the taxonomic identity of both symbionts with molecular analyses. The confirmation of this association would provide evidence to promote the use of *L. squarrosa* as a potential native inoculum in the production of ectomycorrhized *P. pseudostrobus* seedlings in successful reforestation programs in the studied area.

Materials and methods

Study area. The study area is in the state of Tlaxcala, Mexico, on the Trans-Mexican Volcanic Belt (TMVB; [Figure 1](#)). This ecoregion connects the Sierra Madre Oriental and the Sierra Madre Occidental, and serves as a center of biodiversity. The sampling site is in Piedra Canteada, Nanacamilpa municipality ([Figure 1](#)). The dominant climate is temperate subhumid with rains in summer and annual temperatures of 12-18 °C. Mean annual precipitation is < 500 mm (INEGI 1997). The type of vegetation in the study area is dominated by *Pinus-Quercus* (pine-oak) forest and *Abies* (fir) forest. The most representative species in the *Pinus-Quercus* Forest include *Pinus montezumae* Lamb, *P. pseudostrobus*, *P. teocote* Schiede ex Schltdl. & Cham., which usually co-occur with *Quercus* spp. such as *Quercus crassipes* Bonpl., *Q. laurina* Bonpl., and *Q. rugosa* Née. The *Abies* forest is composed of *Abies religiosa* (Kunth) Schltdl. & Cham., *Alnus jorullensis* Kunth., *Salix paradoxa* Kunth., and *Arbustus xalapensis* Kunth., all coexisting in the same area.

Figure 1. Map of the sampling site in Piedra Canteada, Nanacamilpa (brown), Tlaxcala (green), Mexico. Range of the Trans-Mexican Volcanic Belt (red).

Sampling and description. *L. squarrosa* basidiomata were collected in a *Pinus-Quercus* forest area which is known by local mushrooms pickers as a fruiting area for this species of *Laccaria*. The collection was made in a small area where *Pinus pseudostrobus* dominates ([Figure 2](#)). The basidiomata were identified in the field as *L. squarrosa* due to the squarrose surfaces on the stipe which is a diagnostic macromorphological feature of this species. We compared the macroscopic and microscopic characters of our samples with those described for the *L. squarrosa* type specimen (Ramos *et al.* 2017), and as additional evidence we extracted DNA and amplified the internal transcribed spacer (ITS) region (see the following section) from the basidiomata collected in the study area to confirm their identity. Soil samples were collected below the basidiomata following Agerer (1991) and Gardes & Bruns (1996). A stereo-

microscope (Leica S8APO) was used to separate fine roots from the soil samples to select, photograph, and describe the morphology of the ectomycorrhizae that were found (Agerer 1987-2002). From each ectomycorrhiza, hand-made sections were made (cross and longitudinal), mounted in 3 % KOH and observed under a compound light microscope (Leica DM2500) for microscopic characterization (Agerer 1987-2002, DEEMY 2021). The ectomycorrhizae were preserved in 75 % ethanol and tips were stored at -20 °C for DNA extraction to identify the symbionts.

Figure 2. *Pinus pseudostrobus* forest located in the study area.

DNA extraction, PCR, and phylogenetic analyses. Total genomic DNA from both basidiomata and ectomycorrhizae was obtained using a modified cetyltrimethylammonium bromide (CTAB) procedure of Gardes & Bruns (1993). The polymerase chain reaction was performed targeting the nuclear ribosomal ITS regions using the primers ITS1F and ITS4 (White *et al.* 1990). PCR conditions were performed as follows: 94 °C for 5 min; then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; followed by a final elongation step of 72 °C for 7 min. We constructed a dataset of aligned sequences obtained from root tips and basidiomata using the Phylogenetic Data Editor (PhyDE, v.0.997, Muenster, Germany, www.phyde.de). Genbank sequences that showed highest similarity scores (> 97) using the BLAST tool (Altschul *et al.* 1997) and sequences used in previous studies (Osmundson *et al.* 2005, Wilson *et al.* 2013) were included in the analyses. *Cortinarius violaceus* (L.) Gray (DQ486695) and *Psathyrella rhodospora* G. Weaver & A.H. Sm. (DQ486695) were included as outgroups (Table 1). To identify the host tree species at the nucleotide level, the plastid/chloroplastic single-locus *trnL* region was amplified from DNA isolated from the root tip. We amplified the *trnL* region using *trnC/trnD* primer pairs (Tedersoo *et al.* 2008). A second dataset was constructed including the new *trnL* sequences and 10 sequences of *Pinus* species retrieved from BLASTN results in GenBank (Altschul *et al.* 1997) and one of *Pseudolarix amabilis* (NC030631) included as an outgroup. Both datasets (ITS-*trnL*) were separately aligned using the MUSCLE program (Edgar 2004). The

best DNA model was selected using Mega 6.06 (Tamura *et al.* 2013). For the ITS dataset, Maximum likelihood (ML) and Bayesian inference (BI) were used. ML analysis was performed using MEGA 6.06 (Tamura *et al.* 2013) with 500 bootstrap replicates, while the BI analysis was estimated using MrBayes on XSEDE (3.2.6) using the CIPRES portal (Miller *et al.* 2010), employing two independent runs of 1,000,000 generations. For each run, four chains were employed, and one tree was sampled every 1,000 generations. respectively. Only Bootstrap values (BS) higher than 75 and Bayesian posterior probabilities (BPPs) above 0.85 were considered significant. As with the ITS dataset, ML analysis of the *trnL* dataset was performed using MEGA 6.06, and only BS values higher than 65 were considered significant.

Table 1. *Laccaria* species included in this study: voucher specimen, location, and sequence accession numbers. Black asterisk (*) indicates new sequences generated in this study.

Taxon	Voucher Specimen	Location	Genbank ITS
<i>Laccaria</i> sp.	ALB183	Tibet, China	JX504092
<i>Laccaria</i> sp.	GMM6019	Costa Rica	KU685757
<i>Laccaria</i> sp.	GMM6012	Costa Rica	KU685758
<i>L. alba</i> Zhu L. Yang & Lan Wang	AWW438	Yunnan, China	JX504094
<i>L. alba</i>	F1120750	China	JX504126
<i>L. amethystea</i> (Bull.) Murrill	FP-98556	Vorpommern, Germany	DQ499640
<i>L. amethystea</i>	TUB 011464	Germany	AF539737
<i>L. amethysteo-occidentalis</i> G.M. Muell.	AWW556	California, USA	JX504107
<i>L. amethysteo-occidentalis</i>	AWW590	Oregon, USA	JX504112
<i>L. amethystina</i> Cooke	GMM7041	Caucasus, Russia	KU685654
<i>L. amethystina</i>	GMM7621	France	JX504150
<i>L. angustilamella</i> Zhu L. Yang & L. Wang	BAP226	Yunnan, China	JX504118
<i>L. angustilamella</i>	HKAS58714	Yunnan, China	JX504168
<i>L. aurantia</i> Popa, Rexer, Donges, Zhu L. Yang & G. Kos	KUN-F78557 Type	Yunnan, China	JQ670895
<i>L. aurantia</i>	MB-FB-101109	Yunnan, China	JQ681209
<i>L. bicolor</i> (Maire) P.D. Orton	AWW539	Illinois, USA	KM067817
<i>L. bicolor</i>	AWW537	Illinois, USA	JX504105
<i>L. nobilis</i> A.H. Sm.	F1091206	Michigan, USA	KU685636
<i>L. ochropurpurea</i> (Berk.) Peck	JMP0038	Wisconsin, USA	EU819479
<i>L. ochropurpurea</i>	PRL4777	Illinois, USA	KU685733
<i>L. ochropurpurea</i>	KH_LA06_016	Louisiana, USA	KU685721
<i>L. ochropurpurea</i>	PRL3777	Illinois, USA	KU685732
<i>L. proxima</i> (Boud.) Pat.	F1133825	Mississippi USA	KU685642
<i>L. roseoalbescens</i> T.J. Baroni, Montoya & Bandala	LM5042	Veracruz, Mexico	KJ874327

Taxon	Voucher Specimen	Location	Genbank ITS
<i>L. roseoalbescens</i>	LM5099 Type	Veracruz, Mexico	KJ874328
<i>L. salmonicolor</i> A.W. Wilson & G.M. Muell.	GMM7596 Type	Tibet, China	JX504143
<i>L. squarrosa</i> Bandala, Montoya & A. Ramos	DM63 Type	Veracruz, Mexico	MF669958
<i>L. squarrosa</i>	DM93	Veracruz, Mexico	MF669959
<i>L. squarrosa</i>	DM121	Veracruz, Mexico	MF669960
<i>L. squarrosa</i> *	Hernández-Santiago 67	Tlaxcala, Mexico	MT026967
<i>L. squarrosa</i> *	Hernández-Santiago 65	Tlaxcala, Mexico	MT026968
<i>L. squarrosa</i> *	Martínez-Reyes 273	Tlaxcala, Mexico	MT026969
<i>L. squarrosa</i> *	Martínez-Reyes 274	Tlaxcala, Mexico	MT026970
<i>L. trichodermophora</i> G.M. Muell.	GMM7697	Texas, USA	KM067863
<i>L. trichodermophora</i>	GMM7698	Texas, USA	KM067864
<i>L. trichodermophora</i>	GMM7703	Texas, USA	KM067865
<i>L. trichodermophora</i>	GMM7712	Texas, USA	KM067866
<i>L. trichodermophora</i>	GMM7714	Texas, USA	KM067867
<i>L. trichodermophora</i>	GMM7716	Texas, USA	KM067869
<i>L. trichodermophora</i>	TENN42523 Type	Texas, USA	DQ149868
<i>L. trichodermophora</i>	Montoya 4394	Veracruz, Mexico	MF669962
<i>L. trichodermophora</i>	Bandala 4282	Veracruz, Mexico	MF669963
<i>L. trichodermophora</i>	F1111951	Costa Rica	KU685640
<i>L. trichodermophora</i>	KH_LA06_013	Lousiana, USA	KM067881
<i>L. trichodermophora</i>	GMM7733	Texas, USA	JX504157
<i>Psathyrella rhodospora</i> M.G.Weaver & A.H. Sm.	MP133 MN	-----	DQ267129
<i>Cortinarius violaceus</i> (L.) Gray	MTS 4854 (WTU)	Washington, USA	DQ486695

Results

Four soil samples were collected beneath basidiomata of *Laccaria squarrosa* (MT026967 and MT026968) (Figure 3) from different sites in a pure *Pinus pseudostrobus* stand in Nanacamilpa, Tlaxcala, Mexico. A total of eight morphotypes were found in the soil samples and photographed, described, and sequenced. After BLAST analysis (Genbank), the samples labeled as Martínez-Reyes 273 ECM (MT026969) and Martínez-Reyes 274 ECM (MT026970), were confirmed as *L. squarrosa*. In our phylogenetic analysis, these samples clustered together (with high bootstrap and posterior probabilities values, BS = 99, BPPs = 1) with the sequence from the basidiomata of *L. squarrosa* collected in the study area and the sequence of the type specimen (Figure 4). In the case of the host tree the ML analyses clustered together our samples (Martínez Reyes 273, MW082603; Martínez Reyes 274, MW082604) with *Pinus pseudostrobus* sequences with a moderate but significant support value (BS = 65; Figure 5).

Description of the ectomycorrhiza. Laccaria squarrosa (Bandala, Montoya & A. Ramos) + *Pinus pseudostrobus* Linl. (Figure 6). Ectomycorrhizal systems 1.83 mm in length and 3.15 mm wide, dichotomous, with short branches in two levels of ramification, branches 0.54-0.80 mm in length $\times$ 0.38-0.41 mm wide, straight to sinuous, brownish to pale brownish, dark-brownish to the ends, pale at the tip; smooth with some white appressed fibrils that give a glistening aspect, emanating hyphae scattered and loose; tips cylindrical to rounded; mantle opaque at distal end, hydrophilic, hyaline; rhizomorphs and sclerotia absent (Figure 6A-B). *Longitudinal section*: Mantle 15-35 μ m thick, two layered (Figure 6C). Outer mantle 14 μ m wide, loose, plectenchymatous, not gelatinized, with cylindrical cells up 2.5-3.5 μ m, thin-walled, smooth. Inner mantle 18 μ m broad, plectenchymatous, compact, hyphae up to 5 μ m wide, with cylindrical cells 2.5-4 μ m hyaline, thin-walled, tannin cells present, parallel to root axis. Hartig net palmetti, paraepidermal, penetrating the second layer of cortical root cells (Figure 6C-D), the hyphae between the root cells up to 3.5 μ m wide, rounded; cortical cells rectangular, cylindrical 55-75 $\times$ 45-60 μ m (in longitudinal section) and 27-65 $\times$ 27-51 μ m (in cross-section) (Figure 6C-D). Clamp connections absent. *Cross section*: Mantle disposed in a plectenchymatous arrangement (Figure 6D), two layers discernible, hypha disposed in parallel and prostrate, some loose hyphae in the external mantle and compact in the inner mantle, tannin cells cylindrical, cortical cells 3.5 μ m wide rectangular to rounded, hyphae between cortical cells cylindrical. *Plan view*: Outer mantle in a plectenchymatous layer, hyphae in an irregular arrangement composed of cylindrical cells 3-4 μ m wide, sinuous, compact layer thin-walled, smooth hyphae (Figure 6E). Inner mantle in a plectenchymatous layer, in an irregular arrangement composed of cylindrical 2.5-5 (8) μ m wide sinuous, tortuous; compact layer, thin-walled, smooth hyphae (Figure 6F).

Figure 3. *Laccaria squarrosa*. A-B Mature basidiomata in their natural environment in Piedra Canteada, Nanacamilpa, Tlaxcala, Mexico (Hernández-Santiago 65); C-D Scanning electron micrograph of echinulate spores (Hernández-Santiago 65).

Characterization of *Pinus pseudostrobus* with *Laccaria squarrosa* ectomycorrhiza

Figure 4. Phylogenetic relationships of *Laccaria squarrosa* based on ITS sequences from basidiomata and ECM root tips and inferred from the maximum likelihood tree, including bootstrap values (≥ 70) and Bayesian posterior probabilities (≥ 0.95). New sequences obtained here are indicated in bold. Specimen vouchers, accession numbers of ITS sequences, and country of origin are indicated for each branch.

Figure 5. Phylogenetic relationships of *Pinus* species based on *trnL* sequences and inferred from the maximum likelihood tree, including bootstrap values (≥ 65). New sequences obtained here are indicated in bold.

Figure 6. *Laccaria squarrosa* + *Pinus pseudostrobus* ectomycorrhiza. A) Ectomycorrhizal (ECM) system; B) Details of the pruinose surface and tips of ECM roots; C) Tangential section showing mantle (M), Tannin cells (TC), and Hartig net (HN); D) Ectomycorrhiza in cross-section showing Mantle (M), Hartig net (HN), Tannin cells (TC), and hyphae between cortical cells (HCC); E) Plan view of the outer mantle; F) Plan view of the inner mantle. Bars 1 mm in A; 0.5 mm in B; 50 μ m in C) and D); and 20 μ m in E) and F).

Discussion

For the first time, we describe the ectomycorrhiza of *Laccaria squarrosa* + *Pinus pseudostrobus* in natural conditions and extend the distribution and host associations of *L. squarrosa*. *L. squarrosa* was previously known only from the type locality (in Veracruz state located in eastern Mexico) and associated only with *Fagus grandifolia* var. *mexicana*, and from a traditional Purépecha market (in Michoacán, located in western Mexico) without stating the associated host trees. Our new data show that the species is distributed along the Trans-Mexican volcanic belt (TCMV; [Figure 1](#)). Detecting the ECM association in the field (through the morphological and molecular characterization of the mycobiont and host tree) is the first step toward the identification of suitable hosts for forest management and reforestation programs. This is the second record of *L. squarrosa* in Mexico and confirms that this species is distributed along the Trans-Mexican-Volcanic-Belt in association with two different ECM host tree species. *Laccaria squarrosa* can

therefore be used not only as native inoculum for reforestation programs in the study area (Nanacamilpa, Tlaxcala), but also in the type locality of the species (Acatlán, Veracruz, Mexico), where the *Fagus grandifolia* var. *mexicana* forest is endangered. Further sampling should be performed in Mexico to identify additional ECM host tree species associated with this ECM fungus.

The ectomycorrhiza here described displays some similarities with the previously documented ectomycorrhizae of other *Laccaria* species associated with different host trees (Table 2). They display similar colors (except those formed by *L. amethystina* Cooke + *Quercus* sp. and *L. trichodemorphora* G.M. Muell. + *Pinus montezumae*), present emanating hyphae (however, abundance differs among the species), lack of rhizomorphs (except those formed by *L. amethystina* Cooke + *Quercus* sp. and *L. amethystina* Cooke + *Betula* sp.) and has a plectenchymatous outer mantle. Features such as the ramification type, mycorrhizal system length, and mantle thickness vary among all the species. However, more detailed descriptions are needed for further discussion about *Laccaria* ectomycorrhizae morphotypes.

Both the previous record of the holotype of *Laccaria squarrosa* under a pure stand of *Fagus grandifolia* var. *mexicana* and our results suggest that *L. squarrosa* is a generalist species that can be associated either with Angiosperms or Gymnosperms. This record of *L. squarrosa* with both *Fagus* and *Pinus* supports the hypothesis of Wilson *et al.* (2017) who suggest that *Laccaria*'s association with Fagaceae aided their dispersal into the northern hemisphere and its eventual association with *Pinus*. Although in our sampling area there were other ECM host trees (*Abies religiosa*, *Pinus teocote* and *P. montezumae*, and *Quercus* spp.) we only found basidiomata of *L. squarrosa* associated with *P. pseudostrobus*. This result was confirmed by our sequencing and phylogenetic analyses with a good support (BS = 65; Figure 5). Further fieldwork sampling of root tips in the study area would provide valuable ecological information of its possible symbiotic associations with other host species.

Despite the great diversity of *Laccaria* species and their associated host trees in both northern and southern hemispheres, only thirteen descriptions of ectomycorrhizae of six species (*L. amethystina*, *L. bicolor*, *L. proxima*, *L. tortillis*, *L. squarrosa*, and *L. trichodemorphora*) have been described (Table 2). Most of the records are ectomycorrhizae synthesized under artificial conditions and only four of them have described the morphoanatomical features of *Laccaria* ectomycorrhizae collected in natural conditions (Ingleby *et al.* 1990, Cuvelier 1991, DEEMY 2021). Therefore, the ectomycorrhiza here described represent one of the few morphotype descriptions of the ecologically and economically relevant *Laccaria* genus in natural conditions in the world.

Table 2. Diagnostic morphological characteristics of ectomycorrhizas established between species of *Laccaria* associated with different host trees. RT: Ramification type; MSL: Mycorrhizal system length; MC: Mycorrhiza color; MS: mantle surface; EHP: Emanating hyphae presence; Rhiz: Rhizomorphs; MT: Mantle thickness; MO: Mantle organization; D: Dichotomous; M-pin: Monopodial-pinnate; M-pyr: monopodial pyramidal; C: Coralloid; Pl: Plectenchymatous; Ps: pseudoparenchymatous; Nd: No data; N: natural conditions, A: artificial conditions.

Species + host	RT	MSL (mm)	MC	MS	EHP	Rhiz	MT (µm)	MO	Reference	Origin
<i>Laccaria squarrosa</i> + <i>Pinus pseudostrobus</i>	D	1.83	Brownish to pale brownish, dark- brownish	White fibrils glistening aspect	Scattered and loose	Absent	15-35	Pl	*This study	N
<i>L. amethystina</i> + <i>Betula</i> <i>pendula</i>	M-pin	1-2	Ochre, yellowish brown, or orange or red	Loosely woolly	Concentrat- ed distally	Present	36-93	Pl	Cuvelier 1991	N
<i>L. amethystina</i> + <i>Fagus</i> sp.	M-pin M-pyr	1- 6	Brown, ochre, yellowish brown of grey	Loosely woolly	Concentrat- ed distally	Absent	25-70	Pl	DEEMY 2021	N
<i>L. amethystina</i> + <i>Quer-</i> <i>cus robur.</i>	M-pyr	Nd	Violet, dark reddish blue or white	Loosely cot- tony	Abundant	Present	Nd	Pl	DEEMY 2021	N

Species + host	RT	MSL (mm)	MC	MS	EHP	Rhiz	MT (µm)	MO	Reference	Origin
<i>L. bicolor</i> + <i>Pinus montezumae</i>	D	2.5-3.66	Brown to pale brownish and orange brownish	Metallic aspect	Abundant	Absent	23.5-31.4	Pl	Santiago-Martínez <i>et al.</i> 2003	A
<i>L. bicolor</i> + <i>Pinus montezumae</i>	D	Nd	Reddish-brown	Cottony	Scattered	Nd	Nd	Ps	Rodríguez-Gutiérrez <i>et al.</i> 2019	A
<i>L. bicolor</i> + <i>P. patula</i>	D	1-4.5	Brown to orange, reddish brown	Glistening aspect	Nd	Absent	Nd	Pl	Carrasco-Hernández <i>et al.</i> 2010	A
<i>L. laccata</i> + <i>Pinus patula</i>	D	5.5	Whitish orange to greyish brown	Pubescent	Present	Absent	10-50	Pl	Mohan <i>et al.</i> 1993	A
<i>L. laccata</i> var. <i>laccata</i> + <i>P. montezumae</i>	D	Nd	Golden yellow	Cottony	Scattered	Nd		Pl	Rodríguez-Gutiérrez <i>et al.</i> 2019	A
<i>L. proxima</i> + <i>Betula</i> spp. + <i>Picea sitchensis</i> + <i>Pinus</i> spp.	M-pin M-pyr	1-8	Brown to white	Shiny	Present	Absent	15-40	Pl	Ingleby <i>et al.</i> 1990	A
<i>L. proxima</i> + <i>P. pseudostrobus</i>	D	1-8	Pale Brown to dark brown	Nd	Nd	Nd	Nd	Pl	Carrasco-Hernández <i>et al.</i> 2010	A
<i>L. tortilis</i> + <i>Betula pendula</i> and <i>Picea sitchensis</i>	M-pin M-pyr	7	Brown to white	Loosely or cottony	Present	Absent	10-30	Pl	Ingleby <i>et al.</i> 1990	A
<i>L. trichodemorphora</i> + <i>Pinus montezumae</i>	D C	0.4-2.9	White to pale white	Loosely cottony	Abundant	Absent	14.4-81.5	Pl	Galindo-Flores <i>et al.</i> 2015	A
<i>L. trichodemorphora</i> + <i>P. montezumae</i>	D	Nd	Orange, yellowish brown, bright orange	Cottony	Nd	Nd	Nd	Pl	Rodríguez-Gutiérrez <i>et al.</i> 2019	A
<i>L. vinaceobrunnea</i> + <i>P. montezumae</i>	D	Nd	Orange to brownish yellow	Cottony	Nd	Nd	Nd	Ps	Rodríguez-Gutiérrez <i>et al.</i> 2019	A

Laccaria squarrosa was recorded as an edible wild mushroom representing an important food source for local gatherers in the study area, who have traditionally considered the forests as a source of ecosystem services and food. Our robust confirmation of the establishment of ectomycorrhizal symbiosis between *L. squarrosa* and *P. pseudostrobus*, with morphoanatomical, molecular, and phylogenetic evidence, open the way to future biotechnological research to enhance sustainable forest management in the study area. Production of bioinoculants using this pioneer fungal mycobiont in *P. pseudostrobus* plant production (either as mycelial inoculum, dry pilei, or spore slurries) is

a real prospect, as it has been previously demonstrated for other phylogenetically-related Neotropical *Laccaria* species (Pérez-Moreno *et al.* 2020). In doing so, it is hoped that we can protect endangered Mexican forest species and ecosystems by promoting efficient plant production and successful reforestation.

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