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SHORT COMMUNICATION

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Mortierella species from declining *Araucaria araucana* trees in Patagonia, Argentina

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Abstract

Since 2015, *Araucaria araucana*, an ecologically and economically important conifer native to Argentina and Chile, has suffered an unusual partial death of the crown throughout almost all of the distribution range in Argentina. No primary pathogen or pest was evident, associated with the phenomenon. Isolates of *Mortierella*, a poorly studied fungal genus in Patagonia, were obtained from the margins of necrotic phloem tissue of symptomatic trees. Five species of *Mortierella* were isolated from affected tissues. In inoculation tests, *Mortierella alpina* and *M.* aff. *basiparvispora* were pathogenic to *A. araucana*. These species caused necrosis of phloem, leading to chlorosis, foliar desiccation and eventually death, demonstrating that Mortierellales may play a role in the decline of the tree.

KEYWORDS

araucaria, Mortierellales, stem disease, conifer decline, dieback

1 | INTRODUCTION

Araucaria araucana (Mol.) C. Koch (Araucariaceae), also known as pehuén or the monkey puzzle tree, is a key endemic conifer in the austral mountain regions of Argentina and Chile, characterized by being long-lived and large-seeded. The natural distribution is relatively limited, ranging from latitude 37°20′ to 40°20′ S (Herrmann, 2006). Since the European colonization in the 19th century, *A. araucana* has been exploited at alarming rates for its timber, which is used for construction, in furniture manufacture and for paper pulp (Herrmann, 2006). In addition, the indigenous Mapuche people of Chile and Argentina collect and commercialize the Araucaria nut, a crucial component in the diet and economy of these people (Herrmann, 2006).

In Chile, because of the serious threat to survival of the species, pehuén was declared a National Monument in 1990 (supreme Decree 43), prohibiting logging. In Argentina, current regulations protect *A. araucana* from logging and regulate the use of the nuts. International commercialization of *A. araucana* is prohibited in both countries: the species is included in Appendix I of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, online resource, available at http://cites.org). In 2012, the International Union for the Conservation of Nature (IUCN) declared *A. araucana* "endangered" in the native range because of its scarcity and severity of the conservation problems.

Since 2015, *A. araucana* has shown symptoms of an unusual partial death of the crown in Chile (National Forestry Corporation of Chile, CONAF) that has also been detected in almost all of the native range of the species in Argentina. Due to the increasing severity of this possible decline, research was instigated to describe the symptomatology and determine the potential causal agents by professionals of different institutions of Argentina. Decline of *A. araucaria* was observed in 77% of the evaluated area at landscape scale (Vélez et al., 2018). The main symptoms in adult trees and regeneration, at all sites studied, were chlorosis, reddening of branches and defoliation that occurred in different proportions of the crown, leading in

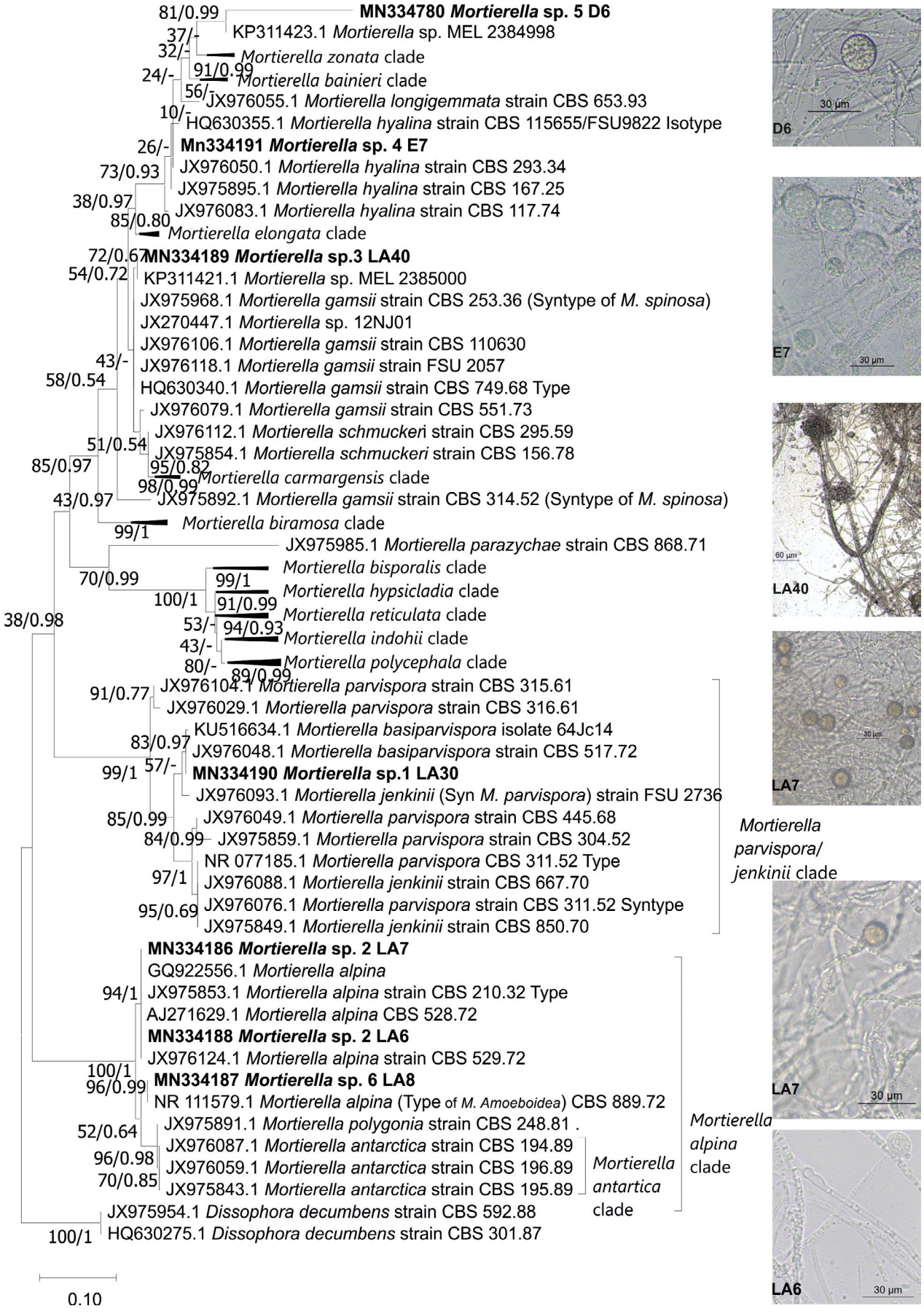
81/0.99
MN334780 Mortierella sp. 5 D6
37/-
KP311423.1 Mortierella sp. MEL 2384998
32/-
Mortierella zonata clade
24/-
91/0.99
Mortierella bainieri clade
56/-
JX976055.1 Mortierella longigemmata strain CBS 653.93
10/-
HQ630355.1 Mortierella hyalina strain CBS 115655/FSU9822 Isotype
26/-
Mn334191 Mortierella sp. 4 E7
73/0.93
JX976050.1 Mortierella hyalina strain CBS 293.34
JX975895.1 Mortierella hyalina strain CBS 167.25
38/0.97
JX976083.1 Mortierella hyalina strain CBS 117.74
85/0.80
Mortierella elongata clade
72/0.67
MN334189 Mortierella sp.3 LA40
54/0.72
KP311421.1 Mortierella sp. MEL 2385000
JX975968.1 Mortierella gamsii strain CBS 253.36 (Syntype of M. spinosa)
JX270447.1 Mortierella sp. 12NJ01
JX976106.1 Mortierella gamsii strain CBS 110630
43/-
JX976118.1 Mortierella gamsii strain FSU 2057
58/0.54
HQ630340.1 Mortierella gamsii strain CBS 749.68 Type
JX976079.1 Mortierella gamsii strain CBS 551.73
JX976112.1 Mortierella schmuckeri strain CBS 295.59
51/0.54
JX975854.1 Mortierella schmuckeri strain CBS 156.78
85/0.97
95/0.82
Mortierella carmargensis clade
98/0.99
43/0.97
JX975892.1 Mortierella gamsii strain CBS 314.52 (Syntype of M. spinosa)
99/1
Mortierella biramosa clade
JX975985.1 Mortierella parazychae strain CBS 868.71
70/0.99
Mortierella bisporalis clade
99/1
100/1
Mortierella hypsicladia clade
91/0.99
Mortierella reticulata clade
38/0.98
53/-
94/0.93
Mortierella indohii clade
43/-
80/-
Mortierella polycephala clade
89/0.99
91/0.77
JX976104.1 Mortierella parvispora strain CBS 315.61
JX976029.1 Mortierella parvispora strain CBS 316.61
KU516634.1 Mortierella basiparvispora isolate 64Jc14
83/0.97
JX976048.1 Mortierella basiparvispora strain CBS 517.72
99/1
57/-
MN334190 Mortierella sp.1 LA30
JX976093.1 Mortierella jenkinii (Syn M. parvispora) strain FSU 2736
85/0.99
JX976049.1 Mortierella parvispora strain CBS 445.68
84/0.99
JX975859.1 Mortierella parvispora strain CBS 304.52
NR 077185.1 Mortierella parvispora CBS 311.52 Type
97/1
JX976088.1 Mortierella jenkinii strain CBS 667.70
95/0.69
JX976076.1 Mortierella parvispora strain CBS 311.52 Syntype
JX975849.1 Mortierella jenkinii strain CBS 850.70
Mortierella parvispora/ jenkinii clade
MN334186 Mortierella sp. 2 LA7
GQ922556.1 Mortierella alpina
94/1
JX975853.1 Mortierella alpina strain CBS 210.32 Type
AJ271629.1 Mortierella alpina CBS 528.72
MN334188 Mortierella sp. 2 LA6
JX976124.1 Mortierella alpina strain CBS 529.72
100/1
96/0.99
MN334187 Mortierella sp. 6 LA8
NR 111579.1 Mortierella alpina (Type of M. Amoeboidea) CBS 889.72
52/0.64
JX975891.1 Mortierella polygonia strain CBS 248.81 .
96/0.98
JX976087.1 Mortierella antarctica strain CBS 194.89
70/0.85
JX976059.1 Mortierella antarctica strain CBS 196.89
JX975843.1 Mortierella antarctica strain CBS 195.89
Mortierella antartica clade
Mortierella alpina clade
JX975954.1 Dissophora decumbens strain CBS 592.88
100/1
HQ630275.1 Dissophora decumbens strain CBS 301.87
0.10
D6
30 µm
E7
30 µm
60 µm
LA40
LA7
30 µm
LA7
30 µm
LA6
30 µm

FIGURE 1 Phylogeny of *Mortierella* isolates illustrating species relationships inferred from joint ML analysis of the ITS region. Sequences generated in this study are represented in bold. The series of two values above internal branches correspond to ML and BI posterior probabilities, respectively. Nodes without support are indicated by a dash and condensed clade with a black triangle. The tree was drawn to scale, with branch lengths corresponding to the number of substitutions per site. Photographs showing typical morphology of the isolates, sporangiophores (LA6, E7, D6 and LA40) and chlamydospores (LA7) are placed on the right side of the figure

some cases to death. In addition, trees presented other signs and symptoms that varied among individuals and sample sites, indicating a complex process overall. Thus, trees could have symptoms of phloem necrosis, rots, resinosis, canker formation and latex-type exudation on stems, leaves with necrotic spots and galls, and seedlings with poor root development (Vélez et al., 2018). In Argentina, *A. araucaria* grows in a region with a great gradient of annual precipitation (from about 3,000 to about 400 mm), from the Andean cordillera in the West to the Patagonian steppe in the East. A tendency for higher incidence of the disease was present in the East, coinciding with decreasing rainfall (Vélez et al., 2018). Varied insects and fungi were detected and isolated from symptomatic trees, but no evident primary pathogen or pest was identified. Among fungal isolates, several isolates of *Mortierella*, a poorly studied genus in Patagonia, were obtained (Vélez et al., 2018).

Mortierella species (Mortierellales: Mortierellomycotina; Hoffmann, Voigt, & Kirk, 2011) are abundant and frequently isolated from soil and plant roots, particularly in soils with pathogenic fungi (Greslebin & Hansen, 2010), but the ecological role of this genus remains unclear. The identification of species of this genus by molecular techniques and their possible role as opportunistic or secondary plant pathogens has received little attention, especially in South America. The aim of the work reported here was to identify the species of *Mortierella* associated with declining *A. araucana* trees and to evaluate the possible role as pathogens in this native conifer.

2 | MATERIALS AND METHODS

Field studies were conducted in February and May of 2017 in Neuquén Province, Argentina, between 38°50′–39°34′S and 71°15′–71°27′W. Within this area, nine different sites were selected for sampling from SW to NE.

Fungal isolations were made from the margin of necrotic tissues, including phloem and xylem, at the root collar and stem, using malt extract agar (MEA), potato dextrose agar (PDA) with penicillin G (100 U/ml), and two selective media, PAR (cornmeal agar supplemented with 10 mg pimaricin, 200 mg ampicillin and 10 mg rifampicin/L) and PARPB (PAR supplemented with 50 mg PCNB and 15 mg benomyl/L; Greslebin & Hansen, 2010). Pure cultures were obtained on MEA, PDA and TA (clarified vegetable juice agar medium; Greslebin & Hansen, 2010) and examined under a Nikon Eclipse E200 microscope (Nikon, Japan). Isolates with morphological characteristics typical of Mortierellales (Figure 1) were subcultured to fresh MEA and synthetic nutrient-deficient agar (SNA; 1 g/L KH_2PO_4, 1 g/L KNO_3, 0.5 g/L $MgSO_4$*$7H_2O$, 0.5 g/L KCl, 0.2 1 g/L glucose, 0.2 1 g/L sucrose) for further identification. Morphology was compared with the original descriptions of the genus and classic identification keys. *Mortierella* isolates were selected and identities confirmed using molecular methods.

DNA was extracted from mycelia grown in TA using ULTRACLEAN Microbial DNA extraction kits (MoBio-Qiagen). Isolates were identified using the Internal Transcribed Spacer (ITS) region (primers ITS1F and ITS4), following Wagner et al. (2013). Amplification was carried out in a total volume of 50 µl containing 1× GoTaq Buffer (Promega), 0.2 mM each dNTP, 1 U Taq, 0.4 µM each primer and 75–100 ng template DNA. PCR conditions were as follows: 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 52°C and 60 s at 72°C, and a final step of 7 min at 72°C. Purification and sequencing of the PCR products were performed by Macrogen Corporation (Korea).

Sequences were aligned using ClustalW, edited manually using MEGA 7 and compared against accessions in GenBank. Poorly aligned portions and divergent positions were automatically deleted using Gblocks software (Instituto de Biologia Evolutiva (CSIC–UPF)) using less stringent options. The aligned matrix was deposited in TreeBASE under submission ID 25514. Phylogenies were reconstructed using two approaches: maximum likelihood (ML) and Bayesian inference (BI), both with partial deletion of gaps (95%). Tamura 3 parameter model with a Γ distribution was established based on the Akaike information criterion implemented in MEGA 7. Heuristic ML bootstrap analysis consisted of 1,000 pseudoreplicates. Bayesian analysis was carried out in MRBAYES v.3.1. Bayesian posterior probabilities (PP) were calculated using metropolis-coupled Markov chain Monte Carlo analysis until the runs converged with a split frequency of 0.01. Analyses were performed with 10,000,000 generations and a simple frequency of 1,000 trees. The first 25% were discarded as "burn-in." The strict consensus tree and posterior probabilities were calculated from 15,000 trees. The tree generated in this analysis was edited in FigTree V1.4.2 software (Yrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh).

All *Mortierella* isolates obtained were tested for pathogenicity using 10 plants of *A. araucana* per treatment. Plants (3 years old), originated from seeds, were procured from a nursery. Plants were 13.8 ± 1.7 cm in height, with stem diameters of 4.0 ± 0.8 mm. Inoculations were performed on the stem with a 3-mm-diameter hyphal plug cut from a 10-day-old culture on TA. An incision, < 10 mm long, was made in the bark and the mycelial plug placed over the incision. Each inoculation point was covered with sterilized, moist muslin cloth and wrapped with aluminium foil (Greslebin & Hansen, 2010). Control plants (N = 10) were inoculated with sterile TA plugs. Three months after inoculation, plants were evaluated: the outer bark was removed, and the length and width (proportion of stem perimeter) of each lesion (discoloured phloem) were measured. An index of tissue affected was calculated as total necrotic lesion length

(cm) × proportion of necrotic stem perimeter at the inoculation point. *Mortierella* colonization within the tissues of the inoculated plants was verified by direct plating of pieces of phloem taken from the margins of the necrotic lesions on PAR medium. Re-isolations were verified as *Mortierella* based on morphological features and identities confirmed using molecular methods. Data were analysed using Kruskal–Wallis and Mann–Whitney U tests (IBM SPSS Statistics 24).

3 | RESULTS AND DISCUSSION

Sixty-six fungal isolates, including 58 Ascomycota and 8 Mucoromycota, were obtained. Seven of the Mucoromycota isolates were identified as *Mortierella* (Figure 1), obtained from 3 of the 9 evaluated sites. *Mortierella* sp.2 LA6, sp.2 LA7, sp6. LA8 and sp.1 LA30 were isolated from one of the evaluated sites with lower annual rainfall; *Mortierella* sp.3 LA40 was isolated in the site with the highest annual rainfall of the evaluated areas; and *Mortierella* sp.4 E7 and sp.5 D6 were obtained from a site with an intermediate amount of annual precipitation. For molecular identification, the matrix of ITS sequences comprised 74 taxa and 613 characters, including 287 conserved sites, 325 variables and 218 parsimony informative sites. Phylogenetic analyses gave robust trees with well-supported clades for each *Mortierella* species (Figure 1). The results of ML and Bayesian analyses were highly concordant. *Mortierella* sp.2 LA6 and LA7 were grouped in the *M. alpina* clade (ML 94, BI 1). *Mortierella* sp.6 LA8 was also placed in the *M. alpina* clade (ML 96, BI 0.99), closely related to *M. alpina* CBS 889.72 (syn. *M. amoeboidea*). The morphologies of isolates LA6 and LA8 were similar to the original description of *M. alpina* (sporangia, at least partly, many-spored; sporangiophores with distinctly widening base, ellipsoidal sporangiospores). Isolate LA7 was also very similar in morphology to the original description but differed from isolates LA6 and LA8 in that abundant numbers of light brown chlamydospores with appendices were present (Figure 1). The sequence of *Mortierella* sp.3 LA40 grouped with *Mortierella* sp. MEL 2385000 (ML 54, BI 0.72) and both were included in one of the clades of the species *M. gamsii* (ML 45, BI-). Despite low support, isolate LA40 was clearly related to the *M. gamsii* paraphyletic group and its morphology agreed with the description of *M. gamsii* (sporangiophores 200–800 µm long, with branches inserted at different levels; spores <10 µm in diameter, globose to subglobose; Figure 1). *Mortierella* sp.1 LA30 grouped with sequences of *M. basiparvispora* (ML 83, BI 0.99); however, this clade is included inside of the parvispora/jenkinii complex clade (ML 85, BI 0.99), which the molecular marker used did not allow to resolve at the species level (Figure 1). Morphologically, *Mortierella* sp.1 LA30 was similar to *M. parvispora*, with sporangiophores longer than 200 µm, branches inserted at different levels, and spores globose to subglobose not exceeding 4 µm in diameter. Finally, *Mortierella* sp.4 E7 grouped with *M. hyalina* (ML 73, BI 0.93), with morphology typical of the species, showing many-spored sporangiophores with repeated basitonous ramification and conspicuous collarette on dehiscence (250–300 µm tall, 25–50 µm in diameter), and smooth-walled spores subglobose to globose (5–6 × 4–5 µm; Figure 1). *Mortierella* sp.5 D6 was placed in a clade with an undefined species, ML 81, BI 0.99 (Figure 1).

Of the seven *Mortierella* isolates tested for pathogenicity, two had a negative impact on the growth of seedlings. From the first month on, seedlings inoculated with *Mortierella* sp.1 LA30 (*M.* aff. *basiparsivora*) and *Mortierella* sp.2 LA7 (*M. alpina*) had symptoms of chlorosis and foliar desiccation. Moreover, 60% of seedlings inoculated with *M.* aff. *basiparvispora* (Figure 2a) and 30% inoculated with *M. alpina* (Figure 2b) died by the end of the trial. In contrast, the control group and seedlings inoculated with other *Mortierella* isolates showed normal growth and no symptoms by the end of the experiment. Significant differences in indexes of tissue affected were observed among treatments ($H = 64.315$, $p < .0001$). Plants inoculated with *M.* aff. *basiparvispora* (sp.1 LA30) and *M. alpina* (sp.2 LA7) showed indexes of tissue affected significantly higher than those of control plants ($p < .0001$ and $p < .05$, respectively; Figure 2c). *Mortierella* aff. *basiparvispora* (sp.1 LA30) was successfully re-isolated from 70% and *M. alpina* (sp.2 LA7) from 80% of inoculated plants. The other isolates tested appeared to be non-pathogenic to *A. araucana*, with lesions restricted to the inoculation points and similar indexes to control plants (Figure 2c).

Generally, *Mortierella* species are assumed to be saprophytic or endophytic, but it has been reported that species of this genus might be pathogenic to plants (Hernández Pérez et al., 2018). We found four known *Mortierella* species, and one possible newly discovered species, associated with *A. araucana*. Two isolates of two different species (*M. alpina* sp.2 LA7 and *M.* aff. *basiparvispora* sp.1 LA30) were able to colonize this tree species, leading to symptoms developed and eventually death of the seedling. The fact that *M. alpina* LA6 and LA8 were not pathogenic might indicate that different isolates of *M. alpina* have different levels of virulence (intraspecific variation). This intraspecific variation was also evident in the micromorphology, particularly regarding chlamydospore formation. Differences in virulence/phenotypic diversity are a known common feature of many fungal pathogens. The other isolates of *Mortierella* might be part of the endophytic mycobiota of *A. araucana*. This paper presents the first report of *Mortierella* species associated with *A. araucana* in Argentina. More work is needed to characterize the Mortierella populations genotypically and phenotypically.

Regional events of dieback and death of forests are being triggered by climate change, including increased temperatures and prolonged droughts that predispose vegetation to attack by pathogens. In Argentina and Chile, there have been a declining trend in rainfall and a sustained increase in average temperatures. This environmental change, occurring mainly during the driest season in Patagonia, represented by spring–summer, is producing a sustained reduction in the radial growth of *A. araucaria* along the gradient of precipitation and the consequent decrease in soil moisture (Mundo, Roig Juñent, Villalba, Kitzberger, & Barrera, 2012). In conclusion, the observed event related to partial desiccation of *A. araucaria* crowns might not be due to a single cause, but rather to a complex process resulting from multiple factors, in which the atypically prolonged drought between 2010 and 2015, and the

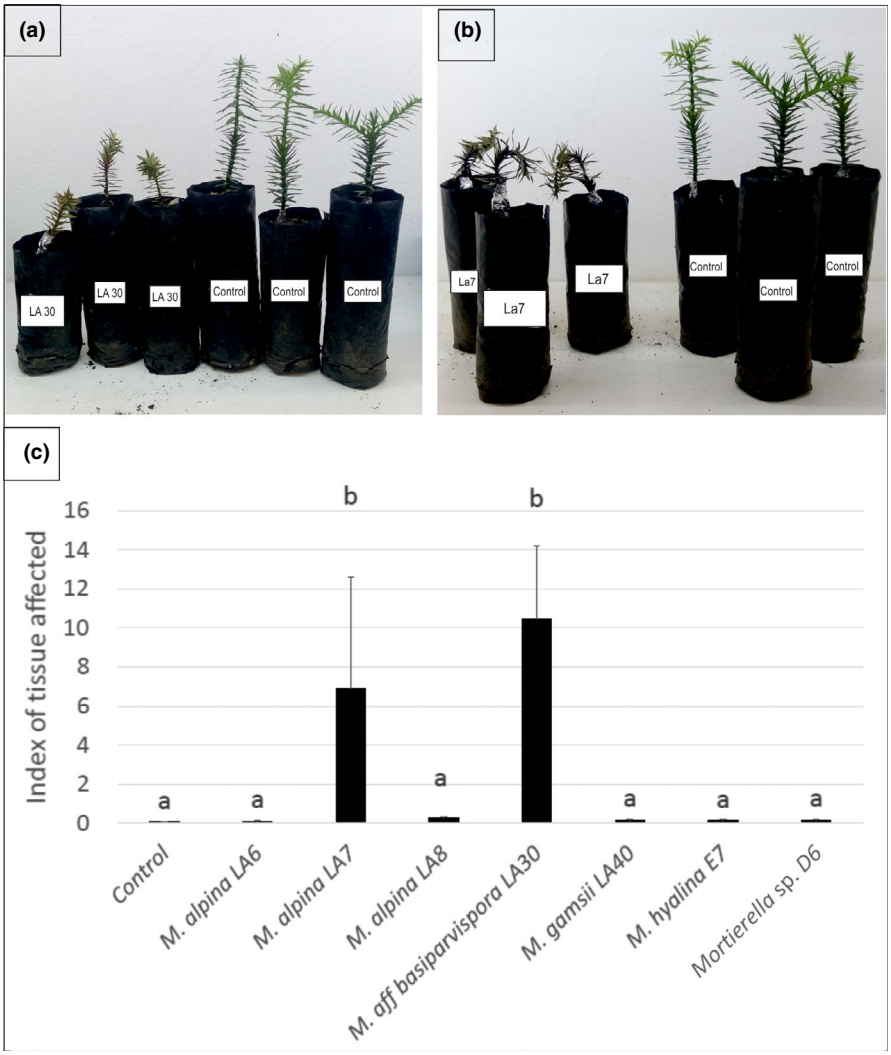

FIGURE 2 Pathogenicity of *Mortierella* isolates to *Araucaria araucana*. Control plants were inoculated with a plug of sterile agar. Symptoms observed on plants of *A. araucana* three months after inoculation with (a) *M.* aff. *basiparsivora* (sp.1 LA30) and (b) *M. alpina* (sp.2 LA7). (c) Pathogenicity of *Mortierella* isolates to *A. araucana* expressed as index of tissue affected. Data are presented as mean ± *SD*. Different letters refer to significant differences ($p < .05$) among treatments

increase in the temperature, could have had a central role in triggering and/or modulating biotic and abiotic interactions for the tree. In this scenario, Mortierellales mycoflora may act as latent pathogens, only expressing full pathogenic potential on stressed *A. araucaria* plants, particularly on sites with low levels of annual precipitation. Further research on the mycoflora of *A. araucaria* forests, the spatial pattern of abiotic variables and the evaluation of ecophysiological parameters, may shed light on the mechanisms involved in the decline process. Such work will improve our understanding of the ecology and pathology of the Mortierellales, including species richness and host–*Mortierella* species–environment interactions. *Mortierella* species are widely distributed, and, under conditions of climate change which increase stress on plants, these organisms might become threats to other forest or agronomic systems.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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