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# Ectomycorrhizas in Lycopodiopsida: their first registry and arbuscular mycorrhiza in *Phlegmariurus saururus* (Huperziaceae)

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**Abstract:** Lycophytes show no instances of ectomycorrhizas. *Phlegmariurus saururus* is the only Huperziaceae that grows in Comechingones biogeographic province, in sunny, exposed surficial rock crevices with poor soil development and very scarce water. As mycorrhizas improve plant fitness in natural ecosystems, it was hypothesized that *P. saururus* can develop multiple types of fungal symbiosis, including ectomycorrhizas. For detecting, identification and description of mycorrhizas, conventional techniques were employed, and samples of roots were cut with an ultra-microtome to obtain thin (3 µm) and ultrathin (0.1 µm) sections. *Phlegmariurus saururus* is the first species of Lycopodiopsida where the ectomycorrhizas are evidenced. Arbuscular mycorrhizas and septate endophytes were also found. Ectomycorrhizas can alter the anatomy and hydrophilic properties of roots, improving the adaptation of the plant hosts to habitats with a marked period of drought, as the novel Andean Comechingones habitats. The ectomycorrhizas detected in *P. saururus* could be considered as an adaptive mechanism related to the successful colonisation of this habitat and can undergo a significant transformation in the lifestyle of fungal symbiosis of lycophytes, which could provide important insights into this morphological and functional evolution.

**Key words:** Ectomycorrhizas, Lycophytes, Mycorrhizas, *Phlegmariurus*.

## INTRODUCTION

Lycopodiales comprise ca. 400 living species in two families: Lycopodiaceae P. Beauv. ex Mirb. and Huperziaceae Rothm. (Zhang & Zhou 2022). The lycophytes, with a history of at least 390 million years (Wikström & Kenrick 2001), is considered the sister to all other extant vascular plants (Kenrick & Crane 1997).

The gametophytes of Huperziaceae are subterranean and rely on endomycorrhizal associations for uptake of nutrients and assimilates (Winther & Friedman 2008). Similarly, many sporophytes have arbuscular mycorrhizal (AM), which allow them to grow on nutrient-poor substrates (Page 2002, Winther & Friedman 2008).

Among Huperziaceae, *Phlegmariurus* Holub is a genus with about 300 mainly pantropical species with a high diversity throughout the tropics in evergreen montane forests, and in the wet Andean grasslands and shrublands in South America. Most species are pendent epiphytes but in the Neotropics, significant number of species also occurs among terrestrial taxa in the high altitude Andean, paramo vegetation, which comprise some 20% of the family. According to Field et al. (2016) and Wikström et al. (1999), it appears that the terrestrial species in *Phlegmariurus* have been derived from epiphytic elements of high montane forests.

The Neotropical *Phlegmariurus* increased the diversification coincidently with the uplift in the Andes (Testo et al. 2019), especially with the transition to terrestrial growth in the "Phlegmariurus reflexus" and "Phlegmariurus crassus" species groups (Testo et al. 2018). The diversification among terrestrial species of *Phlegmariurus* appears to have been driven by increased ecological opportunity as novel habitats became available, and by the proliferation of fragmented high Andean habitats (Testo et al. 2019). One representative of these is the Comechingones biogeographic province in central Argentina, considered a high-altitude habitat island, surrounded by a "sea" of xerophytic, chacoan environments (Arana et al. 2021). In the Comechingones there is only one species that the southernmost limit of distribution of the genus in South America: *P. saururus* (Lam.) B. Øllg., a terrestrial species, which diversified via geographic isolation among the patchily distributed Andean habitat islands (Arana 2016). Additional factors associated with the recent diversification of terrestrial *Phlegmariurus* are almost unknown and deserve further study (Testo et al. 2019). Among them are the ecological and symbiotic relationships, as mycorrhizas which are prevalent in all major terrestrial biomes. Mycorrhizas are symbiotic associations, usually mutualistic, that improve plant fitness and influence plant biodiversity and productivity in natural ecosystems (Baron & Rigobelo 2022, Busby et al. 2016, Card et al. 2016). All members of the Huperziaceae require mycorrhizal symbionts for growth and for the production of gametes (Winther & Friedman 2008). These fungal symbionts are of particular interest as they are reported always as endomycorrhizas (Hoysted et al. 2018) across both free-living generations of these plants: the gametophyte and the sporophyte (Winther & Friedman 2008). In the South American Highlands, arbuscular mycorrhizas (AM) were cited as the most prevailing plant symbiosis followed by dark septate endophytes (DSE), a few orchid and ericoid mycorrhizas and dual and triple associations (Pagano & Lugo 2019). These multiple associations in the highlands of South America could be related to the recent evolutionary plant—fungal symbiont processes as it has been suggested by Brundrett & Tedersoo (2018). Although the ectomycorrhizas (EM) can differ greatly in their anatomy (Agerer 2001), they are distinguished by morphological criteria, i.e., the presence of a sheath of fungal hyphae (mantle) covering the root tips and an intercellular penetration pattern where the hyphae form a network between cortical cells called a Hartig net (Smith & Read 2008, Tedersoo et al. 2010).

The EM are mainly formed by the more derived lineages septate Ascomycota and Basidiomycota but in a few cases also Mucoromycotina (Agerer 2006, Hoysted et al. 2018, 2019). It is very noticeable that, until now, EM have not been found or confirmed for lycophytes or ferns (Lehnert & Kessler 2016). The presence of fungal symbionts in Huperziaceae has been documented by Schmid & Oberwinkler (1993) and Winther & Friedman (2008), among others (Rimington et al. 2020). For the South American representatives of genus *Phlegmariurus*, the presence of arbuscular mycorrhizas was cited in *P. mandiocanus*, *P. reflexus* and *P. saururus* (Krzyzanski et al. 2021, Menoyo et al. 2007, Sánchez Baizabal et al. 2022).

In this study, we hypothesized that *P. saururus*, owing to its recent colonisation in terrestrial high altitude Andean habitats, can develop multiple type of fungal symbiosis, including ectomycorrhizas, combined with the arbuscular mycorrhizas and septate endophytes, wich have been documented in several plant lineages. Thus, the aims of this work are to

document and describe morphologically the presence of ectomycorrhizas in the sporophyte of *P. saururus* and also to describe the arbuscular mycorrhizas and document the presence of septate endophytes present on roots of this species.

## MATERIALS AND METHODS

### Sampling sites and fields collection

Root sampling was conducted in March 2023, at 2100 m asl in high altitude grasslands of Sierras de Comechingones (32° 23' 7.21" S – 64° 55´ 26.39" W). These habitats belong to the Comechingones biogeographic province (belonging to the South American Transition Zone) in central Argentina (Arana et al. 2021), representing biogeographic islands vicariantly fragmented by tectonic events of Andean orogenesis and consequent climate change, such as progressive aridification of South America (Arana et al. 2021, Martínez et al. 2017). This Andean cycle had its effects on the central Pampean ranges from the Eocene, determining that the current structure of the mountains started ca. 12–10 million years ago and had already been formed in the lower Miocene–Pliocene limit, 5.3 million years ago. The habitats are characterised by an endemic biota of diverse lineages with grasslands of Poaceae as *Festuca hieronymi* Hack., *Deyeuxia hieronymi* (Hack.) Türpe, *Nassella filiculmis* (Delile) Barkworth., *Schizachyrium condensatum* (Kunth) Nees and *Eragrostis airoides* Nees, and woodlands with predominance of *Polylepis australis* Bitter (Rosaceae) and *Maytenus boaria* Molina (Celastraceae), and *Escallonia cordobensis* (Kuntze) Hosseus (Escalloniaceae) in mountainous regions of central Argentina, at an altitude above 1000 m a.s.l. (Arana et al. 2021) (Fig. 1a). It is noteworthy that the Comechingones province represents the southern limit of an ancestral biotic component of lycophyte flora, being *P. saururus* the only Huperziaceae (Arana et al. 2011). In this environment, *P. saururus* grows always in sunny, exposed surficial rock crevices with poor soil development (Fig. 1b). The complete substrate was excavated, the plants of *P. saururus* were extracted and only roots attached to the green shoots were used. Root samples from living plants were extracted

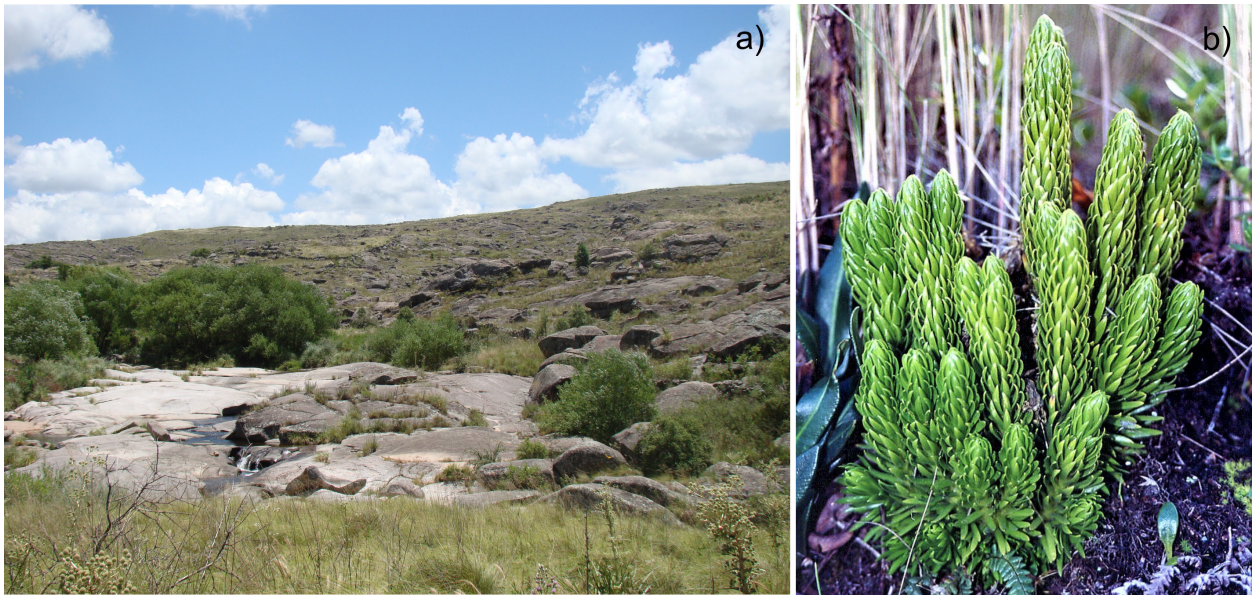


**Figure 1. Natural habitat and general aspect of the plant. a) Habitat from Sierras de Comechingones. b) *Phlegmariurus saururus* habit.**

from five different individual sporophytes of *Phlegmariurus saururus*, collected randomly for analysis (Fig. 2a, b). The samples were immediately fixed with FAA (5:5:90 v/v/v formaldehyde: acetic acid 50%: ethanol 80%), placed in plastic bags and stored at 4°C.

## Ectomycorrhizal analysis

For detecting, identification and description of ectomycorrhizas (EM) transversal and longitudinal sections of the short and swollen root branches (Fig. 2a, b), the typical morphological evidence of the presence of ectomycorrhizal symbiosis (Agerer 1999, Tedersoo et al. 2006) were washed and fixed in Karnovsky's solution (4% formaldehyde, 5% glutaraldehyde, and 0.1 mol·L sodium phosphate buffer, pH 7.4) (Karnovsky 1965), and post fixed with 1/1 solution of sodium phosphate buffer with 2% osmiumtetroxide. After incubation with both fixatives at 4°C overnight, samples were washed, embedded in Spurr resin, cut with

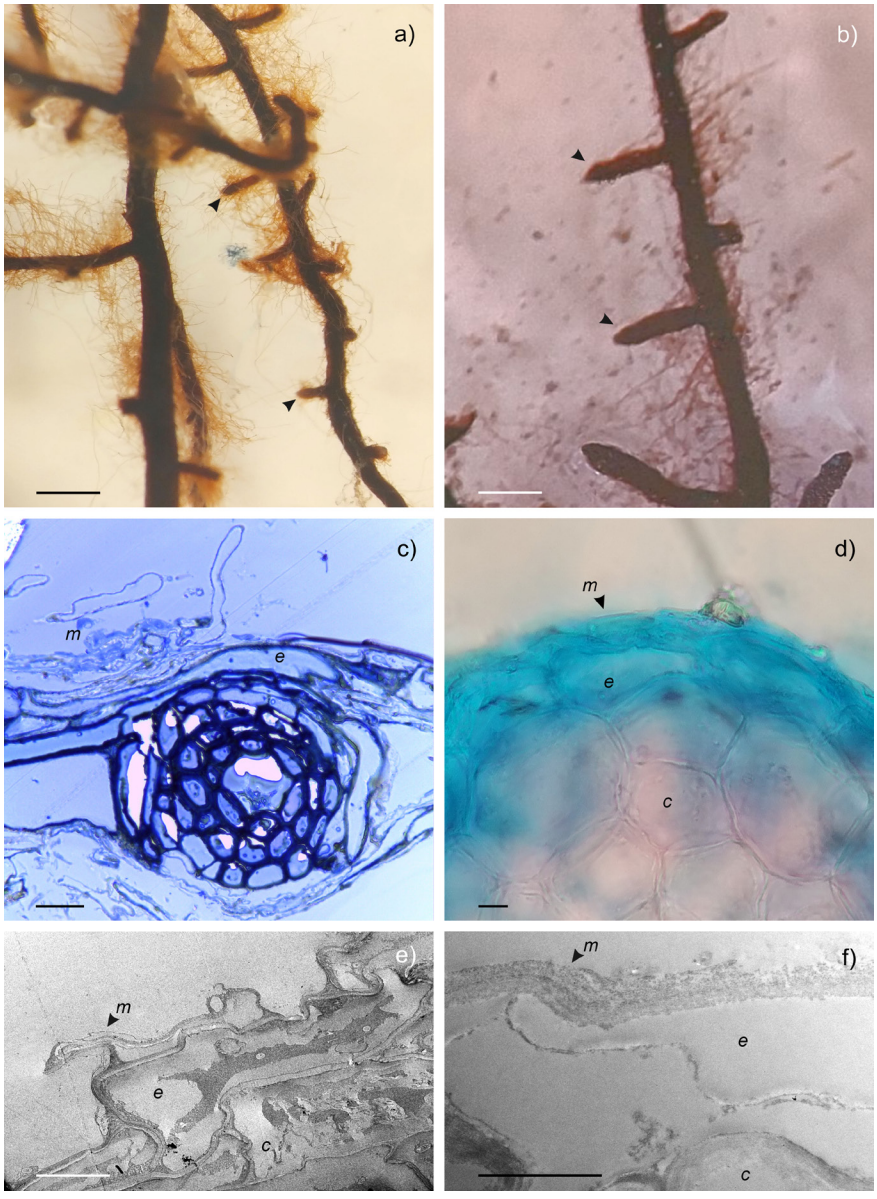


**Figure 2.** ***Phlegmariurus saururus*** **colonization by ectomycorrhizas. a-b) Simple-type morphology of ectomycorrhizas. c-f) Cross sections of roots with ectomycorrhizas. c) and d) Light microscopy. e) and f) Transmission electron microscopy. *e* epidermis, *c* cortex, *m* mantle, Bars: A, B, E 10 µm; C, D, F 30 µm.**

an ultra-microtome to obtain thin (3 µm) and ultrathin (0.1 µm) sections. To characterize the anatomical structure of the *Phlegmariurus* roots, the thin sections were stained with 0.05% toluidine blue (Heslop-Harrison & Heslop-Harrison 1981) and observed using an optical microscope (Carl Zeiss, Axiostar Plus, Göttingen, Germany).

The ultrathin sections of short and swollen root branches were mounted on copper grids and counterstained with uranyl acetate and lead citrate (Venable citrate, Venable & Coggeshall 1965) for observation under a transmission electron microscope (Carl Zeiss, EM109, NTS GmbH, Oberkochen, Germany) of the "Centro Integral de Microscopía Electrónica" (CIME-CONICET, Tucumán, Argentina).

### Arbuscular mycorrhizal fungi analysis

For the identification of arbuscular mycorrhizal fungi (AM) the plants were carefully cleaned of soil and their root systems were clarified and stained using the techniques of Phillips & Hayman (1970) and Grace & Stribley (1991). For each individual, 30 root fragments of 1 cm length were mounted in glycerol (n=150). The technique of McGonigle et al. (1990) was used to estimate MA colonization. Observations were made under an optical microscope (Carl Zeiss, Axiostar Plus, Göttingen, Germany). Microphotographs were taken with a digital camera (Olympus SP350, 8.0MP, Tokyo, Japan).

### Statistical analysis of arbuscular mycorrhizas

The Kruskall Wallis nonparametric test was applied for data analysis. When significant differences were found, Dunn's multiple comparisons test (Zar 1996) was applied a posteriori.

## RESULTS

### Identification and description of EM

The collected roots of the plants exhibited noticeably lateral short and swollen branches, corresponding to simple-type structures of ectomycorrhizas (Fig. 2a, b). A thin mantle and a Hartig net corresponding to the intercellular ectomycorrhizas mycelia was detected in lateral root cross-sections (Fig. 2c-e).

The mantle was (7.5) 52.5-75.5 (120) µm thick and showed an irregular prosenchymatous structure. Mostly, the Hartig net penetrates into the first layer of the cortical parenchyma (Fig. 2c-f, indicated with arrows).

### Determination of the type and percentage of AMF colonization

Root systems show arbuscular mycorrhizal colonization, with *Arum* and *Paris* morphological types. In the *Arum* morphology the intercellular hyphae are thick (2-2.6 µm) forming intracellular arbuscules (Fig. 3a-d). These hyphae present intercellular vesicles of different shapes (cylindrical, ellipsoidal, spherical, subspherical), in terminal position. In addition, there are intracellular vesicles of two shapes, cylindrical and subspherical, whose number varies from 1 to 2 per host cell (Fig. 3e, f). All vesicles have guttules in their interior. In the *Paris* type the intracellular thick hyphae (2.6 - 3.2 µm) form circumvolutions (Fig. 3g).

Statistical analysis of the arbuscular mycorrhizal association showed 10% arbuscular colonization (AC), 20% vesicular colonization (VC) and 68% hyphal colonization (CH).

### Detection of radical endophytes

Dark septate endophytes (DSE) with brown intercellular hyphae connected to intracellular microsclerotia were evident (Fig. 3h). In addition, endophytes with intracellular sclerotia with rounded septate hyphae and elongated hyphae

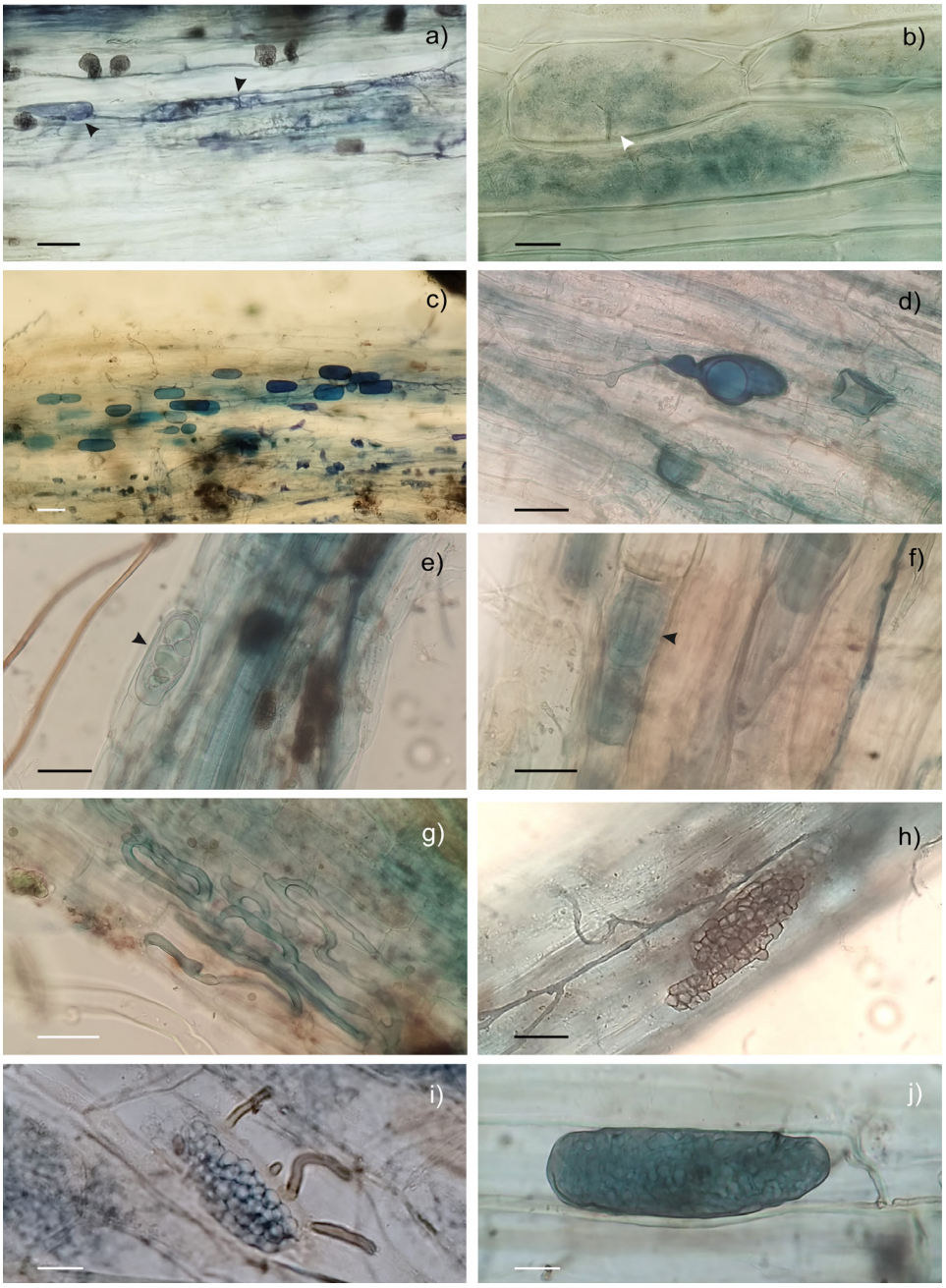


**Figure 3.** ***Phlegmariurus saururus*** **colonization by arbuscular mycorrhizal fungi. a) Intercellular and intracellular hyphae in root fragment. b) Arbuscules in cortex cell (*Arum* type morphology). c) Intercellular vesicles of different shapes. d) Intercellular terminal vesicle with guttule. e-f) Intracellular vesicles. g) Circumvolutions (*Paris* type morphology). h-j) Other endophytes with intercellular hyphae and intracellular sclerotia. h) Dark septate endophytes. i-j) Intracellular sclerotia with rounded septate hyphae and elongated hyphae. Bars: A-J 30 µm.**

(Fig. 3i, j), both in parenchyma cells of the root cortex, were also evident.

## DISCUSSION AND CONCLUSIONS

*Phlegmariurus saururus* is the first species of Lycopodiopsida in which ectomycorrhizas (EM), coexisting with arbuscular mycorrhizas (AM) and dark septate endophytes (DSE) were found. This was evidenced by careful morphological and anatomical observations.

Lycophytes, one of the oldest lineages of vascular plants, have been primarily acknowledged as terrestrial species forming arbuscular mycorrhizas (Winther & Friedman 2008). However, the lycophytes show low levels of colonization by symbiotic fungi (Rimington et al. 2015); and no instances of ectomycorrhizas have

been reported previously from Lycopodiopsida, although there is evidence that ectomycorrhizas already existed for at least 50 million years (Agerer 2006).

Ectomycorrhizas have always been associated with woody perennial seed plants (Sevanto et al. 2023), mainly tree species of the families Pinaceae, Fagaceae, Salicaceae, Betulaceae, Myrtaceae and Fabaceae (Brundrett 2009, Taiz et al. 2018). Our observations show for the first time the presence of ectomycorrhizas (EM) in lycophytes, taking as an example the species *P. saururus*, a saxicolous herbaceous species. The morphology of the ectomycorrhizas and roots modifications found in *P. saururus*, with evident short and swollen branches are characteristic of the simple-type structures of ectomycorrhizas (Agerer 1999). This correspond to the medium-distance smooth exploration (Agerer 2001) were the individual ectomycorrhizas have only a rather limited range of exploration and their rhizomorphs are undifferentiated or, at most, slightly differentiated and ectomycorrhizal mantles appear thin, smooth (Fig. 2c-e) with almost no or only a few emanating hyphae. Soto Acosta et al. (2023) mentioned the presence of EM in two herbaceous species, but belonging to the family Cactaceae with similar morphology of those found in *P. saururus*.

So far, associations with arbuscular mycorrhizal fungi have only been found in sporophytes of ten of the 300 species of the genus *Phlegmariurus* (Rimington et al. 2020). Particularly, in the specimens of *P. saururus* studied here, AM showed the coexistence of *Arum* and *Paris* morphologies. Menoyo et al. (2007) cited *Arum* morphology for the same species. Sánchez Baizabal et al. (2022) mention the presence of *Paris-type* arbuscular mycorrhizas for *P. reflexus*, and Krzyzanski et al. (2021) observed AM in *P. mandiocanus*, without characterising the morphological type.

In relation to the morphological diversity of vesicles, it could be inferred that the roots of *P. saururus* are colonised by more than one species or genus of arbuscular vesicular mycorrhizal fungi, a condition that was cited by Di Barbaro et al. (2017) for the Asteraceae *Helianthus tuberosus* L.

In this study, the presence of Ectomycorrhizal fungi (EM), simultaneously with arbuscular mycorrhizas and dark septate endophytes in the root system of *P. saururus*, was evidenced. The evolutionary and ecological significance of the combination of multiple plant-fungal endosymbiosis was described in many previous studies (Baron & Rigobelo 2022, Brundrett & Tedersoo 2018, and references herein).

Through physical and biochemical interactions, mycorrhizas can influence plant traits and environmental responses (Averill et al. 2019). Colonisation of plant roots can lead to improved water availability and hence improve the ability of their plant hosts to resist and recover from drought (Begum et al. 2019, Gil-Martínez et al. 2018, Lehto & Zwiazek 2011). Particularly, EM can alter the anatomy and hydrophilic properties of roots, influencing the apoplastic water uptake pathway (Xu & Zwiazek 2020), improving the adaptation of the plant hosts to habitats with a marked period of drought, as Andean habitats of Comechingones biogeographic province. The mycorrhizas detected in *P. saururus* could be considered an adaptation mechanism related to the successful colonisation of this species in the recently formed Comechingones Andean habitats.

It is considered that within the lycophytes, there have only been losses of symbiosis and no subsequent gains, with the evolution of non-symbiotic species particularly in Lycopodiales, which suggest that these plants may have a low dependence on their mycorrhizal partners when mature (Field et al. 2015, Rimington et al.

2015, 2020). Although the reciprocal impact of symbiosis interactions on the evolution and distribution of taxa in the lycopod rhizobiome remains enigmatic (Benucci et al. 2020, Bonito et al. 2014, Coleman-Derr et al. 2016), particularly, the demonstrated presence of EM in the root system of *P. saururus* could have favoured its recent colonisation of high Andean rock crevices, microhabitats with scant water availability. The Hartig net developed by EM is a complex organ comprising cortical or epidermal cells of plant roots and fungal hyphae that have ramified intercellularly between the individual root cells to maximize the contact area for reciprocal nutrient transfer (Smith & Read 2008, Tedersoo et al. 2010) specially water (Xu & Zwiazek 2020).

The establishment and survival of the lycophyte *P. saururus* in Comechingones province would have been also favoured by the recent uplifting of central Pampean ranges, which, given their environmental heterogeneity, would allow the development of different microclimates and microhabitats (Arana et al. 2011, Moran 1995).

The discovery of a lycophyte species to develop ectomycorrhizas is the most noteworthy achievement of this study.

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