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Research Article

Identification of arbuscular mycorrhizal fungi in queñua (*Polylepis rugulosa*) in the forest of southern Peru

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Abstract

Queñua (*Polylepis rugulosa*) forests are heavily impacted by anthropogenic activities, necessitating effective reforestation strategies. These efforts often face challenges due to various environmental factors, highlighting the importance of studying soil biotic associations, particularly with arbuscular mycorrhizal fungi (AMF), which play a crucial role in forest ecosystem health by forming symbiotic associations with plants, facilitating nutrient exchange, and promoting growth. This study investigated the AMF community in *P. rugulosa* forests in Arequipa, Peru. Rhizospheric soil was collected from two zones (A and B) differentiated by an altitude gradient. Spore density was measured to analyze colonization percentage; AMF spores were counted per 100 grams of soil, and morphological identification was performed using The International Collection of Vesicular Arbuscular Mycorrhizal Fungi. The findings revealed a 10% colonization rate, with *Acaulospora* as the dominant genus, representing 63.2% of the AMF community in both zones. 41 taxa were identified, including *Acaulospora*, *Glomus*, *Rhizoglomus*, *Claroideoglomus*, and *Racocetra*. The most abundant species was *Acaulospora kentinensis*. Biodiversity indices indicated moderate AMF diversity in both zones, with the highest spore density observed in zone B at the lower altitude. These results suggest that AMF monitoring and study can enhance reforestation efforts for *P. rugulosa* and serve as a valuable bioindicator in forest conservation strategies.

Keywords: *Acaulospora*; Andean Forest; ecosystem restoration*; Glomus*; microorganisms; *Polylepis*; symbiotic association

Introduction

Polylepis is one of the most important and, at the same time, threatened genus in the high mountain forest ecosystems in South America. The genus includes about 27 species distributed in the Andean ecosystem

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of the Andes Mountains, spanning from northern Chile and Argentina to Venezuela (Mendoza and Cano, 2012). Forests in which the genus *Polylepis* is present, hereafter will be denoted as "*Polylepsis* forests" in this document. *Polylepis* forests play a crucial role in the provision of ecosystem services for high Andean populations and communities, such as carbon sequestration, water regulation, erosion control, conservation of soil nutrients and preservation of biodiversity through provisioning refuge (Requena-Rojas *et al.,* 2020). In Peru, *Polylepis* forests are one of the most important ecosystems due to the great biodiversity it stores. Despite this, it is one of the most vulnerable ecosystems due to its fragmentation caused by the influence of anthropogenic activities (Canales-Gutiérrez and Gutierrez-Flores, 2021; Mollohuanca *et al.,* 2022; Zutta *et al.,* 2012). This situation has caused populations to be susceptible to genetic loss (Morales-Aranibar & Morales-Aranibar, 2023), resulting in a direct impact on the viability of the species, especially in the current context of climate change (Zutta *et al.,* 2012). For this reason, the search for alternatives that can improve reforestation efforts for these species has become a topic of research in recent years.

One of the alternatives to carry out future successful reforestation processes of these vulnerable species is through the diagnosis and inoculation of arbuscular mycorrhizal fungi (AMF), which have great application potential thanks to their functionality as a symbiont of plant species. AMF have the potential to intervene in the improvement of processes towards plant growth, such as nutrient absorption, through the improvement of the root system (Hu and Pan, 2023), protection against pathogens (Wall *et al.,* 2020) ando protection of soil quality (Caravaca *et al.,* 2003). It is a well-known fact that mutualism between AMF and their hosts improves the resistance of species to stress conditions such as drought, herbivory, salinity, temperature, presence of pollutants, etc. (Begum *et al*., 2019). Research suggests that native AMF are key factors in facilitating the establishment of plants that will be used in reforestation because they ensure a higher survival rate of seedlings, which, in essence, helps to restore and improve the function of the forest ecosystems to be restored (Li *et al.,* 2021; Manaut *et al.,* 2015).

AMF form mutually beneficial relationships with over 80% of terrestrial plant species. This ancient symbiosis was pivotal in enabling plants to colonize land (Lee *et al.,* 2013). Arbuscular mycorrhizal fungi (AMF) form mutually beneficial relationships with over 80% of terrestrial plant species. This ancient symbiosis was pivotal in enabling plants to colonize land (Rillig and Mummey, 2006). This functional diversity of AMF is essential for maintaining healthy and balanced forest ecosystems.

Understanding the diversity and function of arbuscular mycorrhizal fungi (AMF) within forest ecosystems is crucial for their conservation and restoration. Research worldwide, including studies in the United Kingdom (Guy, 2022) and Brazil (Lee *et al*., 2013), has utilized modeling and restoration experiments to characterize the influence of mycorrhizal fungi. These studies highlight the importance of incorporating mycorrhizal networks into forest management strategies, particularly for mitigating environmental impacts such as drought (Pickles and Simard, 2017). Furthermore, research emphasizes the crucial role of AMF in shaping forest community structure, dynamics, and regeneration following disturbance (Johnson *et al*., 2015), and underscores their contribution to diverse ecosystem services (Rosas-Moreno *et al*., 2023). Given the significant knowledge gaps regarding AMF communities in Peruvian Andean ecosystems, this research presents a critical opportunity to advance our understanding. By investigating AMF diversity in these unique and ecologically important forests, this study will contribute valuable insights into the role of these essential symbionts in supporting the survival and regeneration of these valuable ecosystems.

The objective of this study was to determine the AMF associated with *Polylepis rugulosa* from the forest located in Cacayaco, Arequipa located in the buffer zone of the Salinas and Aguada Blanca National Reserve, as a first step for its isolation and subsequent use in reforestation of the species.

Materials and Methods

Description of the study area

The research was carried out in the queñua (*Polylepis rugulosa*) forest locality of Cacayaco - Arequipa, Peru (within the buffer zone of the Salinas and Aguada Blanca National Reserve), located on the western slope of the Pichu Pichu Volcano. Studied site is with an elevation between 3500 and 4100 meters above sea level; characterized by a temperature between 3 and 8 °C (Soto Huaira *et al.,* 2019). Two areas were selected for the study of the presence of AMF, site A and site B, due to their altitudinal difference (Figure 1). The altitude ranges at sites A and B were established considering the difference of 200 meters between the two areas, with a practical criterion related to their location inside (Zone A) and outside the forest (Zone B). In similar ecological studies, it is common to define altitudinal ranges according to ecosystem characteristics, such as vegetation type, microclimate changes, and environmental gradients (Hazard *et al*., 2013). In this case, the altitudinal range is directly related to the transition from forested to non-forested zones, which is relevant for analyzing how MFA communities vary in response to specific environmental conditions (Hazard *et al*., 2013).

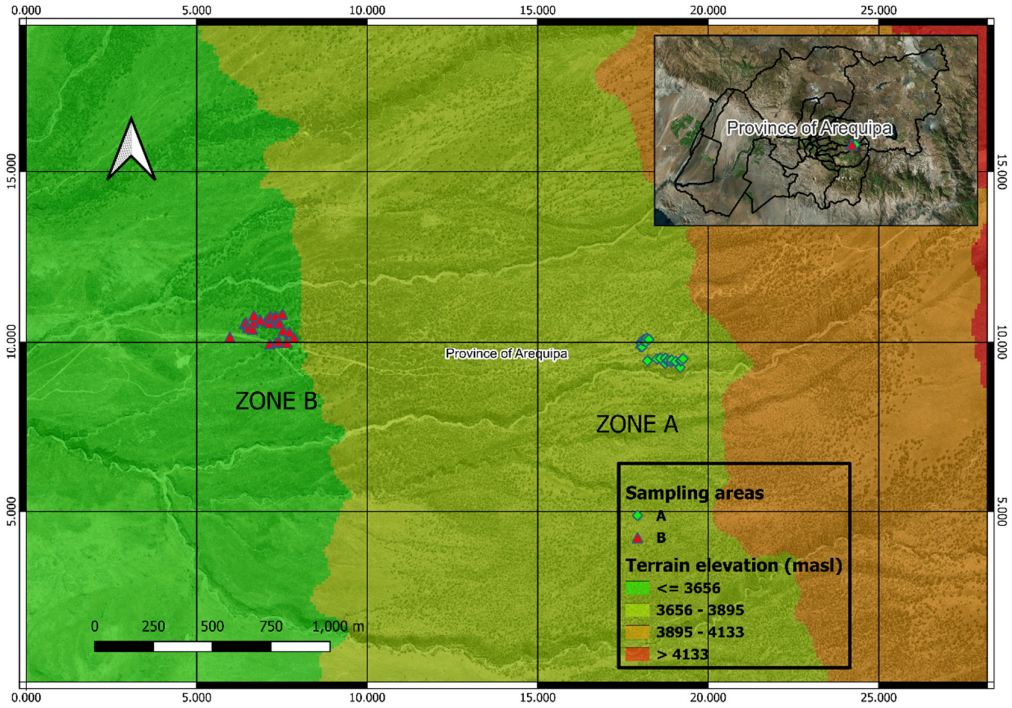


Figure 1. Location of site A and site B in the queñua forest of the town of Cacayaco in the Arequipa region, Peru

Soil sample collection

To investigate the rhizosphere soil microbiome of *P. rugulosa*, forty soil samples were collected from two distinct sites (A and B) within the forest. At each site, twenty individual samples were obtained. To ensure representative sampling, three subsamples were collected within a 2-meter radius around each sampling point. Plant health was assessed by recording key physiological parameters, including plant size, number of branches and leaves, and the degree of wilting observed. Each soil sample, weighing approximately 1 kg, was collected from the 10-30 cm depth and immediately placed in airtight bags to prevent contamination. The samples were then transported to the laboratory for cold storage. Prior to analysis, samples within each site were grouped

into four composite samples based on their spatial proximity. Each composite sample consisted of a pool of five individual samples.

Percentage of mycorrhizal colonization

Mycorrhizal colonization in *P. rugulosa* roots was assessed using established methods (Giovannetti and Mosse, 1980; Phillips and Hayman, 1970; Rodríguez Yon *et al.*, 2015). This involved clearing and staining root segments to visualize and quantify the presence of fungal structures within the root cortex. Root samples were initially bleached in 10% potassium hydroxide (KOH) at 90 °C for 10-60 minutes. Subsequently, they were oxidized in 3% hydrogen peroxide (H_2O_2) at 70-90 °C for 15 minutes. Following thorough washing with distilled water, the samples were acidified with 1N hydrochloric acid (HCl) until they became translucent. Finally, the roots were stained with Parker Quink Blue Ink at 90 °C for 10-30 minutes. Stained root segments were then mounted on slides with lactoglycerol (50%) and examined under a microscope at 40× magnification (Figure 2a). The extent of mycorrhizal colonization was determined by visually assessing the density of fungal structures, including vesicles, hyphae, arbuscules, and spores (Figure 2b). The percentage of colonization was calculated by estimating the proportion of root segments colonized by mycorrhizal fungi.

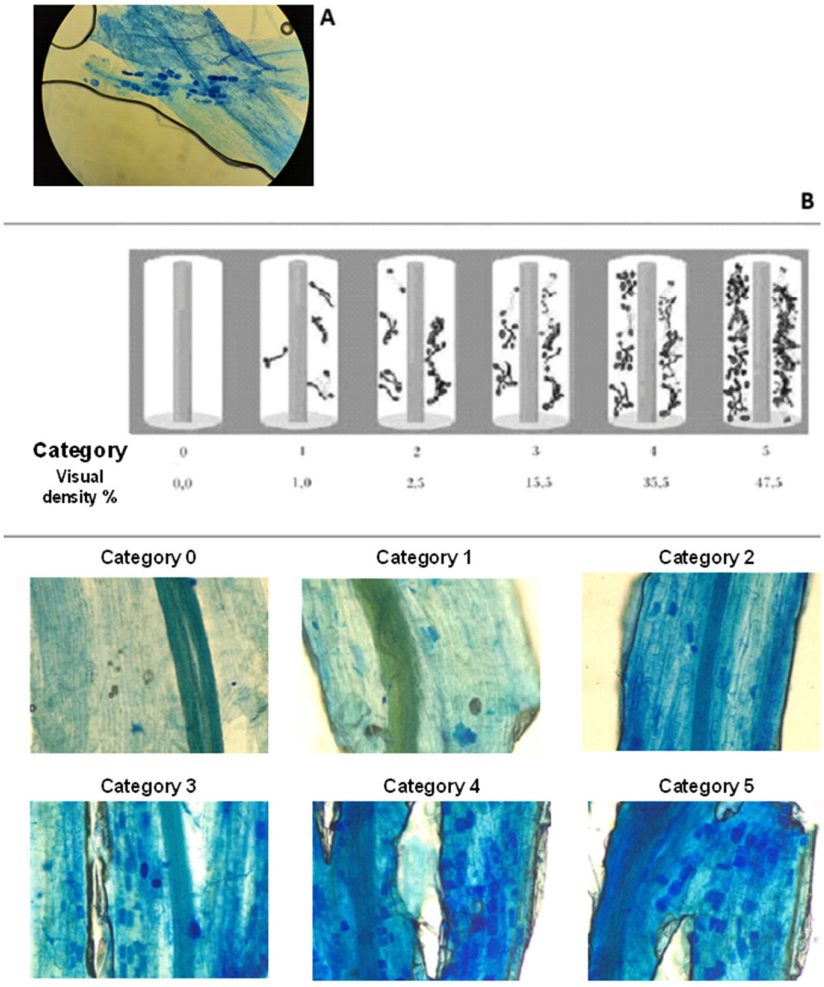


Figure 2. Stained roots and microscope view at 40× A. Visual density by colonization B. Category by visual density

AMF density

The extraction and counting of spores were carried out following the methodology of Gerdemann and Nicolson (1963). Initially, 100 g of soil sample was added to 1 L of Milli-Q ultrapure distilled water, mixed, and allowed to settle. The supernatant was then poured through a series of 600 µm, 425 µm, 106 µm and 75 µm sieves. The material was collected from the 75 µm sieve, using distilled water in a 100 mL beaker, making up the content until it reached 50 ml. Then, 4 ml of collected sample was centrifuged (3000 rpm for 3 min using Ortoalresa Microsem 24 centrifuge) on a sucrose gradient medium (20% and 60%) in 12 ml Falcon tubes. After the centrifugation, the supernatant was poured onto the 75 µm sieve and rinsed with distilled water to remove excess sucrose. The contents of the sieve were transported to Petri dishes with 1.25 cm grids for spore counting in a stereoscope and subsequent density evaluation.

Identification of mycorrhizal fungi

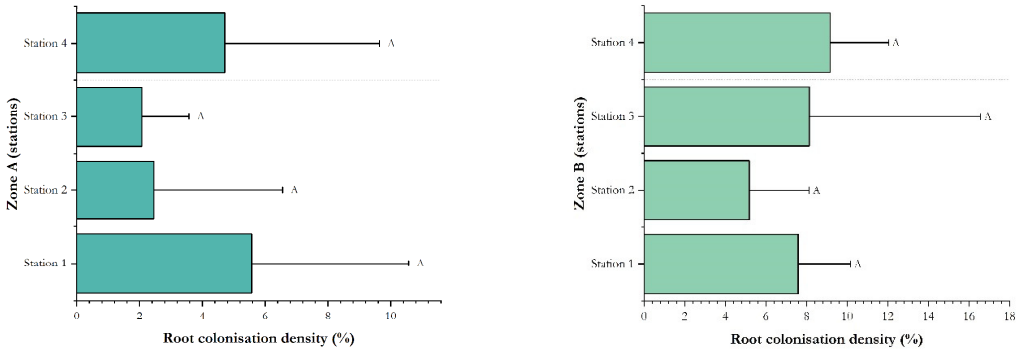
The spores were grouped according to their characteristics such as shape, color, cell wall ornamentation and size. The spores were fixed to a slide with polyvinyl alcohol-glycerol (PVLG) and a mixture of PVLG + Melzer's reagent (1:1 v/v), following the methodology indicated by Schenck & Pérez (1990). They were later analyzed under the microscope. The identification of the genus of the spores was made from the morphological analysis of the spore (such as the cell wall), the Melzer reaction and comparisons based on the INVAM website (The International Collection of Vesicular Arbuscular Mycorrhizal Fungi) (Prieto Benavides *et al.,* 2022).

For both sampling sites, spore abundance (number of spores per 100 g of soil) for each identified species was recorded. Alpha biodiversity was assessed using the Shannon diversity index [H], Simpson's dominance index [1-D], and Pielou's evenness index [J], all calculated using PAST 4.14 software. Species were classified as either 'general' (present in all seasons at both sites) or 'exclusive' (restricted to a single site) (Bonfim *et al*., 2016).

Results

Percentage of mycorrhizal colonization

The percentage of mycorrhizal colonization obtained from the study of roots belonging to the species *P. rugulosa* is showed in Figure 3. To verify the possible existence of statistical differences in zone A and B, data was analyzed. It was found that the data presented a non-parametric distribution. A Kruskal-Wallis test was applied ($p>0.05$), indicating the lack of significant differences between the station means. It is important to note that these tests were performed on samples taken from randomly distributed points in both areas of the forest, so that the test performed can serve as an indicator of the little difference between the two populations of *P. rugulosa*, despite being distributed in an altitudinal gradient. Also, it is seen that the species had a percentage of mycorrhizal colonization of less than 10% in both areas, presenting lower values in zone A (area with higher altitude and presence of younger specimens).



(A) (B)

Figure 3. Percentage of mycorrhizal colonization in *Polylepis rugulosa* roots in the Cacayaco forest, Arequipa, Peru. The values correspond to the average of five repetitions per station in Zone A (A) and Zone B (B)

Counting and evaluation of density of mycorrhizal fungi

The highest mean value for AMF spore density in 100 g^{-1} of rhizospheric soil was 1355 in zone B, and 888 spores g^{-1} of soil in zone A. To identify the existence of differences in spore density between both zones, the data was tested for normal distribution and a t-Student test ($p>0.05$) was performed, indicating equality between the mean of both zones, as can be seen in Figure 4, corresponding to the mean number of total spores of the genera *Acaulospora, Glomus, Rhizophagus, Claroideoglomus, Racocetra*, etc. The highest mean spore density occurred in zone B, specifically at station B02, and the lowest in zone A, at station A03, with 1355 and 619 spores, respectively (Table 1).

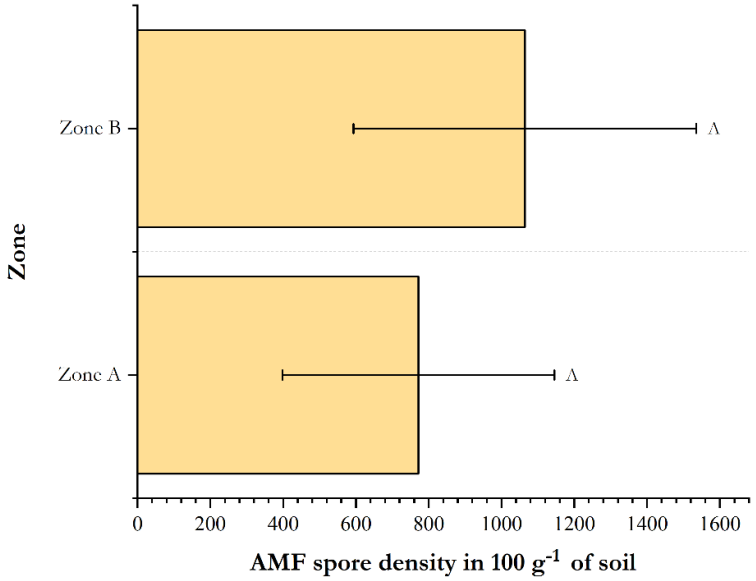


Figure 4. Average total arbuscular mycorrhizal fungi spores by zones obtained from 100 g^{-1} of soil

Table 1. Spore density per season in Zone A and Zone B

Zone	Stations	Average density of AMF spores in 100 g^{-1} of soil
Zone A	Station A01	760
	Station A02	820
	Station A03	619
	Station A04	888
Zone B	Station B01	1148
	Station B02	1355
	Station B03	1024
	Station B04	730

In both zones, the genus *Acaulospora* presented the highest density of spores, recording a total of 120 in Zone A and 210 in Zone B. On the other hand, the genus that presented a lower density in Zone A was *Diversispora* (1 spore), while in Zone B, *Dentiscutata* and *Funneliformis* were found to be the lowest (1 spore respectively) (Table 2). It is important to highlight that in certain cases genera were found in one zone that were not present in the other, as was the case of *Scutellospora* was found only in Zone B, and *Racocetra* was found only in Zone A.

Table 2. Identified arbuscular mycorrhizal fungi species in the present research

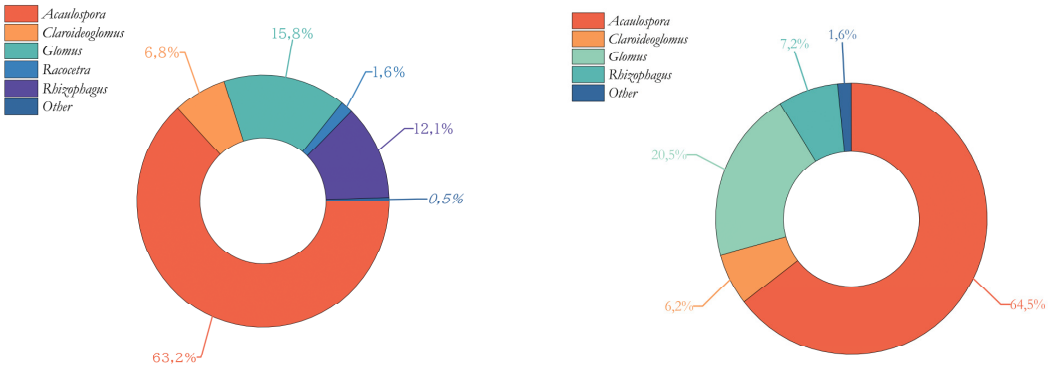
Species	Zone A stations				Zone B stations			
	A01	A02	A03	A04	B01	B02	B03	B04
Acaulospora bireticulata	-	-	5	-	-	-	-	-
Acaulospora capsicula	-	-	-	-	1	-	-	-
Acaulospora colombiana	5	6	6	9	6	5	2	1
Acaulospora denticulata	-	-	8	14	10	3	7	5
Acaulospora elegans	-	-	-	-	-	1	-	-
Acaulospora foveata	-	-	2	-	-	-	-	-
Acaulospora kentinensis	-	-	20	21	14	4	29	31
Acaulospora koskei	1	-	-	-	-	-	-	-
Acaulospora laevis	-	-	-	-	1	-	-	-
Acaulospora mellea	-	1	-	-	-	-	-	-
Acaulospora morrowiae	-	1	-	-	3	-	-	-
Acaulospora rehmii	-	1	-	2	7	5	-	-
Acaulospora sp.	-	-	-	-	28	8	17	19
Acaulospora sp.1	1	6	1	1	-	-	-	-
Acaulospora sp.2	-	2	1	-	3	-	-	-
Acaulospora sp.3	-	-	5	-	-	-	-	-
Acaulospora sporocarpia	1	-	-	-	-	-	-	-
Diversispora globifera	1	-	-	-	-	-	-	-
Claroideoglomus claroideum	-	2	-	-	-	-	-	-
Claroideoglomus etunicatum	1	2	2	1	1	-	-	-
Claroideoglomus lamellosum	1	-	3	-	2	5	-	1
Claroideoglomus lateum	-	-	-	-	1	-	-	-
Claroideoglomus sp.	-	-	-	-	1	-	-	-
Claroideoglomus sp.1	-	-	-	1	8	-	-	-
Dentiscutata heterogamous	-	-	-	-	1	-	-	-

Racocetra coralloidea	-	1	-	-	-	-	-	-
Racocetra verrucosa	2	-	-	-	-	-	-	-
Scutellospora calospora	-	-	-	-	-	1	2	-
Funneliformis mosseae	-	-	-	-	-	1	-	-
Glomus fistulosum	-	-	1	-	-	-	-	-
Glomus Hoi	-	-	1	-	-	-	-	-
Glomus pansihalos	1	-	-	-	-	1	-	1
Glomus sp.3	-	2	-	-	-	1	-	-
Glomus sp.	-	-	-	-	5	5	1	9
Glomus sp.1	1	-	1	4	-	1	1	-
Glomus sp.2	5	2	8	4	13	5	12	4
Glomus sp.4	-	-	-	-	1	1	-	-
Glomus sp.5	-	-	-	-	-	1	-	1
Rhizophagus aggregatus	-	1	-	-	-	-	-	-
Rhizophagus clarus	3	-	-	2	-	-	-	-
Rhizophagus intraradices	2	2	7	6	13	4	4	1

Identification of mycorrhizal fungi

For both sampling areas, a total of 41 taxa were identified up to the species level (Table 2), belonging to 5 predominant genera, such as *Acaulospora, Glomus, Rhizoglomus, Claroideoglomus,* and *Racocetra*. Likewise, the species with the greatest presence was *Acaulospora kentinensis,* reaching 29 individuals.

Based on the information provided by *The International Collection of Vesicular Arbuscular Mycorrhizal Fungi* (INVAM), the AMF species present in the Cacayaco forest were identified. In Figure 5a, it can be seen that in Zone A of the forest the genus with the highest abundance is *Acaulospora* (63.2%), followed by *Glomus* (15.8%), *Rhizophagus* (12.1%), *Claroideoglomus* (6.8%) and *Racocetra* (1.6%). Similar results were found in Zone B (Figure 5b), where the most abundant AMF was *Acaulospora* (64.5%), *Glomus* (20.5%), *Rhizophagus* (7.2%) and *Claroideoglomus* (6.2%). This indicates that, despite the difference in altitude and age of the *P. rugulosa* seedlings, the mycorrhizal populations in both areas were similar, both in the presence of species and in abundance.



(A) (B)

Figure 5. AMF genera present in *Polylepis rugulosa* in the Cacayaco forest, Arequipa, Peru. The genera that were considered have an abundance greater than 1.6% and correspond to Zone A (A) and Zone B (B)

In Figure 6 we present the results with respect to the biodiversity indices for both sampling areas. In both cases, the average values of the Shannon index [H] are in the range of medium diversity, with average values of 2.371 and 2.113 for zone A and zone B respectively.

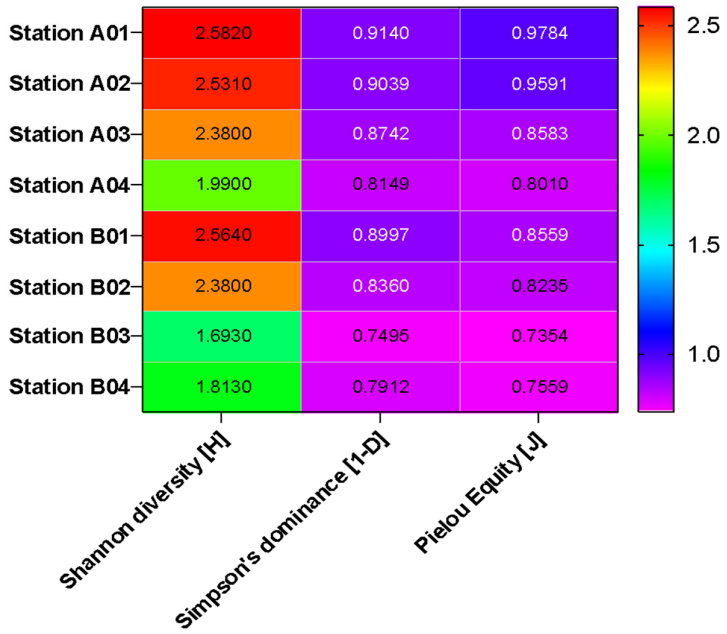


Figure 6. Biodiversity indices of studied arbuscular mycorrhizal fungi

Discussion

Mycorrhizal colonization rates observed in this study were lower than those reported by Soteras *et al*. (2015) and Becerra *et al*. (2019) in Argentinian *P. australis* forests, which exhibited colonization percentages ranging from 4% to 95% and 11.4% to 70%, respectively. Soteras *et al*. (2015) noted potential seasonal variations in colonization levels. However, the observed colonization rates in this study were also lower than those reported for other *Polylepis* populations, which typically range from 2.078% to 9.164%.

A potential link between altitudinal differences and AMF density was observed between the two sampling sites, mirroring findings from Peruvian puna grasslands (Koske, 1987; Lugo *et al*., 2008). These results highlight the influence of environmental factors, particularly flooding, on AMF distribution within queñua forests. Notably, Solís-Rodríguez *et al*. (2020) demonstrated high AMF diversity in less disturbed ecosystems.

The results of density of mycorrhizal fungi were similar to those found by Menoyo *et al.* (2009), who reported AMF number, which was between 360 and 825 spores per 100 g of soil, in the mountains of Central Argentina ("Quebrada del Condorito" National Park); and the AMF were belonging to 5 genera. Cofré *et al.* (2019), reported that the species richness of AMF is in a range between 26 and 40, so finding 41 species in this study affirms a significant content of taxa present in the Queñua Forests.

The identification results of mycorrhizal fungi are consistent with those obtained by Soteras *et al.* (2015, 2016), who studied *P. australis* populations in the high mountains of central Argentina and found that the most representative genera of AMF were composed of *Acaulospora, Glomus* and *Rhizophagus*. Likewise, similar results were found by Rodrigues *et al.* (2021), who found that in the South American Atlantic Forest biomes

the most prominent genera were composed of *Acaulospora, Claroideoglomus, Gigaspora, Glomus,* and *Septoglomus*, presenting lower values in native forests, compared to regenerating areas. Consistently, Becerra *et al.* (2019), when evaluating the AMF associated with *P. australis species* in the first restoration of montane forests in central Argentina, discovered that of the 33 morphospecies found, 20 belonged to the genera *Acaulospora, Ambispora, Claroideoglomus, Entrophospora, Funneliformis, Gigaspora, Glomus, Rhizophagus* and *Scutellospora*. The abundance of the genus *Acaulospora* and *Glomus* is consistent with the literature; for instance, Haug *et al.* (2019), who investigated AMF in the ecosystems that are between 3000 to 4000 meters above sea level and reported a predominance in the abundance of the genus *Acaulospora*, followed by *Glomus*.

The biodiversity indices observed in this study align with those reported for conserved and secondary forests by Ruiz (2020). While zone B exhibited higher spore density, zone A demonstrated greater biodiversity. This is expected, as indices like Shannon's increase with species richness and evenness. Our findings exceed those reported by Moreira *et al*. (2007) in Brazilian Araucaria forests and Zhang *et al*. (2022) in Chinese mountain ecosystems. Consistent with Solís-Rodríguez *et al*. (2020), who observed a link between AMF abundance and vegetation, soil properties, and species identity in low-flood tropical forests, we found that the site with less disturbance (likely zone A) exhibited higher AMF diversity. This supports the hypothesis that mycorrhizal biodiversity can serve as a valuable bioindicator of negative environmental impacts, such as those associated with the irrigation canal crossing the study area.

The morphological analysis of mycorrhizae, with Acaulospora as the predominant genus, revealed the most common arbuscular mycorrhizal fungi (AMF) in the *P. rugulosa* forest. This information is highly relevant for restoration efforts. Firstly, it enhances our ecological understanding of the crucial role AMF play in promoting plant growth, particularly under the challenging conditions faced by *P. rugulosa* at high altitudes. Secondly, identifying these mycorrhizal species suggests that their introduction into degraded soils could significantly improve the ability of *P. rugulosa* individuals to acquire nutrients, withstand water stress, and establish successfully. Finally, this knowledge provides a valuable restoration tool: If the most promising AMF species, particularly those with the greatest potential for soil improvement and enhancing the adaptation of *P. rugulosa* to its high-altitude environment, are isolated and reproduced under controlled conditions, they can be used as effective bioinoculants in restoration programs, optimizing reforestation efforts.

Conclusions

In the study, mycorrhizae were found in the roots of *Polylepis rugulosa*, showing a colonization rate of 10%. This highlights the symbiotic relationship between these fungi and the host plant. While this percentage is consistent with the study's findings, it is relatively low compared to similar ecosystems, which may indicate potential limitations on the functionality and health of the forest ecosystem. Such limitations could stem from environmental factors like soil nutrient availability, climatic conditions, or human disturbances, suggesting a need for further investigation.

The spatial distribution of mycorrhizal spores displayed a clear pattern, with Zone B exhibiting higher spore densities than Zone A, which has a steeper altitudinal gradient. Although this observation points to significant spatial variability, additional analysis is required to determine a causal link between spore density and altitude. For example, the higher spore density in Zone B might be influenced by factors other than altitude, such as soil characteristics or the types of vegetation within the zone.

The prevalence of the genus *Acaulospora*, which makes up 63.2% of the identified mycorrhizal taxa, highlights its ecological significance in this high-altitude forest ecosystem. *Acaulospora kentinensis* was notably found in both zones, suggesting its ability to adapt to different environmental conditions. This genus likely plays a crucial role in nutrient cycling, soil stabilization, and enhancing host plant resilience, which aligns with

findings from similar studies. Further exploration of these ecological functions would offer valuable insights into the adaptive strategies of arbuscular mycorrhizal fungi in these ecosystems.

Biodiversity indices indicated a moderate diversity of mycorrhizal fungi, reflecting the intricate interactions and adaptations within the forest community. When compared to similar high-altitude ecosystems, these diversity levels align with expectations, showcasing a stable yet sensitive balance of fungal communities. This highlights the necessity of preserving these ecosystems to sustain their ecological functionality. Although the dominance patterns of certain AMF taxa and the observed spatial distributions may imply environmental changes, the study does not provide direct evidence of such dynamics. Therefore, these findings should be viewed as potential indicators of ecosystem shifts rather than confirmed changes. Future research could include longitudinal monitoring or focus on anthropogenic and climatic stressors to gain a deeper understanding of these dynamics. In summary, this study enhances our understanding of AMF dynamics in high-altitude *Polylepis* forests, providing insights that are vital for conservation and ecosystem management. By documenting fungal diversity, spatial distribution, and dominant taxa, the research emphasizes the significance of mycorrhizal associations in maintaining forest health. These findings can guide reforestation efforts, soil restoration initiatives, and long-term monitoring strategies, highlighting the need for proactive measures to protect these unique and vulnerable ecosystems.

Authors' Contributions

A.C., B.C., and O.P. jointly led the study's conceptualization and design. All authors participated in the study's development and drafting, as well as data collection and analysis. A.S., L.Y., and M.M. conducted the methodological aspects of the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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