JEE Journal of Ecological Engineering

Journal of Ecological Engineering, 2026, 27(11), 39–51
https://doi.org/10.12911/22998993/226041
ISSN 2299–8993, License CC-BY 4.0

Received: 2026.06.30
Accepted: 2026.09.15
Published: 2026.10.01

Isolation and identification in trap-cultures of arbuscular mycorrhizal fungi occurring within the Selva Pisana Biosphere Reserve, Italy

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ABSTRACT

The objective of this research was to isolate and identify native communities and perform individual isolates of arbuscular mycorrhizal fungi (AMF) from an experimental field with diversity of symbiont fungi, located near San Piero a Grado (Pisa), in the northwest of Tuscany, a natural reserve on the east coast of Italy. The isolation and identification procedures of native AMF obtained from trap cultures established with soils collected from different experimental sites are describes. A total of 36 trap cultures were prepared using various host plants, including *Zea mays*, *Petroselinum crispum* and spontaneous species emerging from the native soil seed bank. Several morphotypes were propagated and subsequently characterized through morphological and molecular analyses. The isolates were identified as *Funneliformis mosseae*, *Claroideoglomus etunicatum*, *Racocetra fulgida*, *Diversispora spurca*, *Funneliformis geosporum*, and *Septoglomus constrictum*. These isolates were preserved in pure cultures to obtain spores for functional characterization. The study of native communities revealed the impact of local AMF species richness.

Keywords: functional diversity, AMF diversity, morphotypes, pure culture.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are ubiquitous root symbionts that play essential roles in plant nutrition, stress tolerance, and ecosystem functioning, making them key components of both natural ecosystems and sustainable agricultural systems (Folli-Pereira et al., 2012). Molecular analyses of AMF communities inhabiting host plant roots have shown that optimal plant performance is not necessarily associated with high AMF richness and diversity, but may instead be related to the identity of the symbiotic partners (Camuy-Velez et al., 2025; Turrini et al., 2018). Indeed, AMF isolates may differentially activate symbiosis-associated genes, regulating nutrient transport, defense mechanisms and plant metabolic process (Campos-Soriano et al., 2012; Zhou et al., 2021), and thus affecting symbiosis functionality. Moreover, because host plants may preferentially allocate carbon to the most beneficial fungal partners (Brígido et al., 2017; Buil et al., 2023), it has been hypothesized that they selectively recruit the most efficient AMF assemblages from native soil communities.

The UNESCO Biosphere Reserve "Selva Pisana" is recognized as a Mediterranean biodiversity hotspot, harbouring diverse native AMF communities associated with coastal dune vegetation (Tsiknia et al., 2021; Turrini et al., 2008). Previous investigations carried out in this protected ecosystem demonstrated that differences in the composition of native AMF communities significantly influence plant growth and nutrient acquisition (Turrini et al., 2018). These findings highlighted the ecological importance of

preserving native AMF diversity and suggested that individual fungal species may differ considerably in their functional performance.

Despite these advances, an important knowledge gap remains. Previous studies primarily focused on describing AMF community composition and evaluating the effects of native AMF assemblages on plant performance, whereas comparatively little information is available on the successful isolation, propagation and characterization of the individual AMF species composing these communities. The recovery of native isolates is essential for investigating the biology and ecological traits of individual species, preserving fungal biodiversity, and providing well-characterized inocula for future studies and practical applications in sustainable agriculture and ecological restoration.

The results obtained by Turrini et al. (2018) represented the starting point for selecting native AMF species occurring in this biodiversity hotspot and identifying those with the greatest functional potential. Trap cultures provide an effective strategy for recovering native AMF species, particularly those that sporulate poorly under field conditions, allowing their multiplication and subsequent establishment in pure culture. Obtaining pure cultures is a fundamental step for accurate morphological and molecular identification, long-term preservation of native isolates, functional characterization, and the future development of native inocula.

Therefore, unlike previous studies conducted in the Selva Pisana Biosphere Reserve, which focused on the ecological role of native AMF communities, the present study aimed to isolate individual native AMF species through trap cultures, establish representative pure cultures, and identify the recovered isolates using complementary morphological and molecular approaches. This study provides a collection of well-characterized native AMF isolates that constitutes a valuable biological resource for biodiversity conservation and future ecological and agricultural applications.

MATERIALS AND METHODS

Establishment of trap-cultures of native AMF

In the experiment, a soil sampling was carried out (9 × 114 m size), where 12 plots of 3 × 4 m size were established in the same position of plots where species identification by morphological analysis of AMF spores was previously performed (Njeru et al., 2015). Twelve composite soil samples, one for each plot, were obtained by using a "X" shaped sampling pattern, in which five points were defined, one at the center of the plot, identified using GPs coordinates, and four additional points positioned at a distance of 1 m from the cental point. The geographical coordinates of the field experiment were taken in an area of 1026 m^2 (Table 1). Soil from each point was mixed and labeled as a single sample. The soil material was allowed to air dried before sieving through a 5 mm mesh size sieve.

For each experimental plot, three trap cultures, each with different host plants, were established, for a total of 36 trap cultures. The host plants utilized were: *Zea mays* (maize), *Petroselinum crispum* (parsley) and spontaneous plants from the seed bank of the original site. These species were used because of their susceptibility to mycorrhizal colonization and frequent use for trap cultures; the use of different host plants or of a consortium of natural plants has been suggested as appropriate to detect a high number of AMF species (Oehl et al., 2003; Selvakumar and Thangavel, 2026; Vályi et al., 2015). Each soil sample was mixed, 1:1 by volume, with a calcined attapulgite clay (Oil Dri, Chicago, IL), and poured into surface sterilized plastic pots (1800 cm^3). After planting, trap cultures were transferred in greenhouse under natural light and temperature and supplied with water by an automated irrigation system. To check the sporulation of AMF species, seasonal samplings were scheduled for three years. Since maize is an annual plant, it was seeded each year.

Assessment of native AMF sporulation in trap-cultures

Starting from September of the first year periodical checks of all the 36 pots were carried out. Results were summarized for each year of trap culture growth, in the fall of the first and second year and pring third year. For each trap-culture, a soil sample was obtained (33 g), which was wet-sieved through a set of nested (400, 200, 100 and 50 μm mesh) sieves. After sieving, the material was transferred to five 25-mL suspensions. The suspensions were under-layered with 25 mL of a 70% w/v sucrose solution, and the water/sucrose solution density gradient was centrifuged at 2.000 rpm for 2 min. In the material from the 400 μm sieve, no

Table 1. Geographical coordinates of the experimental field plots

Plot	Latitude	Longitude
1	Lat. N 43°40'19.488"	Long. E 10°20'39.887"
2	Lat. N 43°40'19.553"	Long. E 10°20'39.710"
5	Lat. N 43°40'20.269"	Long. E 10°20'39.602"
6	Lat. N 43°40'20.528"	Long. E 10°20'39.556"
9	Lat. N 43°40'20.705"	Long. E 10°20'39.674"
10	Lat. N 43°40'20.914"	Long. E 10°20'39.707"
13	Lat. N 43°40'21.162"	Long. E 10°20'39.541"
14	Lat. N 43°40'21.439"	Long. E 10°20'39.386"
17	Lat. N 43°40'21.464"	Long. E 10°20'39.358"
18	Lat. N 43°40'21.695"	Long. E 10°20'39.149"
21	Lat. N 43°40'22.382"	Long. E 10°20'39.152"
22	Lat. N 43°40'22.530"	Long. E 10°20'39.318"

spores or sporocarps were observed. After centrifugation, the supernatant was passed through a 45 μm sieve and washed with tap water. The trapped material, largely containing spores, spore clusters and sporocarps, was flushed into 9-cm diameter plastic plates and observed under a stereomicroscope (Wild, Leica). Spores and sporocarps were retrieved and separated according to their size and colour, to be morphologically characterized.

Isolation in pure culture of major morphotypes

In order to maintain the retrieved morphotypes in pure cultures, 10 to 30 spores or sporocarps were collected for each of them, and put in a pair of cellulose membranes together with two 15 days old chicory plantlets, and then transferred into 50 mL plastic tubes filled with a mix of sterilized soil and calcined attapulgite clay. After 6 weeks growth in a growth chamber at 25 °C, colonization was checked by gently removing the plants from the tubes, and the occurrence of spores/sporocarps or extraradical mycelium was assessed under a stereomicroscope. If colonization check was positive, plants were transferred to 700 cm^3 plastic pots, and moved to the greenhouse.

Morphological characterization of the isolated AMF

The morphological characterization was performed on the AMF morphotypes isolated in pure cultures. When no pure culture attempts were successful, the morphological description was performed on homogeneous spores from trap cultures. After wet sievings of soil aliquots of pure cultures, or trap cultures, spores were manually isolated by using capillary pipettes, while sporocarps were dissected with forceps to release spores. Spores were placed in Eppendorf tubes, submitted to sonication as needed in a B-1210 cleaner (Branson Ultrasonics, Soest, The Netherlands), washed three times in sterile distilled water, prepared on microscope slides in polyvinyl alcohol lactoglycerol (PVLG) and examined under a Polyvar light microscope incorporating with Nomarski differential interference contact optics (Reichert-Young, Vienna, Austria). In addition, spores were mounted in PVLG+Melzer's reagent (1:1, v/v), which is used as a diagnostic tool in morphology-based identification, differently reacting with components of AMF spore walls. Qualitative characteristics of the spore, such as shape, colour, size, wall ornamentation, wall structure, and the shape, colour, and size of the subtending hypha, were evaluated in at least 20 spores for each morphotype using a micrometric eyepiece. Spore morphotypes were compared with original diagnoses of AMF species and the culture descriptions by Błaszkowski (2012).

Molecular identification of AMF

In order to support the presumptive identification of the morphotypes obtained in trap cultures and/or in pure cultures, DNA was extracted from single spores or multi-spore samples (from 5 to 10 spores) and sequenced. The ribosomal region comprising the SSU-ITS-LSU fragment of extracted DNA was amplified by a nested protocol obtaining fragments of about 1500 bp.

DNA extraction from spores

Healthy and intact spores belonging to an identified morphotype were manually collected with a capillary pipette under the dissecting microscope and cleaned by sonication for 120 s. The spores were rinsed three times with sterile distilled water (SDW) and surface sterilized for 20 min with a 2% chloramine T solution supplemented with streptomycin (400 μg/mL). After sterilization, the spores were washed five additional times with SDW. Intact spores were selected under the dissecting microscope and transferred into Eppendorf tubes before DNA extraction. Spores were immediately

crushed into PCR tubes using a glass pestle, and then the DNA was extracted by using MasterPure yeast DNA purification KIT (Epicenter, Madison, USA) according to manufacturer's instructions.

PCR conditions of full-length SSU-ITS-LSU sequences

DNA extracts from single and multiple spores were used to analyze a DNA region covering partial SSU, ITS and partial LSU sequences by a nested PCR protocol. In the first PCR reaction DNA extracts (1 µL) were amplified in 25 µL of PCR reaction mix using 0.125 U of GoTaq Flexi DNA Polymerase (Promega, Milan, Italy), 0.4 µM each primer (Af1, Af2 and Ar1, Ar2, Ar3, Krüger et al., 2009), 0.2 mM (each) dNTPs, 1.5 mM $MgCl_2$ and 1× manufacturer's reaction buffer. The thermal cycler was programmed as follows: a manual hot start at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min. Nested PCR reactions were performed using a 1:100 dilution of the first PCR amplicons and using 2 µL of dilutions as template for the second reaction in a final volume of 50 µL. Each primer (0.4 µM), Cf1, Cf2, Cf3 and Br1, Br2, Br3, Br4 (Krüger et al., 2009), was added to the PCR mix. The concentration of Taq DNA polymerase, dNTPs, buffer and $MgCl_2$ were described above. Amplification conditions were as follows: a manual hot start at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 63 °C for 45 s, 72 °C for 1.5 min and a final extension step at 72 °C for 10 min. PCR products (10 µL) were separated on 0.8% agarose gels containing ethidium bromide (0.5 µg/mL).

Cloning and sequencing

Amplified DNA fragments of SSU-ITS-LSU regions were purified by Wizard SV Gel and PCR Clean-Up System according to the manufacturer`s instructions (Promega), with a final elution volume of 20 µL and purified products (2 µL) were quantified by a BioPhotometer (Eppendorf). After purification, amplicons were cloned into pGem®-T Easy vector according to the manufacturer`s instructions (Promega) to transform competent *E. coli* cells. Presumptive positive clones were screened through SP6/T7 PCR amplification, followed by a nested PCR using primer pairs by Krüger et al. (2009). Concentration of PCR mix components and PCR conditions were the same described above for PCR reactions. Up to six recombinant clones obtained from single or multiple spores were selected and purified using Wizard® Plus SV Minipreps (Promega). Recombinant plasmids were sequenced forward and reverse at GATC Biotech (AG European Custom Sequencing Centre, Cologne, Germany).

Sequence analyses and data analyses

The sequences were reworked using the MEGA6 program, to eliminate portions of the carrier. Forward and reverse sequences obtained from the same clone were assembled using Clustal Omega (EMBL) and sequences similarities were evaluated through BLASTn provided by NCBI. Chimeric sequences were identified using USEARCH 6.0. Sequences were aligned with the closest corresponding sequences retrieved from GenBank, together with represetnative sequences from the principal clades of Glomeromycota using MUSCLE implemented in MEGA6. Phylogenetic tree was inferred by neighbour-joining method analysis, while evolutionary distances were computed using the maximum composite likelihood method. The robustness of the phylogenetic branches was tested through 1000 bootstrap replicates.

Statistical analysis

Non-parametric Kruskall-Wallis analysis was used to determine differences in the diversity indices among AMF communities colonizing the roots of the three different plant species grown in treatments 2 and 22. Rarefaction curves were determinated with PAST 3.0 software to estimate whether the number of screened sequenced were sufficient to capture AMF diversity of each host. AMF communities were also evaluated by two-way permutational multivariate analysis of variance (PERMANOVA), to test the effects of soil microcosm and the host plant species, performed in PAST 3.0. Multivariate analyses (PCA and RDA) were performed using Canoco 5.0.

RESULTS AND DISCUSSION

The use of three different host plants probably contributed to the recovery of a broader spectrum of native AMF species. Although many

AMF exhibit low host specificity (Matinizadeh et al., 2026), differences in root morphology, growth dynamics and rhizosphere chemistry among maize, parsley and wild grasses may influence fungal colonization, carbon allocation and sporulation (Bicharanloo et al., 2020; Wang et al., 2025). Consequently, each host plant may create a distinct ecological niche that favours the multiplication of particular fungal taxa. Employing multiple host species therefore increases the likelihood of recovering both dominant and less competitive members of the native AMF community, improving the representativeness of the isolates obtained.

Arbuscular mycorrhizal fungal morphotypes recovered from trap cultures over the three-year period are shown in Figure 1.

At the first sporulation check, in the first year, overall 21 trap cultures showed abundant production of spores or sporocarps: 8 traps with maize plants, 5 with parsley and 8 with wild grasses as host plants. Different morphotypes were described, the most frequent being a sporocarpic one (SPC), occurring in 11 different trap cultures. In trap cultures from plot 10, the highest number of morphotypes (6) was found, whereas in trap cultures from plot 9 and 21 no sporulation was observed.

In the second year, spores of two new morphotypes were found, and the occurrence of other morphotypes already found in the previous year was confirmed. On the contrary, two morphotypes retrieved in the first year, named "cluster yellow" and "cluster hyaline" were not found.

The detection of two additional morphotypes during the second year indicates that long-term cultivation may reveal fungal species that were initially overlooked because of their low abundance or delayed sporulation. Not all native AMF species allocate resources to spore production at the same stage of root colonization, and some require prolonged interaction with the host or before completing their reproductive cycle (Buade et al., 2021; Tian et al., 2011). Therefore, extending the duration of trap cultures may increase the probability of recovering species that remain undetected during the initial propagation period.

In the third year, the assessment of sporulation allowed the identification of a smaller number of morphotypes than in previous years, in 13 trap cultures. No new morphological types appeared, while all the morphotypes already found in both in the first y second year occurred.

The reduction in morphotype richness observed during the third year indicates that, under

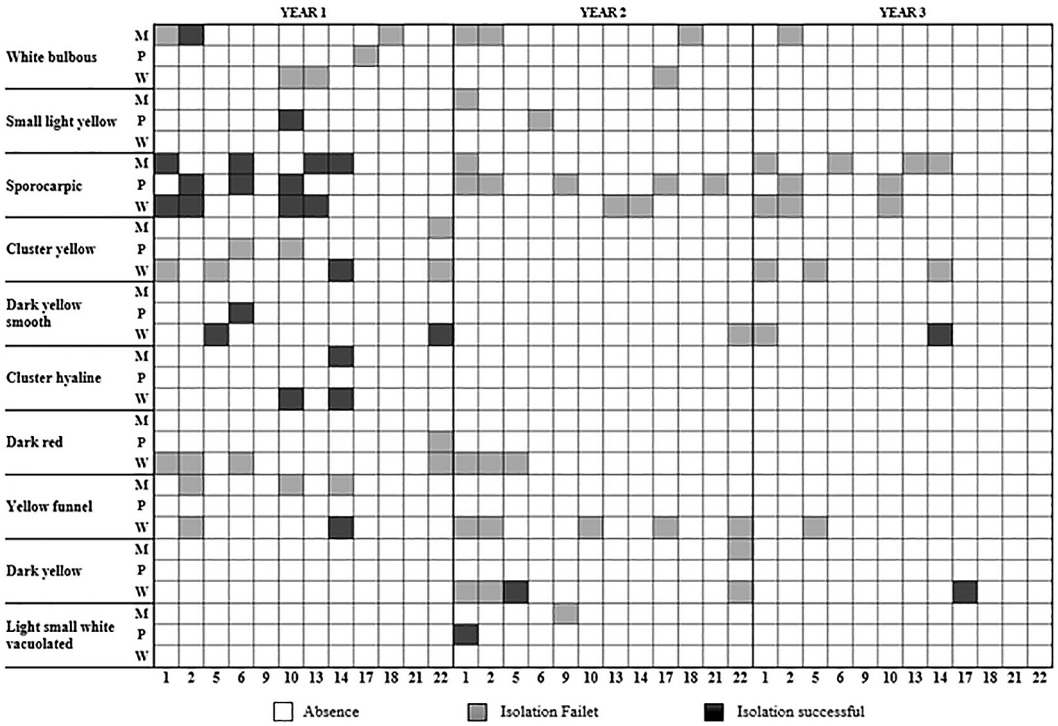

Figure 1. Occurrence and isolation outcome of AMF morphotypes recovered from trap cultures during three consecutive years. Columns represent plots of origin grouped by cultivation year, and rows represent morphotype–host plant combinations (M = maize, P = parsley, W = wild plants)

the relatively stable conditions of trap cultures, highly competitive species that rapidly colonize roots and sporulate abundantly tend to dominate the available niche, while slower-growing or more host-dependent taxa gradually decline (Davison et al., 2022). This pattern reflects the selective pressure imposed by ex situ cultivation, rather than the actual diversity present in the original ecosystem. While prolonged trap culture favors the recovery of certain species, it can simultaneously reduce community complexity through competitive exclusion. Consequently, trap cultures should be regarded as a selective tool that enriches for the most adaptable members of the native AMF community while allowing the recovery of isolates suitable for subsequent biological and molecular characterization.

Successful sporulation of several AMF species in trap cultures established from the experimental hot-spot field demonstrates that this method can provide suitable conditions for the completion of the AMF life cycle (Oehl et al., 2004; Pedersen et al., 2017; Sakha et al., 2025), particularly when long periods of cultivation are utilized (Oehl et al., 2009; Yao et al., 2010), as in our case. However, as the *ex-situ* reproduction of AMF does not allow to recover all the species occurring in native AMF communities, the *in-situ* conservation of such beneficial symbionts appears a fundamental step to be considered in the years to come (Turrini and Giovannetti, 2012).

The recovery of only part of the native AMF community suggests that trap cultures preferentially favor species capable of adapting to the environmental conditions imposed by ex situ cultivation. Differences in germination requirements, colonization efficiency, sporulation capacity and host compatibility probably determined which species became established and persisted during propagation (De La Cruz et al., 2026; Srinivasan et al., 2014).

In total, 41 isolation attempts were carried out starting from the trap culture material. Some attempts failed, as no germination/colonization was found after 6 weeks growth. A total of 21 pure cultures were established in larger pots (900 cm^3). In June of the second year, 18 cultures turned out to have sporulated, mostly representing the sporocarp producing morphotypes (13 cultures). Other four morphotypes were successfully reproduced in pure culture. Interestingly, a well recognizable morphotype ("white bulbous", ascribed to the genus *Racocetra*) produced abundant, large white spores in the original trap culture, but did not reproduce in pure culture during the first year; therefore, spores from trap cultures were utilized for morphological description and molecular analysis. In the second and third year, only attempts to isolate the new morphotypes (in the second year) and the "white bulbous" were performed. After checking plant colonization and sporulation, both new morphotypes and eventually white bulbous morphotypes were successfully reproduced.

In Table 2, some morphometric data of the morphotypes retrieved and reproduced in pure culture are summarized.

The morphometric data (spore shape, colour and size) are reported for each of the following retrieved morphotypes: bulbous white, small light yellow, sporocarpic, yellow cluster, smooth dark yellow, dark yellow, funnel yellow, hyaline cluster (Table 3).

The white bulbous spores possessed a bilayered spore wall structure comprising layers L1 and L2. In immature spores, both wall layers were tightly adherent and exhibited comparable thicknesses. As the spores matured, L2 underwent progressive thickening and structural differentiation. The outer wall layer (L1) was permanent, smooth, and rigid, displaying an off-white to pale-yellow coloration and a thickness ranging from 0.7 to 1.8 µm. L2 consisted of pale yellow, laminated layers that increased in number during development. In mature spores, L2 was 4.5–6.0 µm thick and changed from bright yellow to golden when it reacted positively to Melzer's reagent.

When mounted on PVLG and Melzer's reagent (1:1, v/v), the light yellow spores showed a wall composed of four distinct layers (L1–L4). Immature spores only had L1 and L2, which were continuous with the juvenile wall of the underlying hypha. During spore development, L3 was subsequently formed in both the subtending hypha and the spore, progressively increasing in thickness and ultimately constituting the major portion of the mature spore wall. The innermost layer (L4) developed at a later stage, originating from the subtending hyphal wall in the region adjacent to the spore base.

L1 constituted as a hyaline mucilaginous layer 0.6 to 1.8 µm thick in immature spores. This layer produced a pink to dark-pink reaction in Melzer's reagent and was often difficult to distinguish from L2 because of their close adherence. As the spores matured, L1 became granular during degradation and was usually shed together with L2.

Table 2. Morphological characterization of spores belonging to selected arbuscular mycorrhizal fungal isolates from the experimental field

Morphotypes	Code name	Sporocarp occurrence	Colour	Diameter mean±standard error of mean (µm)	Hyphal attachment	Presumptive identification
SPC	1W1	YES	Yellow	196.5±4.6	FS	*Funneliformis mosseae*
SPC	1W3	YES	Yellow	202.6±5.9	FS	*Funneliformis mosseae*
WB	2M1	NO	White	253.7±2.1	B	*Racocetra fulgida*
SPC	2W3	YES	Yellow	189.8±4.7	FS	*Funneliformis mosseae*
DYS	5W1	NO	Yellow	116.9±3.3	FS	*Claroideoglomus etunicatum*
DYS	5W2	NO	Yellow	111.4±0.9	FS	*Claroideoglomus etunicatum*
DYS	6P1	NO	Yellow	118.6±2.4	FS	*Claroideoglomus etunicatum*
SLY	10P2	NO	Yellow	107.9±1.6	C	*Claroideoglomus claroideum*
CY	14W1	NO	Hyaline	51.6±0.94	C	*Dominikia aurea*
YF	14W3	NO	Brown	125.0±1.59	FS	*Funneliformis geosporum*
DY	17W	NO	Brown	130.3±1.85	FS	*Septoglomus constrictum*
DY	17W1	NO	Brown	147.3±2.01	FS	*Septoglomus constrictum*
DY	22M1	NO	Brown	133.5±1.7	FS	*Septoglomus constrictum*
DYS	22W3	NO	Brown	117.8±2.4	FS	*Claroideoglomus etunicatum*

Note: Morphotypes: SLY – Small light yellow, SPC – Sporocarpic, WB – White bulbous, DYS – Dark yellow smooth, CY – Cluster yellow, YF – Yellow funnel, DY – Dark yellow. Hyphal attachment: C – Cylindric, FS – Funnel-shaped, B – Bulbous.

Table 3. Morphological description of the reproduced AMF

Morphotypes	Similar to	Color	Shape	Size distribution
White bulbous	*Racocetra fulgida*	Pale to slightly darker cream color	Globose, subglobose	155–310 µm, mean = 253 µm. (n = 124)
Small light yellow	*Claroideoglomus claroideum*	Cream to light yellow	Globose to subglobose	90–124 µm, mean = 108 µm. (n = 31)
Sporocarpic	*Funneliformis mosseae*	Straw to dark orange-brown, but a majority are yellow-brown	Globose to subglobose, some irregular	124–295 µm, mean = 203 µm. (n = 53)
Cluster yellow	*Dominikia aurea*	Light orange to orange	Irregular in shape; without a peridium; composed of rather closely packed spores.	38–63 µm, mean = 52 µm. (n = 45)
Dark yellow smooth	*Claroideoglomus etunicatum*	Orange to red brown	Globose, subglobose	98–133 µm, mean = 111 µm. (n = 81)
Dark yellow	*Septoglomus constrictum*	Fairly wide range, from red-brown to almost black.	Globose to subglobose, rarely irregular	105–180 µm, mean = 147 µm. (n = 60).
Yellow funnel	*Funneliformis geosporum*	Yellow-brown to dark orange-brown	Globose to subglobose, some irregular	99–152 µm, mean = 125 µm (n = 49).
Cluster hyaline	*Septoglomus viscosum*	Subhyaline to pale straw at darkest	Globose to subglobose, rarely irregular. Many spores are formed in loose aggregates branching from a common hypha	50–120 µm, mean = 82 µm. (n = 160).

L2 was also hyaline and developed simultaneously with L1 during the early stages of spore formation. Its thickness ranged from 0.6 to 2.0 µm. This layer showed no reaction in Melzer's reagent and gradually degraded during maturation. Accumulation of organic debris on the spore surface was frequently observed as L2 deteriorated.

The L3 layer was composed of pale yellow lamellae tightly adhered to each other, with a thickness of 2.8 µm that increased during development, reaching 6.2 µm in mature spores. This laminated layer becomes the main structural component of the mature spore wall.

L4 exhibited a similar color to L3 and its thickness ranged from less than 0.5 µm to almost 3.0 µm. When thin, it frequently appeared folded. Its morphology resembled that of a flexible germ wall; however, it did not participate in germination. This layer extended into the underlying hypha as a continuation of the hyphal wall. L4 was interpreted as the innermost spore wall layer rather than as a component of L3. This layer commonly separated partially or completely from the remainder of the spore wall and occasionally retained a small projection at the point of attachment to the subtending hypha.

Sporocarpic spores exhibited a tripartite wall structure consisting of layers L1, L2, and L3, which developed sequentially throughout wall differentiation. The outermost layers were frequently subject to degradation and sloughing in mature and older spores, particularly those obtained from natural soils. In some isolates, spores occurred in compact clusters surrounded by a peridial envelope. However, this character is considered of limited diagnostic value because of its variable occurrence and lack of stability.

The cluster-yellow spores showed a spore wall composed of two distinct layers (L1 and L2). The subtending hypha possessed a light-orange to orange wall that was continuous with both spore wall layers. At the spore base, the hyphal wall was markedly thickened, with layer L2 reaching up to 35 µm in thickness and remaining between 10 and 30 µm thick below the point of attachment before gradually tapering to 1–3 µm and becoming hyaline distally.

L1 was a hyaline, evanescent layer less than 1.5 µm thick, forming the spore surface as the outermost layer of the wall. In mature spores, this layer usually detached and was often absent. Layer L2 was the structural layer of the wall, with a finely laminated organization. Its color ranged from light orange to orange, and it was between 1.5 and 3.0 µm thick across most of the spore wall, increasing to 6.0 µm in the region adjacent to the spore base. When mounted in Melzer's reagent, only L1 displayed a staining reaction, developing a pastel-red to orange-red coloration, whereas L2 remained unchanged.

The dark-yellow smooth spores possessed a bilayered spore wall consisting of layers L1 and L2, which developed sequentially during spore maturation. At the earliest developmental stages, only L1 was present and was continuous with the wall of the subtending hypha. As the spores matured, L2 was formed beneath L1, initially as a single layer and subsequently increasing in thickness through the deposition of additional laminae with similar structural characteristics.

L1 constituted the outermost layer of the cell wall and appeared as an irregular, hyaline, mucilaginous, moderately plastic surface, with a thickness between 0.6 and 2.8 µm in immature spores. This layer reacted positively to Melzer's reagent, producing a pink to reddish-purple coloration. As spore development progressed, L1 gradually deteriorated and commonly detached, leaving behind discontinuous patches or a granular residue on the spore surface. In some cases, this layer appeared to facilitate microbial colonization, and spores stored in tap water developed a distinct halo after prolonged incubation at room temperature or under refrigeration.

L2 constituted the permanent structural wall layer and was composed of tightly adherent laminae displaying a light orange-brown to reddish-brown coloration. The thickness of this layer generally ranged from 4.4 to 6.4 µm, although in the region adjacent to the subtending hypha it occasionally increased to approximately 7.6 µm due to additional laminar deposition.

The dark yellow spores had a cell wall composed of two distinct layers (L1 and L2). The outer layer remained attached to the underlying wall during development; however, this layer gradually deteriorated and eventually detached as the spores aged.

L1 was a hyaline outer cell wall layer 2–4 µm thick. No staining reaction was observed in the spores with Melzer's reagent. In mature and senescent spores, this layer was frequently degraded, and remnants were occasionally found near the base of the spore in the region of the underlying hypha. L2 constituted the permanent laminated cell wall layer, forming the main structural component of the mature spore wall and was continuous with the inner cell wall layer of the persistent underlying hypha. L2 was colored orange-brown to dark reddish-black and was 5–9 µm thick.

The yellow-funnel spores possessed a tripartite spore wall structure (L1, L2, and L3), which developed sequentially during spore maturation. The thickness of three layers together of the wall ranged from 8 to 16 µm.

L1 was hyaline and formed the outermost layer of the wall, less than 1 µm thick, and was non-reactive to Melzer's reagent. In mature

spores, this layer was degraded or appeared as a granular residue detached from the spore surface. The L2 layer constituted the main structural layer of the wall and consisted of firmly adhered lamellae. The color varied from yellowish-brown to orange-brown, with lighter tones on the outside and darker tones toward the inner surface. The thickness of this layer ranged from 6 to 14 µm. L3 was a semi-rigid to rigid inner wall layer measuring approximately 1.0–2.5 µm in thickness. Although commonly adherent to L2, it could usually be distinguished by its slightly darker yellow to orange-brown pigmentation. This layer extended into the lumen of the subtending hypha, where it formed a continuous recurved septum. In some spores, the inner surface of L3 exhibited small wart-like projections; however, because their occurrence was inconsistent, these structures were not considered taxonomically or phylogenetically informative.

The cluster-hyaline spores showed a wall composed of three distinct layers (L1, L2, and L3). L1 was the outermost layer and separated partially from the two inner layers. L2 and L3 remained closely adherent.

L1 was a semi-flexible hyaline layer 1 to 2 µm thick when it lacked surface deposits and up to 5 µm thick in the presence of adhering exudates or debris. No staining reaction with Melzer's reagent was observed, suggesting that the material present on the surface did not correspond to a carbohydrate-rich mucilaginous coating.

L2 was a very thin, semi-flexible hyaline layer, less than 0.5 µm thick. Due to its well-defined margins, this layer appeared as a slightly raised peripheral edge when attached to layer L3. Layer L2 was consistently observed in the examined spores, despite not being described in the original diagnosis of the species. L2 occasionally separated from L3, being detected in less than 10% of the crushed spores.

L3 represented the innermost structural wall layer and increased in thickness during spore development through the successive deposition of laminae. This layer exhibited greater rigidity than L1 and L2, while retaining a moderate degree of flexibility. Compared with the brittle structural walls of many glomeromycotan species, L3 was relatively pliable; however, it was less flexible than the wall reported for species such as *Diversispora spurca*. Consequently, folds were commonly observed on its inner surface following spore crushing.

The inability to establish pure cultures from several morphotypes should not be interpreted as evidence of fungal inviability. Arbuscular mycorrhizal fungi exhibit marked interspecific differences in spore dormancy, germination behaviour, nutritional requirements and dependence on specific host plants (Pepe et al., 2016; Selvakumar et al., 2023). These biological characteristics may considerably reduce the establishment success of certain taxa under standardized propagation conditions.

DNAs from samples of spores/sporocarps from pure isolates or from trap cultures, belonging to five morphotypes, were successfully amplified and sequenced, while DNA samples of four morphotypes did not yield amplicons. Several biological and technical factors may account for the absence of PCR amplicons in some morphotypes, and this should not be interpreted as evidence of incorrect identification. These factors include the recovery of empty, senescent, or non-viable spores containing degraded DNA; insufficient DNA yields from certain morphotypes; sequence variation in primer-binding regions, which may reduce amplification efficiency; the multinucleate nature of AMF spores, and structural characteristics of the spores that hinder DNA extraction (Kryukov and Yurkov, 2018). Together with the biological characteristics of AMF, these factors provide a plausible explanation for the unsuccessful isolation or molecular identification of some morphotypes despite their successful recovery from trap cultures.

BLASTn analysis of sequences confirmed membership to the phylum of Glomeromycotina. The sequence obtained are reported in Table 4, along with the percentage of identity with database sequences, and their taxonomic affiliation. Isolate 2M1, which was morphologically ascribed to *Racocetra* sp., actually turned out to produce sequences of *Racocetra fulgida*, while sequences from 2P2, 2W3 and 6M2 were homologue (99%) to *Funneliformis mosseae*. A dark red morphotype was sequenced using spores from trap culture 22M, and spores isolated in the pure culture 17W1, but the latter failed to give consistent sequences. However, 22M phylotype showed 97–99% identity with *Septoglomus constrictus* sequences. *Claroideoglomus claroideum* was the species whose sequences in public databases matched to 10P2 sequences, thus confirming the morphological identification. Spores from 14W1, produced sequences which fitted (95–98%) with those belonging to an uncultured fungus, that could possibly be

referred to a Glomeraceae genus, branching close to *Septoglomus*, although morphologically was similar to *Dominikia aurea*, due to the clustered mode of spore aggregation. A phylogenetic tree including all sequences obtained in this study is reported in Figure 2.

The occurrence of *R. fulgida* (formerly *Scutellospora fulgida*) in the experimental hot spot site, confirms previous data, since it was retrieved as a regular component of the rhizosphere of dunes plants growing in the same area (Grassi et al., 2026; Turrini et al., 2008). As *R. fulgida*

Table 4. Sequences obtained from spores of arbuscular mycorrhizal fungi selected morphotypes, identified using SSUmCf/LSUmBr primers pair

Isolate	Sequence code	Identity (%)	Taxonomic affiliation
2M1	2M-1	FR750148 (99)	*Racocetra fulgida*
	2M-2	FR750140 (99)	*Racocetra fulgida*
	2M-3	FR750141 (99)	*Racocetra fulgida*
	2M-4	FR750146 (99)	*Racocetra fulgida*
	2M-5	FR750144 (99)	*Racocetra fulgida*
	2M-6	FR750146 (99)	*Racocetra fulgida*
2P2	2P-2	FR750026 (99)	*Funneliformis mosseae*
	2P-4	FR750026 (99)	*Funneliformis mosseae*
	2P-5	FR750026 (99)	*Funneliformis mosseae*
	2P-6	FR750026 (99)	*Funneliformis mosseae*
	2P-8	FN547474 (99)	*Funneliformis mosseae*
	2P-9	FN547474 (99)	*Funneliformis mosseae*
6M2	6M2-10	JF439135 (99)	*Funneliformis mosseae*
	6M2-13	FN547474 (99)	*Funneliformis mosseae*
	6M2-14	FR750031 (99)	*Funneliformis mosseae*
	6M2-15	JF439130 (99)	*Funneliformis mosseae*
	6M2-16	JF439130 (99)	*Funneliformis mosseae*
	6M2-18	JF439135 (99)	*Funneliformis mosseae*
2W3	2W3-1	FN547484 (99)	*Funneliformis mosseae*
	2W3-2	FR750026 (99)	*Funneliformis mosseae*
	2W3-3	FR750026 (99)	*Funneliformis mosseae*
	2W3-4	FR750026 (99)	*Funneliformis mosseae*
	2W3-5	FR750026 (99)	*Funneliformis mosseae*
	2W3-6	FR750026 (99)	*Funneliformis mosseae*
22M	22M-1	JF439167 (97)	*Septoglomus constrictus*
	22M-2	FJ461826 (98)	*Septoglomus constrictus*
	22M-3	FJ461826 (98)	*Septoglomus constrictus*
	22M-4	FJ461827 (99)	*Septoglomus constrictus*
	22M-5	JF439167 (97)	*Septoglomus constrictus*
	22M-6	JF439167 (98)	*Septoglomus constrictus*
10P2	10P2-3	FR750059 (97)	*Claroideoglomus claroideum*
	10P2-5	FR750059 (97)	*Claroideoglomus claroideum*
	10P2-6	FR750059 (97)	*Claroideoglomus claroideum*
14W1	14W1-1	JX276907 (98)	*Uncultured Glomeromycota*
	14W1-2	JX276907 (98)	*Uncultured Glomeromycota*
	14W1-3	JX276907 (96)	*Uncultured Glomeromycota*
	14W1-4	JX276907 (95)	*Uncultured Glomeromycota*
	14W1-5	JX276907 (98)	*Uncultured Glomeromycota*
	14W1-6	JX276907 (98)	*Uncultured Glomeromycota*

was reported to be threatened by anthropogenic disturbance (Giovannetti and Gianinazzi-Pearson, 1994; Helgason et al., 1998), its occurrence in a site which is under the control of national and international authorities represents an important step towards its conservation as a rare and endangered microorganism. Moreover, its isolation and reproduction in trap culture is an important progress towards its characterization and possible utilization in the years to come. On the other hand, the isolation of many isolates of *F. mosseae* represents a further demonstration on its wide occurrence in natural and arable sites (Al-Qarawi et al., 2013; Guardiola-Márquez et al., 2022; Parihar et al., 2025). Beyond *R. fulgida*, the AMF species isolated and identified from the experimental protected site are AMF 'generalists' (Oehl et al., 2010), as they are presumed to adapt and thrive in different soil environments, from natural to tilled soils, as a result of their ability to sporulate quickly and massively (Batool et al., 2026; Soka and Ritchie, 2015).

Phylogenetic tree generated using the neighbour-joining method based on Glomeromycota sequences, showing the relationships among the four isolated AMF species (Figure 2). Bootstrap values above 65% obtained from 1000 resamplings are displayed at the corresponding branches. The analysis was conducted using partial sequences of the nuclear SSU-ITS-LSU rDNA region (~1500 bp). Sequences obtained in this study are presented in bold and labeled according to the AMF isolate code, trap-culture code, and clone identifier. The tree is rooted with a reference sequence of Archaeospora trappei FR750036.

Beyond the taxonomic identification of native AMF species, the establishment of well-characterized isolates represents an important contribution to the conservation of microbial biodiversity within the Selva Pisana Biosphere Reserve. These isolates constitute a valuable biological resource for future studies aimed at understanding plant–fungus interactions, evaluating functional diversity and developing native inocula adapted to Mediterranean environments. Compared with commercial inoculants originating from different geographical regions, native isolates are expected to exhibit greater ecological compatibility with local environmental conditions, thereby increasing their potential value for ecological restoration and sustainable agricultural practices.

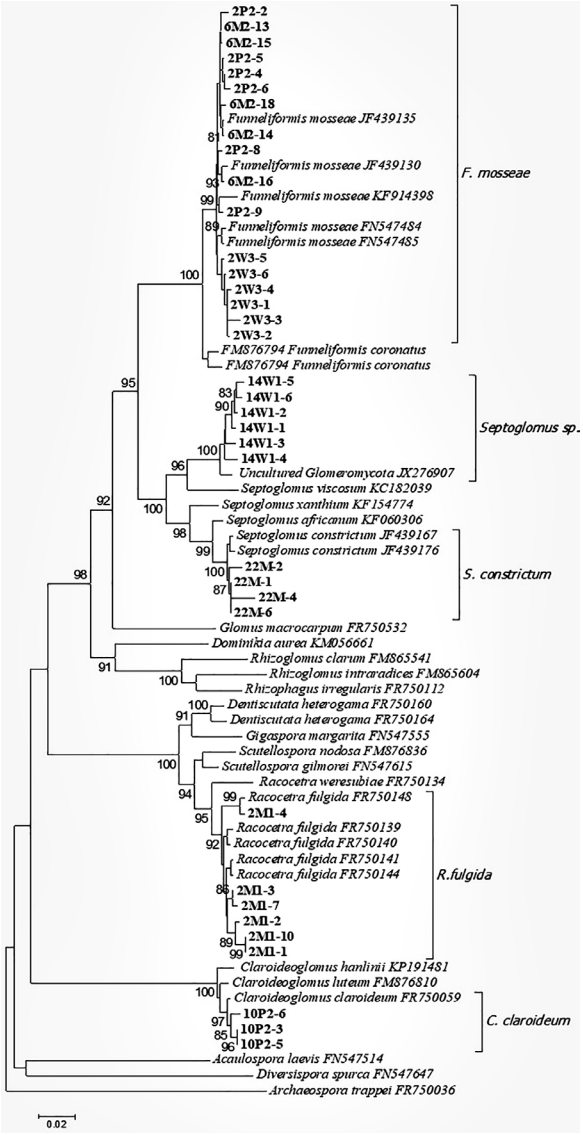

Figure 2. Phylogenetic tree generated using the neighbour-joining method based on Glomeromycota sequences

CONCLUSIONS

This study demonstrated that long-term trap cultures are an effective, although selective, approach for recovering and propagating native arbuscular mycorrhizal fungi from the Selva Pisana Biosphere Reserve. The use of different host plants and repeated monitoring over three years enabled the detection of morphotypes with contrasting sporulation patterns, persistence, and capacity to establish in pure culture. The progressive reduction in morphotype richness observed during prolonged propagation indicates that ex situ cultivation favoured the most adaptable and competitive taxa, while other members of the native community were not successfully maintained.

The combined use of morphological and molecular tools allowed the identification and preservation of representative native AMF isolates, including both widely distributed generalist taxa and ecologically valuable fungi associated with this protected Mediterranean ecosystem. The native AMF isolates recovered and characterized in this study represent a valuable biological resource that complements previous ecological research conducted in the Selva Pisana Biosphere Reserve. The successful establishment of these isolates in pure culture constitutes the main scientific contribution of the study, because it provides living biological material for future investigations on host compatibility, symbiotic efficiency, functional diversity, environmental adaptation, ecological restoration and sustainable agricultural systems.

Acknowledgements

The authors wish to thank the University of Pisa, Professors Luciano Avio, Alessandra Turrini and Cristiana Sbrana for their scientific support, especially Professor Manuela Govannetti, supervisor of this research, for guidance and continuous monitoring.

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