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# Morphological and Molecular Identification of Culturable Arbuscular Mycorrhizal Fungi (AMF) Associated with *Pternandra azurea* from Martabe Batang Toru Forest, North Sumatra, Indonesia

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## ABSTRACT

Arbuscular Mycorrhizal Fungi (AMF) form a mutualistic symbiosis with almost all host plant species and exist in various ecosystems. Studies of AMF diversity in Sumatra tropical rainforest plants are still limited. This research aimed to isolate and identify AMF associated with *Pternandra azurea* from Martabe Batang Toru forest, North Sumatra. The spores were obtained from the rhizosphere of *P. azurea* and propagated by trap culture using *Pueraria javanica*, *Sorghum vulgare*, and *Zea mays*. AMF spores were isolated using wet sieving and decanting techniques, then inoculated into roots of *P. javanica* grown in sterile zeolite to obtain a single-species culture. The AMF were identified based on spore morphology and molecular analysis using AML1/AML2 specific primers. A total of 13 AMF single-species cultures were obtained, and based on morphological characteristics, they were identified as *Claroideoglomus lamellosum*, *C. claroideum*, *Acaulospora rehmii*, *A. longula*, and *Glomus ambisporum*. Further identification using molecular analysis, the cultures were identified as *C. etunicatum*, *A. spinosa*, *A. longula*, and *G. ambisporum*. Molecular identification resulted in different AMF species from morphological identification. *Claroideoglomus* was dominant AMF observed. All cultures formed internal hyphae, arbuscules, and vesicles within the roots. The AMF cultures obtained can be used as biofertilizers to restore degraded ecosystems.



## 1. Introduction

Arbuscular Mycorrhizal Fungi (AMF) are members of the Glomeromycota phylum forming a mutualistic symbiosis with host plant roots (Rosendahl 2008). The fungi are obligate symbionts of host plants. Around 70-90% of plant species in the world form a mutualistic symbiosis with AMF (Shi *et al*. 2023). AMF has an important role in increasing plant growth and soil productivity. AMF forms a hyphal network system in

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the rhizosphere to extend water and nutrient absorption area by plant roots and explore the surrounding soil's volume (Khaliq *et al*. 2022). AMF can also protect host plant roots from pests and diseases, increase host plant resistance to abiotic stresses such as drought, salinity, and heavy metals as well as improve soil structure and aggregation (Diagne *et al*. 2020). The distribution of AMF in nature varies widely. AMF can be found in various habitats or ecosystems ranging from grasslands (Goldmann *et al*. 2020), agricultural lands (Zhu *et al*. 2020), forests (Shi *et al*. 2019), mountains (Zhang *et al*. 2021), deserts (Vasar *et al*. 2021) to post-mining lands (Tuheteru *et al*. 2022).

The effectiveness of AMF in improving host plant growth is influenced by fungi and host plant species forming associations (Chen *et al*. 2017). Some AMF species have host preferences to form the association or to promote host plant growth and are relatively easy to isolate, whereas some are difficult to isolate, requiring certain specifications to make them culturable or even unculturable. AMF identity could determine the functional symbiosis of the fungi, therefore AMF identification is an important step in obtaining optimum benefits from the fungal application as a biofertilizer. Traditionally, the identification of AMF species relies on spore morphological characteristics. Morphological analysis has limitations due to minimal variation in spore morphotypes between AMF species, and some species tend to produce spores with different morphotypes or dimorphisms (Spruyt *et al*. 2014). Molecular characteristics are more reliable tools for AMF identification. Therefore, combining morphological and molecular tools in AMF spore identification is required.

As technology advances, molecular approaches like Polymerase Chain Reaction (PCR) with primers targeting ribosomal RNA (rRNA) gene sequences have been used to determine AMF species. rRNA region used as a molecular study marker for Glomeromycota is the small subunit (SSU) or 18S, the internal transcribed spacer (ITS) including 5.8S, and the large subunit (LSU) or 28S. Use of three regions, individually or in combination, because the optimal rRNA region to be used as a universal marker for AMF is unknown until now (Delavaux *et al*. 2022). The SSU rRNA gene is often used to identify AMF because it has lower intraspecific variability than ITS and LSU (Kohout *et al*. 2014). A total of 355 species are identified morphologically (http://www.amf-phylogeny.com/), and around 348 to 1600 species are identified molecularly (Ohsowski *et al*. 2014). Molecular analysis will provide more accurate and specific results.

Studies on the diversity of AMF in Indonesian tropical rain forests are still limited, especially from molecular aspects (Maulana *et al*. 2017; Faad *et al*. 2018; Edy *et al*. 2022). Martabe forest is located on the border of Batang Toru forest, rich in biodiversity and a habitat for various plant species (Nugraha *et al*. 2023). One type of plant often found in Martabe Batang Toru forest is *Pternandra azurea*, locally known as “kayu baja” in South Tapanuli. Based on information from local people, the *P. azurea* fruit is one of the important food sources for primates. The *P. azurea* grows naturally in secondary forests and regrows easily after logged for wood construction. Therefore, it contributes to forest regeneration (Amirta *et al*. 2016). The *P. azurea* plays an important role in Batang Toru's primary and secondary forest ecosystems through ecological function, animal sustainability, and human welfare. There is no research on AMF diversity associated with the plant. Therefore, this research aimed to identify culturable AMF associated with *P. azurea* from Martabe Batang Toru forest, North Sumatra, using morphological and molecular approaches. The benefits of this research are the availability of tropical AMF culture collection and their DNA sequence database for fungal identification and provide tropical AMF inoculum to support the reclamation process of degraded land.

## 2. Materials and Methods

### 2.1. Soil Sampling

Rhizosphere soil sampling was conducted in 9 ha out of 84.7 ha selected area of Martabe Batang Toru forest, PT Agincourt Resources Martabe, North Sumatra (1°31' 39.4" N, 99°03' 31.0" E). The soil samples were taken from four sides based on cardinal points of *P. azurea* (minimum height 2 m), with a 0-30 cm depth below the soil surface, and collected from the base of the tree until the edge of a canopy, depending on size of the tree. There were 18 *P. azurea* trees selected. As much as ±250 g of the soil sample was collected from each tree’s cardinal point, then composited into 1 kg. The composite soil samples derived from 18 trees were divided into nine parts and used as replicates. The average AMF root colonization in the field of *P. azurea* was 20%, with arbuscules as the dominant colonization structure.

## 2.2. Trap Culture Development

The development of trap cultures was carried out based on a method from Brundrett *et al*. (1996). As much as 500 g of composite soil samples were mixed with 1 kg of sterile zeolite. The soil sample was planted with *Pueraria javanica*, *Sorghum vulgare*, and *Zea mays* seedlings in a pot by forming three layers: sterile zeolite as the top and bottom layers and composite soil samples as the middle layer. Trap culture plants were maintained in the greenhouse for at least 3 months. Maintenance was done by watering weekly, fertilizing twice a week, and manual pest control. The fertilizer application was 1 g/L containing 25 % nitrogen, 5% phosphoric acid, and 20% potassium (Brundrett *et al*. 1996). The trap cultures were used as spore banks for spore isolation.

## 2.3. Spore Extraction

Extraction of AMF spores used wet sieving and decanting techniques (Brundrett *et al*. 1996). The technique followed a 50 g trap culture medium mixed with 250 ml of distilled water. The mixture was filtered using sieve of 50, 106, 125, 180, and 250 µm. Soil aggregates from each sieve size were washed and then put into a 50 ml centrifuge tube and given distilled water until it formed a suspension. The suspension was centrifuged at 2000 rpm, 27°C, 5 minutes. The supernatant was discarded, and the pellet was added with 25 ml of 50% sugar solution. The suspension was centrifuged at 2000 rpm, 27°C, 2 minutes. The supernatant was filtered using a 50 µm sieve and rinsed with distilled water. The filtered spores were transferred to a petri dish for single-species culture isolation.

## 2.4. Single-Species Culture Isolation

Extracted AMF spores were inoculated into *P. javanica* root system as host. *P. javanica* seedlings having two pairs of leaves grown in 9 cm plastic petri plates containing sterile zeolite media. A healthy single spore from each morphotype or genus of trap cultures was inoculated on the *P. javanica* young root surface and then wrapped in aluminium foil to protect it from sunlight. Isolation for each genus was replicated 20 times. A total of 180 isolation plates were made. Single-species culture plants were maintained in the greenhouse for 3 months to observe root colonization and continue until 8 months to produce a spore bank for DNA analysis. Maintenance was carried out by watering twice a week, fertilizing once every two weeks, and manual pest control. The fertilizer application was 1 g/L containing 25% nitrogen, 5% phosphoric acid, and 20% potassium. Observations of the AMF colonization and sporulation were carried out weekly using a stereo microscope. Each culture was harvested after producing at least 200 spores. The spores were then observed for morphological characteristics and DNA isolation for molecular analysis.

## 2.5. Spore Morphological Identification

Single-species culture spores from each genus replicate were placed on a slide and then given PVLG and Melzer's reagent (Brundrett *et al*. 1996). The slide was covered with a cover glass. Spore identification based on morphological characteristics follows the manual INVAM (https://invam.ku.edu/) and combination with Glomeromycota Species List (http://www.amf-phylogeny.com/). The AMF spore morphological characteristics observed include shape, color, size, ornament, and spore wall under an Olympus CX 33 compound microscope equipped with a Sony Exmor CMOS Sensor camera using 1000× magnification. At least 30 spores were observed for morphological characters of each species.

## 2.6. Spore Molecular Identification

Two hundred spores were collected from each single-species culture plate and placed in a 2 ml centrifuge tube containing 300 µl of sterile distilled water. DNA extraction of AMF spores using Geneaid Genomic DNA Mini Kit (Plant) according to the company's instructions. Spores were crushed with a micropestle. PCR amplification used forward primer AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and reverse primer AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') (Lee *et al*. 2008). Total PCR reaction volume carried out 10 µl consisting of 5 µl master mix, 0.25 µl AML1 and AML2 primers each, 1 µl DNA template, and 3.5 µl $ddH_2O$. The PCR reaction was started with one cycle of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for 1 minute, elongation at 72°C for 2 minutes, and one cycle of final elongation at 72°C for 5 minutes. Amplicons were verified using electrophoresis and then sent to PT Genetika Science Indonesia for sequencing. DNA sequences were identified based on similarity in the MaarjAM database using the Basic Local Alignment Search Tool (BLAST) followed by phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) 11 software.

### 2.7. Root Colonization Analysis

Root colonization analysis was carried out on single-species cultures following the method of Brundrett *et al*. (1996). Root samples from each culture were washed clean and soaked in 10% KOH solution at 90°C for 10 minutes. Roots were rinsed with distilled water and soaked in 1 N HCl solution for 15 minutes. Roots were soaked in 0.05% trypan blue dye solution at 90°C for 10 minutes and stored in 50% glycerol solution. After being stained, roots were cut 1 cm and placed on a glass slide with 50% glycerol solution as mounting. Observation of AMF colonization structures such as arbuscules, vesicles, and internal hyphae was carried out under a compound microscope.

## 3. Results

The results of single spore isolation of AMF associated with *P. azurea* using *P. javanica* as the host were 24 single-species cultures from a total of 180 isolation plates made. All 24 single-species cultures produced AMF colonization characteristics of external mycelium and spores on the roots and rhizosphere at 3 months after inoculation (Figure 1A; 2A; 3A; 4A; 5A). Spore production of the 24 single-species cultures was verified from 49 to 506 at 7-8 months after inoculation. A total of 13 single-species cultures producing around 200 spores were used for molecular identification. The culture numbers are P02C01, P02C03, P02C06, P02C12, P02C18, P02C20, P03C05, P03C13, P04C01, P04C07, P05C02, P08C01, and P08C06. Most single-species cultures of AMF produced high arbuscules structures and few vesicles with imperfect round to oval shapes (Figure 1F; 2F; 3F; 4F; 5F).

### 3.1. Morphological Identification

Based on morphological characteristics, a total of 13 single-species cultures belonging to five morphospecies with three genera were obtained. The fungi are five cultures of *Claroideoglomus lamellosum* (P02C01, P02C03, P02C06, P02C20, P05C02), two cultures of *C. claroideum* (P02C12, P02C18), two cultures of *Acaulospora rehmii* (P03C05, P03C13), two cultures of *A. longula* (P04C01, P04C07), and two cultures of *Glomus ambisporum* (P08C01, P08C06). The detailed spore morphology description of each AMF species is presented in Table 1. Spores of *Claroideoglomus* and *Glomus* are characterized by subtending hyphae (Figure 1E; 2E; 5E), while *Acaulospora* is characterized by sporiferous saccule (Figure 3B; 3C; 3E; 4E).

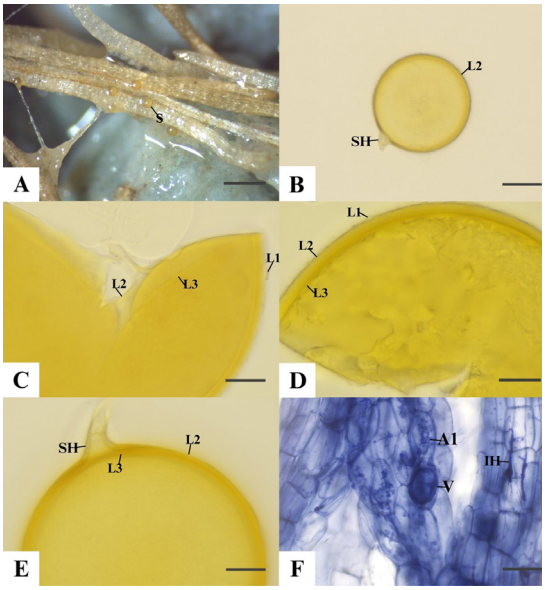


Figure 1. *Claroideoglomus lamellosum.* (A, F) Colonization on the outer and inner of roots, (B) Single spore (S), (C) Spore in PVLG, (D) Spore in PVLG + Melzer's reagent, (E) Subtending hyphae (SH). Spore wall layer (L1–3), Arbuscule (A1), Vesicle (V), Internal hyphae (IH). Scale bars = 750 µm (A), 50 µm (B, F), 20 µm (C–E)

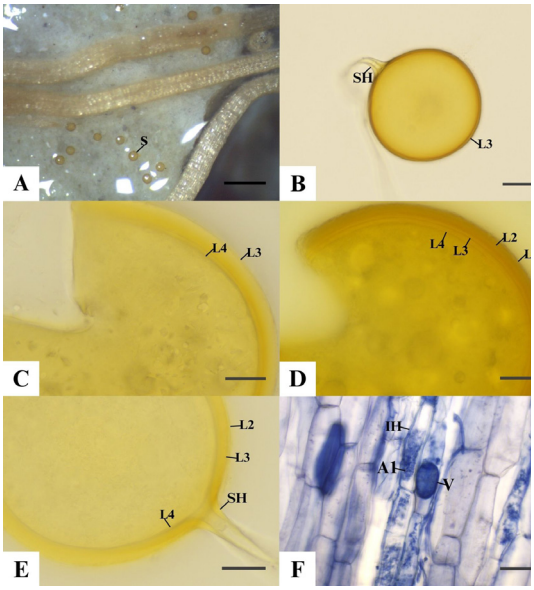


Figure 2. *Claroideoglomus claroideum.* (A, F) Colonization on the outer and inner of roots, (B) Single spore (S), (C) Spore in PVLG, (D) Spore in PVLG + Melzer's reagent, (E) Subtending hyphae (SH). Spore wall layer (L1–4), Arbuscule (A1), Vesicle (V), Internal hyphae (IH). Scale bars = 750 µm (A), 50 µm (B, F), 20 µm (C–E)

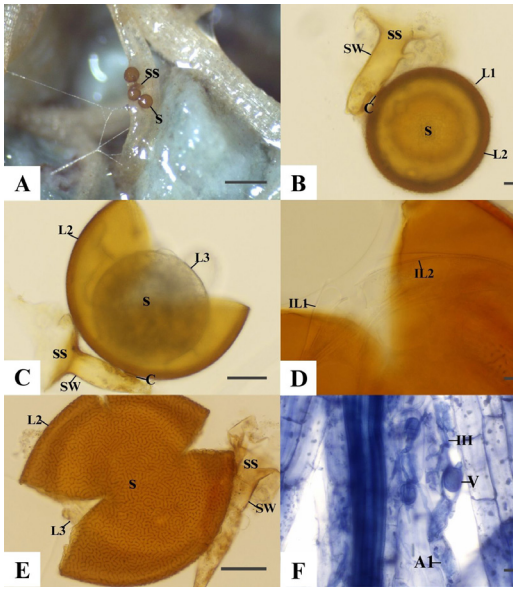


Figure 3. *Acaulospora rehmii.* (A, F) Colonization on the outer and inner of roots, (B) Single spore (S), (C) Spore in PVLG, (D) Spore in PVLG + Melzer's reagent, (E) Labyrinthiform ornamentation. Sporiferous saccule (SS), Spore wall layer (L1–3), Spore wall inner layer (IL1–2), Cicatrix (C), Sporiferous saccule wall (SW), Arbuscule (A1), Vesicle (V), Internal hyphae (IH). Scale bars = 750 µm (A), 50 µm (B–C, E–F), 20 µm (D)

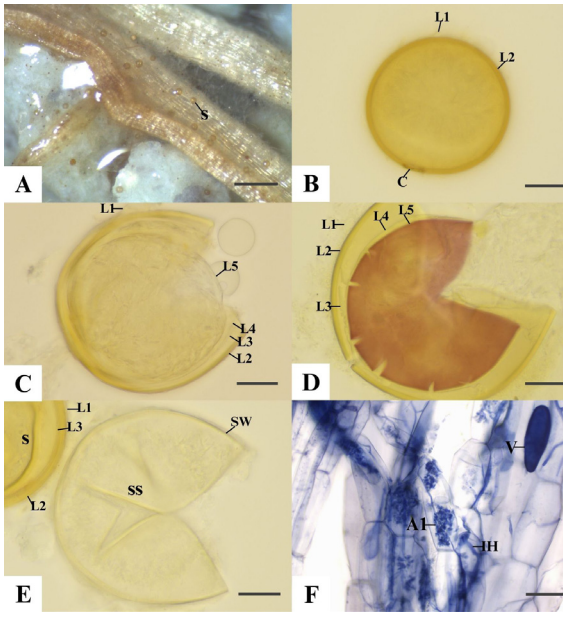


Figure 4. *Acaulospora longula.* (A, F) Colonization on the outer and inner of roots, (B) Single spore (S), (C) Spore in PVLG, (D) Spore in PVLG + Melzer's reagent, (E) Sporiferous saccule (SS). Spore wall layer (L1–5), Cicatrix (C), Sporiferous saccule wall (SW), Arbuscule (A1), Vesicle (V), Internal hyphae (IH). Scale bars = 750 µm (A), 50 µm (F), 20 µm (B–E)

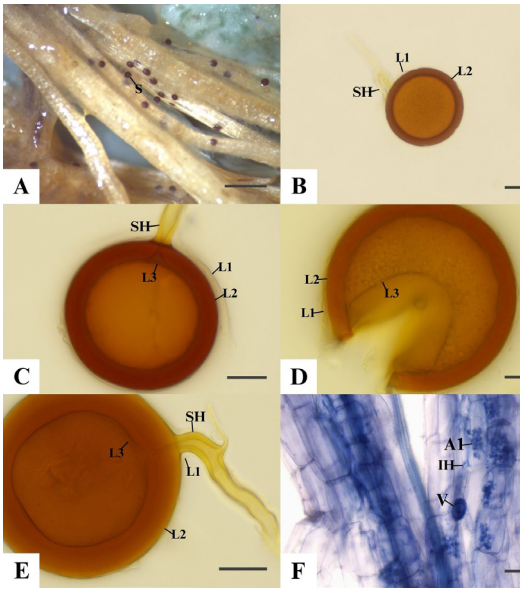


Figure 5. *Glomus ambisporum.* (A, F) Colonization on the outer and inner of roots, (B) Single spore (S), (C) Spore in PVLG, (D) Spore in PVLG + Melzer's reagent, (E) Subtending hyphae (SH). Spore wall layer (L1–3), Arbuscule (A1), Vesicle (V), Internal hyphae (IH). Scale bars = 750 µm (A), 50 µm (B, F), 20 µm (C–E)

## 3.2. Molecular Identification

Successful confirmation of SSU rRNA gene amplification from each AMF culture was carried out using electrophoresis gel. The electrophoresis result of PCR products showed that all single-species cultures had DNA bands measuring around 800 bp (Figure 6). Identification of AMF species begins with homology analysis between obtained DNA sequences and reference sequences in the MaarjAM database. The results of homology analysis show that all single-species culture sequences had similarity values ranging from 98-99% (Table 2). Three sequences (P02C01, P02C03, P02C06) had similarity with *C. lamellosum*, four sequences (P02C12, P02C18, P02C20, P05C02) had similarity with two species *e.g.*, *C. lamellosum* and *C. claroideum*, two sequences (P03C05, P03C13) had similarity with *A. spinosa*, two sequences (P04C01, P04C07) had similarity with *A. longula*, and two sequences (P08C01, P08C06) were identified as *Glomus* sp. at genus level. Furthermore, molecular analysis of the SSU rRNA sequences using a phylogenetic tree resulted in four species. They were *C. etunicatum*, *A. spinosa*, *A. longula*, and *G. ambisporum* (Figure 7). Two morphospecies, *C. lamellosum* and *C. claroideum* were identified as *C. etunicatum* based on molecular phylogenetic tree. Furthermore, molecular analysis resulted in morphospecies of *A. rehmii*, which

Table 1. Spore morphological characteristics of each AMF species

| Culture number | Species | Description |
|---|---|---|
| P02C01<br>P02C03<br>P02C06<br>P02C20<br>P05C02 | *Claroideoglomus lamellosum* | Spores pale yellow to light yellow, globose to subglobose, 93.46–127.10 x 127.98–151.94 µm diameter. Spore wall 5.31–13.63 µm thick with three layers. Layer 1 tends to degrade on the surface and appears flaky, hyaline, 1.11–4.38 µm thick. Layer 2 laminate, pale yellow to yellow, 3.16–13.41 µm thick. Layer 3 sometimes adherent and difficult to distinguish from layer 2, membranous, hyaline, 0.50–1.11 µm thick. Subtending hyphae hyaline to pale yellow, cylindrical to slightly flared or curved, 5.97–15.69 µm diameter, 1.41–3.50 µm thick. Arbuscular-vesicular root colonization type |
| P02C12<br>P02C18 | *Claroideoglomus claroideum* | Spores yellow to light yellow, globose to subglobose, 103.24–121.22 x 121.93–153.69 µm diameter. Spore wall 8.64–14.80 µm thick with four layers. Layers 1 and 2 usually present in young spores. Layer 1 difficult to distinguish from layer 2, mucilagenous, ephemeral, hyaline to yellow, 1.46–3.13 µm thick. Layer 2 ephemeral, hyaline to yellow, 2.27–6.77 µm thick. Layer 3 laminate, smooth, pale yellow to yellow, 2.92–8.91 µm thick. Layer 4 membranous, pale yellow to yellow, 0.67–1.29 µm thick. Subtending hyphae hyaline to pale yellow, cylindrical to slightly flared, 4.54–20.30 µm diameter, 1.30–6.59 µm thick. Arbuscular-vesicular root colonization type |
| P03C05<br>P03C13 | *Acaulospora rehmii* | Spores yellowish brown to dark brown, globose to subglobose, 117.95–171.18 x 179.74–184.05 µm diameter. Spore wall 9.49–15.29 µm thick with three layers. Layer 1 connected to the neck wall of sporiferous saccule, ephemeral, usually absent in mature spores or only fragments, hyaline, 1.32–5.66 µm thick. Layer 2 laminate, yellow to yellowish brown, 3.13–5.11 µm thick, surface forms a labyrinthian pattern 1.25–4.42 µm wide. Layer 3 membranous, hyaline, 0.35–0.50 µm thick, inner consists of two layers with layer 1 sometimes difficult to distinguish from layer 3 spore wall, hyaline, 0.41–1.16 µm thick and layer 2 hyaline, 0.46–1.07 µm thick. Cicatrix circular to ovoid, 14.25–17.09 µm diameter. Sporiferous saccule hyaline to pale yellow, globose to subglobose, neck 33.02–37.64 µm diameter. Sporiferous saccule wall 1.51–1.75 µm thick with single layer. Sporiferous saccule will be released after spores mature or remaining sporiferous saccule neck. Arbuscular-vesicular root colonization type |
| P04C01<br>P04C07 | *Acaulospora longula* | Spores subhyaline to pale yellow, globose to subglobose, sometimes ellipsoid, 62.45–74.06 x 74.90–79.69 µm diameter. Spore wall 2.66–4.07 µm thick with five layers. Layer 1 connected to the neck wall of sporiferous saccule, mucilagenous, ephemeral, hyaline, 3.16–3.54 µm thick. Layer 2 inseparable from layer 3, laminate, smooth, pale yellow, 2.76–3.83 µm thick. Layer 3 hyaline, 0.50 µm thick. Layer 4 usually attached to layer 5, hyaline, 0.52–0.74 µm thick. Layer 5 membranous, hyaline, turning light purple in Melzer's reagent, 0.50–0.79 µm thick. Cicatrix circular to oval, 7.10–10.70 µm diameter. Sporiferous saccule hyaline, globose to subglobose, 70.80–73.10 µm diameter. Sporiferous saccule wall 1.69–2.32 µm thick with single layer. Arbuscular-vesicular root colonization type |
| P08C01<br>P08C06 | *Glomus ambisporum* | Spores brown to dark brown, globose to subglobose, 70.76–82.11 x 82.88–105.92 µm diameter. Spore wall 8.85–11.44 µm thick with three layers. Layer 1 reticulate, ephemeral, subhyaline to hyaline, 0.80–5.68 µm thick. Layer 2 confluent with hyphal attachment, laminate, brown to dark brown, 7.24–11.69 µm thick. Layer 3 separated from subtending hyphae, membranous, hyaline, 0.50–1.51 µm thick. Subtending hyphae hyaline to pale yellow, cylindrical, 5.66–8.90 µm diameter, 1.08–2.51 µm thick. Arbuscular-vesicular root colonization type |

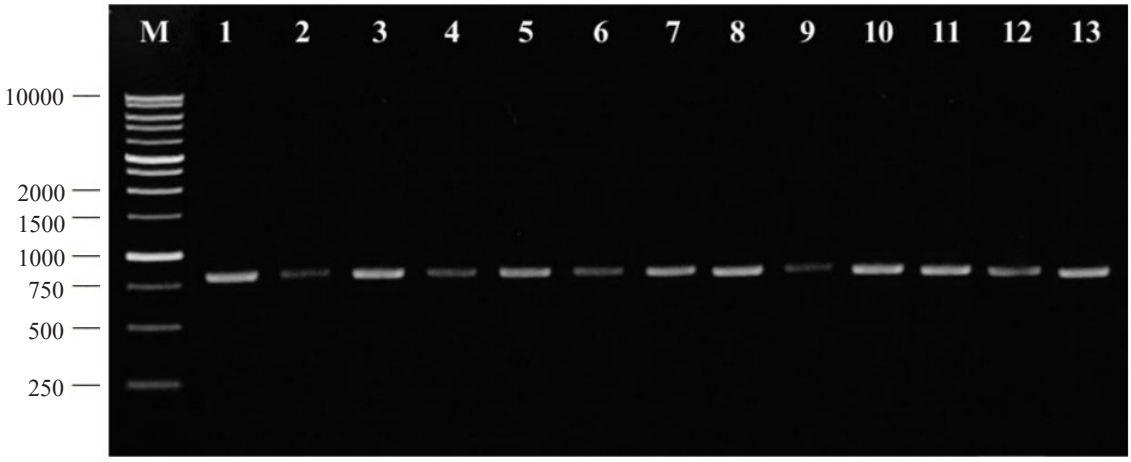


Figure 6. PCR product electrophoresis results of AMF single-species culture (~800 bp). (1) P02C01, (2) P02C03, (3) P02C06, (4) P02C12, (5) P02C18, (6) P02C20, (7) P03C05, (8) P03C13, (9) P04C01, (10) P04C07, (11) P05C02, (12) P08C01, (13) P08C06. (M) Markers (1 kb ladder)

Table 2. Homology analysis results of AMF single-species culture

| Culture number | Species homology | Accession | Query coverage (%) | Percent identity (%) |
|---|---|---|---|---|
| P02C01 | *Claroideoglomus lamellosum* | FR750221.1 | 100.0 | 99.87 |
| P02C03 | *Claroideoglomus lamellosum* | FR750221.1 | 99.5 | 99.87 |
| P02C06 | *Claroideoglomus lamellosum* | FR750221.1 | 99.0 | 99.87 |
| P02C12 | *Claroideoglomus lamellosum* | FR750221.1 | 100.0 | 99.86 |
| | *Claroideoglomus claroideum* | AJ276080.2 | 100.0 | 99.86 |
| P02C18 | *Claroideoglomus lamellosum* | FR750221.1 | 98.9 | 99.87 |
| | *Claroideoglomus claroideum* | AJ276080.2 | 98.9 | 99.87 |
| P02C20 | *Claroideoglomus lamellosum* | FR750221.1 | 100.0 | 99.87 |
| | *Claroideoglomus claroideum* | AJ276080.2 | 100.0 | 99.87 |
| P03C05 | *Acaulospora spinosa* | JX461239.1 | 99.1 | 99.33 |
| P03C13 | *Acaulospora spinosa* | JX461239.1 | 99.1 | 99.33 |
| P04C01 | *Acaulospora longula* | AJ306439.1 | 100.0 | 98.64 |
| P04C07 | *Acaulospora longula* | AJ306439.1 | 100.0 | 98.76 |
| P05C02 | *Claroideoglomus lamellosum* | FR750221.1 | 100.0 | 99.86 |
| | *Claroideoglomus claroideum* | AJ276080.2 | 100.0 | 99.86 |
| P08C01 | *Glomus* sp. | HF559328.1 | 99.9 | 98.52 |
| P08C06 | *Glomus* sp. | HF559328.1 | 100.0 | 98.50 |

was identified as *A. spinosa*. *A. longula*, on the other hand, is consistent with the result of morphological identification. Morphospecies of *G. ambisporum* is also consistent identified as *G. ambisporum* based on molecular phylogenetic tree. Homology analysis was not able to identify *G. ambisporum* at the species level (Table 2). However, molecular phylogenetic analysis was able to identify into species level even though the bootstrap value was 51% (Figure 7).

## 4. Discussion

This is the first report on culturable AMF derived from *P. azurea* as one of the important host plants grown in Martabe Batang Toru tropical rain forests. A total of seven AMF species were obtained, five species based on morphological characteristics, namely *C. lamellosum*, *C. claroideum*, *A. rehmii*, *A. longula*, *G. ambisporum*, and four species were based on molecular analysis, namely *C. etunicatum*, *A. spinosa*, *A. longula*, *G. ambisporum*. *P. javanica* was selected as the host plant because it is a responsive plant to AMF colonization, has dense fine roots, is a fast-growing plant, and is tolerant to drought stress (Lapanjang *et al*. 2023). Therefore, most of the AMF associated with *P. azurea* are able to colonize the *P. javanica*.

Spores are reproductive organs of AMF. AMF can only reproduce asexually with spore formation (Corradi and Brachmann 2017). Knowing whether AMF species are culturable or unculturable can be carried out by

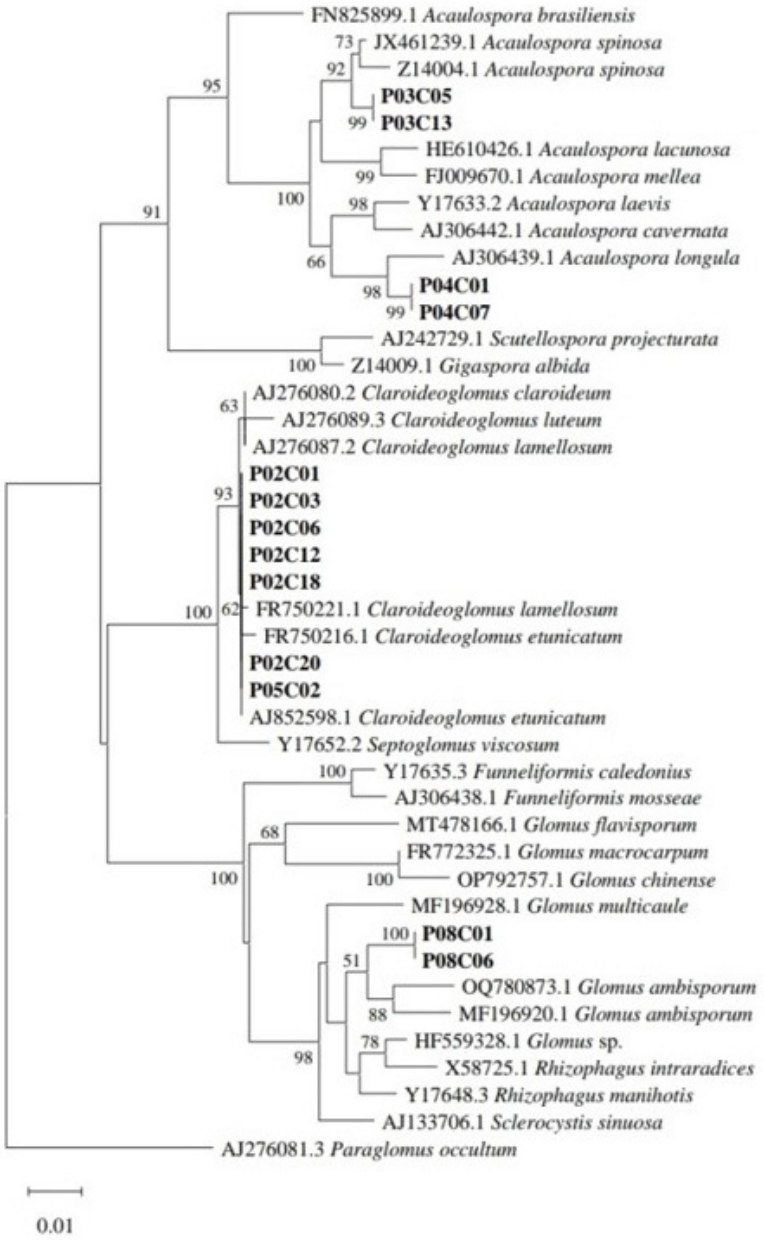


Figure 7. Phylogenetic tree of AMF single-species culture constructed using Neighbor-Joining method with 1000× bootstrap value

propagation through single spore isolation. A total of 24 single-species cultures were successfully obtained from 180 isolation plates indicated that AMF propagation using the single-spore technique had a relatively low success rate. Spore quality, such as spore viability and maturity, are important aspects determining the success of making AMF single-species culture (Paré *et al*. 2022). In addition, other factors contributing to spore germination are the suitability of AMF species in new environments, host plant type, and soil microbes (Berruti *et al*. 2016). The natural AMF community generally consists mainly of unculturable species. This is influenced by their native habitat and the wild plant species occupied in the natural forest (Ohsowski *et al*. 2014).

Morphologically, *Claroideoglomus* and *Glomus* have a glomoid spore morphotype. The spores are formed from the development of hyphae tips (subtending hyphae) called chlamydospores (Crişan *et al*. 2019). *Acaulospora* has an acaulosporoid spore morphotype. The spores are produced on the outside of sporiferous saccule's neck (Souza 2015). Molecular identification resulted in different AMF species compared to morphological identification, except *A. longula* and *G. ambisporum*. This study used AMF-specific primers AML1/AML2

from SSU rRNA region measuring approximately 800 bp (Lee *et al*. 2008). The results of the homology analysis showed that the four cultures were similar to two species, and two cultures were only known at the genus level (Table 2). AML primers (small amplicon size) targeting the SSU rRNA region provide relatively low resolution in accuratey determining species levels (Spruyt *et al*. 2014). Therefore, molecular identification of AMF single-species cultures was based on constructing a phylogenetic tree. Utilization of the SSU region is supported by the AMF database availability well-managed to form a phylogenetic tree for clade determination (Davison *et al*. 2015; Stefani *et al*. 2020).

*C. lamellosum*, *C. claroideum*, and *C. etunicatum* have similar spore colors that are difficult to distinguish. Therefore, the determination of *Claroideoglomus* species in this study also used other additional morphological characters such as the spore ornamentation and wall structure. The layer number of spore walls of *C. lamellosum*, *C. claroideum*, and *C. etunicatum* are 3, 4, and 2 layers (Becker and Gerdemann 1977). Although the most similar sequences with P02 and P05 are *C. lamellosum* or *C. claroideum*, the cultures were placed in phylogram as *C. etunicatum* because there is only a difference of 1-2 base pairs. *A. rehmii* has a labyrinthiform ornament on the spore wall, distinguishing it from other *Acaulospora* species (Sieverding and Toro 1987). The unavailability of *A. rehmii* genome information in the world database makes P03C05 and P03C13 cultures classified into *A. spinosa* with similarities in the AML region. DNA sequence differences may be present in other genes, so primer selection is important because each rRNA region has varying abilities in distinguishing AMF species (Ilkhan *et al*. 2021). Further research can be conducted to analyze using a primer with targets covering three rRNA gene regions (SSU, ITS, and LSU). Rosas-Moreno *et al*. (2023) and Aguilar-Paredes *et al*. (2024) used nested PCR with primer pairs SSUmAf/LSUmAr and SSUmCf/LSUmBr to identify several members of *Acaulospora* and *Claroideoglomus* genera. These primers consist of SSU and LSU partial regions and the entire ITS region to produce long sequences with higher AMF species coverage (Krüger *et al*. 2009).

All single-species cultures colonized *P. javanica* roots by forming internal hyphae, arbuscules, and vesicles. Colonization of AMF on plant roots begins with a pre-infection stage, where spores influenced by environmental factors will germinate to form an appressorium (Souza 2015). The infection begins when appressorium penetrates roots and forms structures like internal hyphae, arbuscules, and vesicles. Internal hyphae function as nutrient transfer organs from external hyphae into the host plant. Arbuscules provide nutrient exchange between AMF and host plants, while vesicles serve as fungal food reserve storage (Giovannini *et al*. 2020). After the infection stage, hyphae grow widely outside roots (external hyphae), responsible for transporting water and nutrients from the soil to the host plant's roots.

*Glomus* and *Acaulospora* dominate AMF single-species cultures. *Claroideoglomus* is a synonym of *Glomus*. Schüßler and Walker (2010) revised Glomeromycota taxonomy based on molecular phylogenetic analysis of the SSU rRNA gene, where several *Glomus* species were reclassified as *Claroideoglomus*. *Glomus* (syn. *Claroideoglomus*) and *Acaulospora* have high adaptability to various environmental conditions and host plant species (Tapwal *et al*. 2023). The presence of these genera in AMF single-species culture may be due to shorter spore production times than other genera, such as *Gigaspora* and *Scutellospora* (Ogoma *et al*. 2021). The success in creating single-species cultures that have been characterized adds to the availability of indigenous Indonesia AMF cultures from tropical rainforest plants that can be used to develop biofertilizers for agriculture, forestry, and ecosystem restoration.

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