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Molecular identification of indigenous arbuscular mycorrhizal fungi in the rhizosphere of black pepper (*Piper nigrum*) in West Lampung, Indonesia

RAHMAT HIDAYAT, MARIA VIVA RINI♥, RUSDI EVIZAL, AGUS KARYANTO,
PAUL BENYAMIN TIMOTIWU, SALWA AZZAHRA
Department of Agronomy and Horticulture, Faculty of Agriculture, Universitas Lampung. Jl. Prof. Dr. Ir. Soemantri Brojonegoro No. 1, Bandar Lampung 35141, Lampung, Indonesia. Tel./fax.: +62-721-781822, ♥email: maria.vivarini@fp.unila.ac.id

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Abstract. *Hidayat R, Rini MV, Evizal R, Karyanto A, Timotiwu PB, Azzahra S. 2026. Molecular identification of indigenous arbuscular mycorrhizal fungi in the rhizosphere of black pepper (*Piper nigrum*) in West Lampung, Indonesia. Biodiversitas 27 (5): d270526. https://doi.org/10.13057/biodiv/d270526.* Black pepper (*Piper nigrum*) serves as a vital economic spice commodity in Indonesia, yet its agricultural productivity is frequently constrained by suboptimal soil conditions, particularly in acidic marginal drylands. The use of indigenous Arbuscular Mycorrhizal Fungi (AMF) offers a sustainable, environmentally friendly strategy to mitigate these abiotic stresses. However, the specific diversity of AMF communities in the black pepper plantations of West Lampung remains largely underexplored. Therefore, this study aimed to conduct a species-level molecular identification of indigenous AMF isolated from the black pepper rhizosphere in this region. Five distinct spore isolates were successfully recovered using a trap culture method and were initially characterized based on morphological attributes. For accurate taxonomic resolution, molecular analysis was performed using nested Polymerase Chain Reaction (PCR) targeting the 18S Small Subunit (SSU) rRNA gene with AML1/AML2 primers. This was followed by DNA sequencing and phylogenetic reconstruction using the Neighbor-Joining method. The phylogenetic analysis confirmed the presence of four distinct evolutionary lineages: *Ambispora leptoticha* (SP1), *Acaulospora* cf. *spinosa* (SP2), *Acaulospora colombiana* (SP4), and unclassified *Glomeromycotina* sp. (SP3 and SP5). Interestingly, the typically ubiquitous genus *Glomus* was entirely absent from the analyzed isolates, while the sampled soil exhibited extreme acidity (pH 4.13) and unexpectedly high available phosphorus (77.17 ppm). This study provides a crucial molecular baseline for indigenous AMF, confirms the first molecular record of the *Ambispora* lineage, and supports the future development of site-specific bioinoculants tailored for acidic soils.

Keywords: Acidic soils, *Ambispora leptoticha*, arbuscular mycorrhizal fungi, black pepper, West Lampung

INTRODUCTION

Black pepper (*Piper nigrum* L.), globally known as the "king of spices," is a vital commodity for the economy of tropical regions, particularly Indonesia. As a major producer, Indonesia relies heavily on Lampung Province as a main production center (Direktorat Jenderal Perkebunan 2025). However, black pepper productivity is frequently constrained by suboptimal edaphic conditions inherent to tropical marginal drylands (Zu et al. 2014). In West Lampung, pepper is predominantly cultivated on marginal lands dominated by ultisols and inceptisols (Addharu et al. 2022). These soils are typically acidic (low pH), have toxic levels of aluminum saturation, and exhibit strong phosphorus (P) fixation, rendering essential nutrients unavailable even with fertilizer application (Zu et al. 2014; Varghese and Ray 2024; Van et al. 2025). To address these challenges sustainably and reduce dependency on agrochemical inputs, the utilization of Arbuscular Mycorrhizal Fungi (AMF) has emerged as a crucial biological strategy (Wahab et al. 2023).

AMF establish symbiotic associations with over 80% of terrestrial plants, including black pepper (Bilgili 2025). In this symbiosis, AMF function as extensions of the root, significantly enhancing the uptake of immobile nutrients, particularly fixed P in acidic soils (Sahur et al. 2020). Through an extensive extraradical hyphal network, AMF increase the absorption surface area and secrete phosphatases to solubilize bound phosphorus (Battini et al. 2017; Ferrol et al. 2019; Gill et al. 2025). Beyond nutrient acquisition, AMF modulate plant physiological resilience. Specific strains induce systemic resistance against foot rot caused by *Phytophthora capsici* (Cheng et al. 2021; Sarathambal et al. 2023) and mitigate abiotic stresses, such as drought (Wahab et al. 2023). However, the efficacy of these benefits depends on the compatibility between the host genotype and specific AMF species (Enebe and Erasmus 2023). Although commercial biofertilizers exist, exotic AMF strains may fail to persist under extreme edaphic conditions, such as the highly acidic soils in West Lampung (Salomon et al. 2022; Boussageon et al. 2025). Indigenous AMF populations, conversely, have co-evolved with local edaphic factors, developing adaptive traits that enable them to function optimally under local stress conditions (Delavaux and Bever 2022). Thus, utilizing locally adapted indigenous AMF consortia is strategically more valuable than relying on generic inoculants.

This host-symbiont specificity underscores the critical need for precise taxonomic identification, as different isolates within the same genus can elicit significantly different growth responses. For instance, Rini et al. (2017) reported that a specific *Glomus* sp. isolate promoted superior growth in oil palm (*Elaeis guineensis* Jacq.) compared to another isolate. Consequently, applying unidentified or mismatched AMF consortia may result in suboptimal agronomic outcomes. Historically, AMF communities in black pepper rhizospheres were characterized primarily through morphological spore identification (Susila et al. 2022). While cost-effective, morphological methods are prone to ambiguity due to homoplasy, phenotypic variations, and "cryptic" species that possess similar spores but distinct genetic makeups (Crossay et al. 2017). Furthermore, degraded field-collected spores often lack diagnostic features, making reliable identification impossible (Crossay et al. 2017; Maússe-Sitoe and Dames 2024). Relying solely on morphology limits identification to the genus level and fails to distinguish closely related species, necessitating the use of molecular markers for accurate taxonomic resolution (Rini et al. 2021; Susila et al. 2022). The Small Subunit (SSU) 18S rRNA gene is preferred, offering a stable phylogenetic backbone for *Glomeromycota* and overcoming high intragenomic polymorphism issues encountered with Internal Transcribed Spacer (ITS) markers (Thiéry et al. 2016).

In Indonesia, particularly in West Lampung, information regarding indigenous AMF biodiversity remains fragmentary. While recent studies reported the presence of genera such as *Acaulospora*, *Glomus*, *Gigaspora* (Akib et al. 2022), *Scutellospora*, and *Entrophospora* (Hajoeningtijas et al. 2024) in black pepper rhizospheres, these relied largely on morphology. To date, there is a scarcity of data providing species-level identification of indigenous AMF from this region verified through molecular sequencing. This knowledge gap hinders the development of targeted, site-specific biofertilizers adapted to local acidic soil conditions. Therefore, this study aimed to molecularly identify indigenous AMF species from the black pepper rhizosphere in Gunung Terang, West Lampung, using nested PCR targeting the 18S rRNA gene, and to provide a validated inventory that can underpin the development of site-specific bioinoculants.

MATERIALS AND METHODS

Study site and sample collection

This study utilized five AMF isolates (SP1 to SP5). The spores were originally isolated from the rhizosphere of black pepper (*Piper nigrum* L. cv. Jambi) grown in Gunung Terang Village, Air Hitam Sub-district, West Lampung District, Indonesia (5°06'21"S 104°26'28"E) (Figure 1). To accurately represent the study site, a composite soil sample was prepared by pooling seven random subsamples collected from the rhizosphere of seven, healthy black pepper plants. These plants were spaced at least 10 m apart to account for spatial variability within the plantation. From each selected plant, approximately 500 g of soil was collected at a depth of 0-20 cm after removing the surface litter. The soil characteristics from the sampling site are presented in Table 1, representing a single measurement from this composite sample. Soil pH was measured electrometrically (1:2.5 H_2O). Organic carbon was analyzed using the Walkley and Black method, total nitrogen by the Kjeldahl method, available phosphorus and exchangeable potassium by the Bray I method and flame photometry, and soil texture was determined using the Bouyoucos hydrometer method.

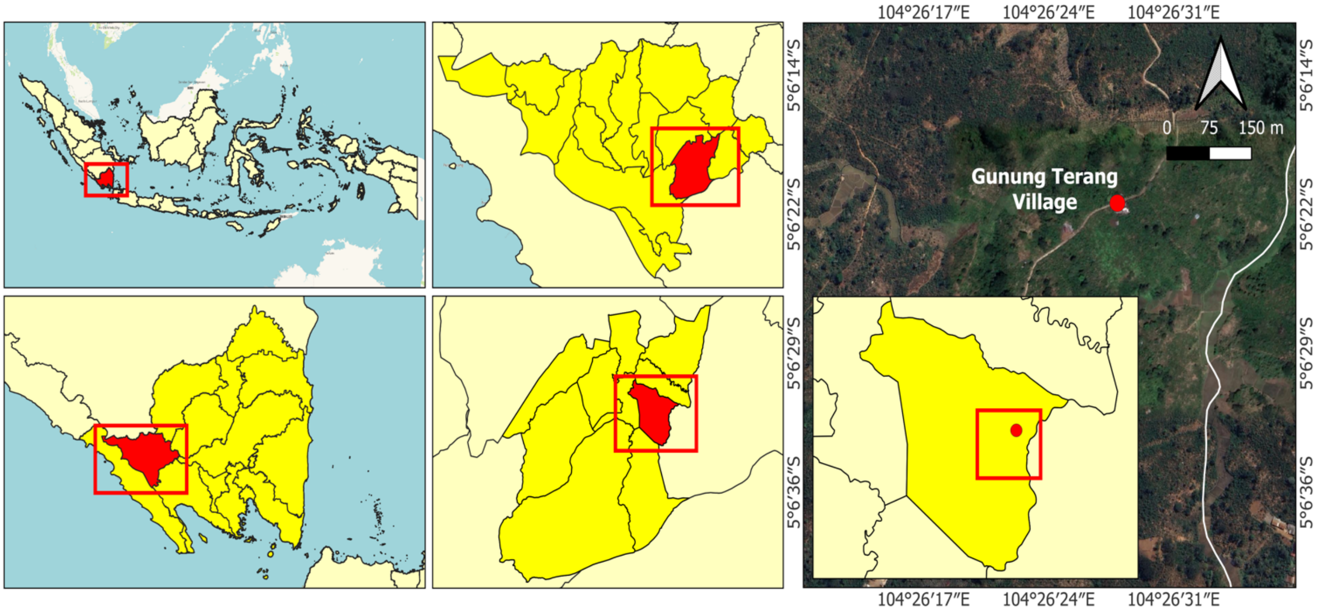

Figure 1. Map of sampling site in Gunung Terang Village, Air Hitam Sub-district, West Lampung District, Indonesia

Table 1. The soil characteristics

Variable	**Value**
pH	4.13
Total N (%)	0.308
Exchangeable K (cmol(+) kg^{-1})	0.66
Available P (ppm P_2O_5)	77.17
Organic carbon (%)	2.83
Soil texture (clay loam)	
Clay (%)	31.60
Silt (%)	32.00
Sand (%)	36.40

Spore propagation via trap culture

To ensure isolate purity and viability, spores were not extracted directly from the field soil but were first propagated using a trap culture method (Azzahra et al. 2024). The planting medium consisted of a 2:1 (v/v) mixture of river sand and zeolite. The sand was sterilized twice at 121°C (1 atm) for 60 min and washed with running water, while the zeolite was thoroughly washed prior to mixing. The homogenized media (~700 g) were placed into polybags measuring 14 × 20 cm. The composite soil sample (~250 g) from the black pepper rhizosphere served as the inoculum and was placed inside the polybags. Maize (*Zea mays* L.) was selected as the host plant. Before planting, the maize seeds were surface-sterilized with a 10% Clorox solution for 15 min and pre-germinated on moistened straw paper at room temperature for three days. Five seedlings were planted in each polybag on top of the soil inoculum, and the polybags were subsequently covered with the sterile sand-zeolite mixture.

The trap cultures were maintained in a greenhouse for 3 months. Plant maintenance included daily watering, manual weeding, and flower pruning. Fertilization was strictly controlled: Urea fertilizer was applied twice a week from week 2 to week 6 at a concentration of 2 g/L (20 mL per polybag), while NPK fertilizer was applied once at week 6 at a dose of 0.3 g per polybag. Crucially, to stimulate sporulation, watering was discontinued during the final two weeks of the culture period to induce drought stress. Following the harvest, the polybags were cut, and the lower section of the planting media (~700 g of the sand-zeolite mixture) was processed for spore isolation.

Isolation and morphological identification

Morphological identification of the AMF was performed by isolating spores from the lower section of the trap culture media using the wet sieving and decanting method (Pacioni 1992) with various sieve sizes (45 µm, 90 µm, 150 µm, 250 µm, and 500 µm). The spores were transferred to Petri dishes, manually counted, and observed under stereo- and compound microscopes (at 100× to 400× magnification). The spores were initially characterized by observing their color (based on the Cyan, Yellow, Magenta/CYM color chart), size, shape, the presence or absence of spore ornaments such as a bulbous suspensor, sporiferous saccule, cicatrix, and their reaction to Melzer's solution.

From each of the five identified AMF isolate types (SP1-SP5), a pool of approximately 10 healthy spores was isolated and surface-cleaned with sterile water. From this pool, a single, intact spore free from soil debris was rigorously selected for molecular analysis to ensure specific DNA amplification. The remaining spores from the same pool were mounted on glass slides with Melzer's reagent and gently pressed for detailed morphological verification under a compound microscope. The morphological assignments were cross-referenced with the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, Kansas, USA) online database alongside specific monographs (Schenck and Perez-Collins 1990; Brundrett et al. 1995).

DNA extraction

DNA extraction was performed using the InstaGene Matrix commercial kit (Bio-Rad Laboratories, Hercules, CA, USA). The single selected spore from each AMF isolate type was placed in a sterile 0.2 mL microtube. Prior to transfer, each spore was carefully rinsed with sterile water to remove any adhering soil particles and potential PCR inhibitors. Subsequently, 20 µL of InstaGene Matrix solution was added to each tube. The spore was crushed using a sterile pipette tip. Incubation was performed in a thermal cycler (SensoQuest GmbH, Göttingen, Germany), starting at 56°C for 30 min, followed by 95°C for 10 min. The samples were then briefly centrifuged at high speed. A clear supernatant of 15 µL was transferred to a new microtube to serve as the DNA template (Schwarzott and Schüßler 2001; Redecker 2020). To monitor potential contamination, an extraction blank containing only the InstaGene Matrix solution without a spore was processed simultaneously. Due to the extremely low DNA yield from single-spore extraction, DNA quality was verified by successful amplification of the target gene rather than by spectrophotometric quantification.

Polymerase Chain Reaction (PCR)

PCR was performed on a SensoQuest thermal cycler to amplify a partial fragment of the 18S rRNA gene by nested PCR (Lee et al. 2008). The first stage utilized the primer pair NS1 (5'-`GTAGTCATATGCTTGTCTC`-3') and NS4 (5'-`CTTCCGTCAATTCCTTTAAG`-3') (White et al. 1990). The reaction mixture (25 µL total volume) consisted of 9 µL sterile water, 12.5 µL 2× MyTaq HS RedMix (Bioline, Meridian Bioscience), 1.25 µL each of the NS1 and NS4 primers (10 µM), and 1 µL of the DNA template. A PCR negative control (sterile water) was included in every run. PCR amplification for each isolate was performed in technical duplicates. The extraction blank and PCR negative controls consistently show no bands on the gel, confirming the absence of contamination. Furthermore, both technical duplicates yielded consistent amplification results. The thermal cycling conditions were an initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C (1 min), annealing at 56°C (1 min), and extension at 72°C (1 min), with a final extension at 72°C for 5 min. The PCR product from the first stage served as the template for the second stage (nested PCR) using the primers AML1 (5'-`ATCAACTTTCGATGGTAGGATAGA`-

3') and AML2 (5'-`GAACCCAAACACTTTGGTTTCC`-3') (Lee et al. 2008). The conditions for the nested PCR were identical to the first stage, except that the annealing temperature was increased to 58°C.

Electrophoresis

The PCR products were verified via electrophoresis on a 0.8% agarose gel stained with 1 µL Ethidium Bromide (EtBr). A total of 3 µL of the PCR product was mixed with 1 µL 6X DNA Loading Dye (Thermo Scientific, USA) and loaded into the wells. A 100 bp DNA ladder (Thermo Scientific, USA) was used as a molecular size marker. Electrophoresis was conducted at a constant voltage of 55 V for 60 min to minimize heat generation and ensure high-resolution separation of the ~800 bp bands. The DNA bands were visualized using a Gel Documentation System under UV light. Successfully amplified products were purified and submitted for sequencing.

Sequencing and phylogenetic analysis

Purified PCR products were sequenced by a commercial service provider (1st BASE, Malaysia). Raw chromatograms were inspected using CodonCode Aligner; low-quality ends were trimmed, and ambiguous bases were manually corrected. The cleaned sequences were aligned using the Clustal W algorithm in MEGA X software. Species identification was initially performed using the Basic Local Alignment Search Tool (BLAST) against the NCBI database to assess similarity to reference sequences. Non-target sequences (e.g., plants or non-Glomeromycota fungi) were excluded.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The Neighbor-Joining method was selected for its computational efficiency in providing a robust initial phylogenetic backbone for SSU rRNA sequences, which is a standard approach for preliminary AMF taxonomic placement. To ensure the reliability of the branching patterns, the analysis was performed with 1,000 bootstrap replicates and the Tamura 3-parameter model with a Gamma distribution (+G), which effectively accounts for nucleotide substitution biases and rate variation among sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-Parameter method (Tamura 1992) with a discrete Gamma distribution (+G) and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences together with reference sequences. All positions containing gaps and missing data were eliminated (Partial Deletion option). There was a total of 678 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The partial SSU rRNA gene sequences of the indigenous AMF isolates generated in this study have been officially deposited in the NCBI GenBank database under the accession numbers PZ225325, PZ225326, PZ225327, PZ225328, and PZ225329.

Statistical and analytical methods

No additional formal statistical analyses (such as diversity metrics or root colonization percentages) were performed, as the primary objective of this study was restricted to the sequence-based taxonomic identification of the five targeted AMF isolates.

RESULTS AND DISCUSSION

Results of morphological identification of AMF spores

Morphological identification presented challenges due to the lack of intact diagnostic features in many field-collected spores. However, careful observation revealed distinct variations among the five isolates recovered from the black pepper rhizosphere. Detailed morphological characteristics are summarized in Table 2, and individual spore visualizations are presented in Figures 2-6.

Morphologically, isolate SP2 (Figure 3) was the only isolate that could be confidently assigned to the genus *Acaulospora* based on the presence of a distinct sporiferous saccule and spine-like ornamentation on the spore wall. Isolate SP1 (Figure 2) exhibited a pedicel attachment; however, the structure appeared truncated, likely representing a remnant separated from a saccule, which prevented definitive genus-level assignment based solely on morphology. Meanwhile, isolates SP3, SP4, and SP5 (Figures 4, 5, and 6) lacked distinctive genus-specific features such as auxiliary cells or germination shields that were observable under the current conditions.

The application of Melzer's reagent revealed a positive reaction in all isolates, providing crucial initial clues for their taxonomic placement. For instance, the rust-brown staining in SP1 and the strong dextrinoid reaction in SP4 were key distinguishing features. Furthermore, the specific CYM color coordinates (ranging from pale yellow to orange) helped categorize the spores based on pigment accumulation. These preliminary morphological assignments were further tested using SSU rRNA sequences.

Results of molecular identification of AMF spores

Successful amplification of an approximately 800 bp DNA fragment for all five isolates (SP1-SP5) was confirmed by agarose gel electrophoresis (Figure 7). The observed band sizes matched the expected amplicon length for the AML1/AML2 primer products. The clear, single bands indicated specific amplification of the SSU rRNA gene region, and no non-specific bands or contamination were detected. In this study, PCR was performed in separate batches to optimize amplification for each isolate; consequently, the displayed gel visualizations are presented separately (Figure 7).

BLAST analysis of the sequenced DNA samples revealed high homology with reference sequences in the NCBI database. All isolates showed percent identity ranging from 95.00% to 100.00% with query coverage >99.00% (Table 3). Isolate SP1 showed the highest similarity to *Archaeospora leptoticha* (reclassified as *Ambispora leptoticha*), while isolate SP2 was identified as *Acaulospora* cf. *spinosa* based on its 95% sequence identity with the reference strain. Isolates SP3 and SP5 matched with uncultured *Glomeromycotina* sp., and isolate SP4 showed high homology with *Entrophospora colombiana* (reclassified as *Acaulospora colombiana*).

The phylogenetic relationships of these isolates relative to reference sequences are depicted in the Neighbor-Joining tree (Figure 8). The analysis grouped the five isolates into four distinct clades corresponding to their BLAST identifications, placing SP1 within the Ambisporaceae, SP2 and SP4 within the Acaulosporaceae, and SP3/SP5 within unresolved *Glomeromycotina* lineages.

Table 2. Morphological characteristics of spore isolates SP1-SP5

Description	**SP1**	**SP2**	**SP3**	**SP4**	**SP5**
Spore size	>200-<235 µm	>175-<205 µm	>65-<85 µm	>65-<85 µm	>75-<120 µm
Color C-Y-M (%)*	Pale yellow (0-20-0)	Yellowish brown (20-100-60)	Yellow (0-50-10)	Cream (0-30-15)	Orange (0-90-30)
Sporocarp	None	None	None	None	None
Sporiferous saccule	None	Present	None	None	None
Bulbous suspensor	None	None	None	None	None
Germination shield	None	None	None	None	None
Melzer reaction	Positive	Positive	Positive	Positive	Positive
Suspected genus	-	*Acaulospora*	-	-	-

Note: *: C-Y-M (%) based on the color scheme for glomalean fungi by Brundrett et al. (1995), expressed as Cyan-Yellow-Magenta ratios (e.g., 0-20-0)

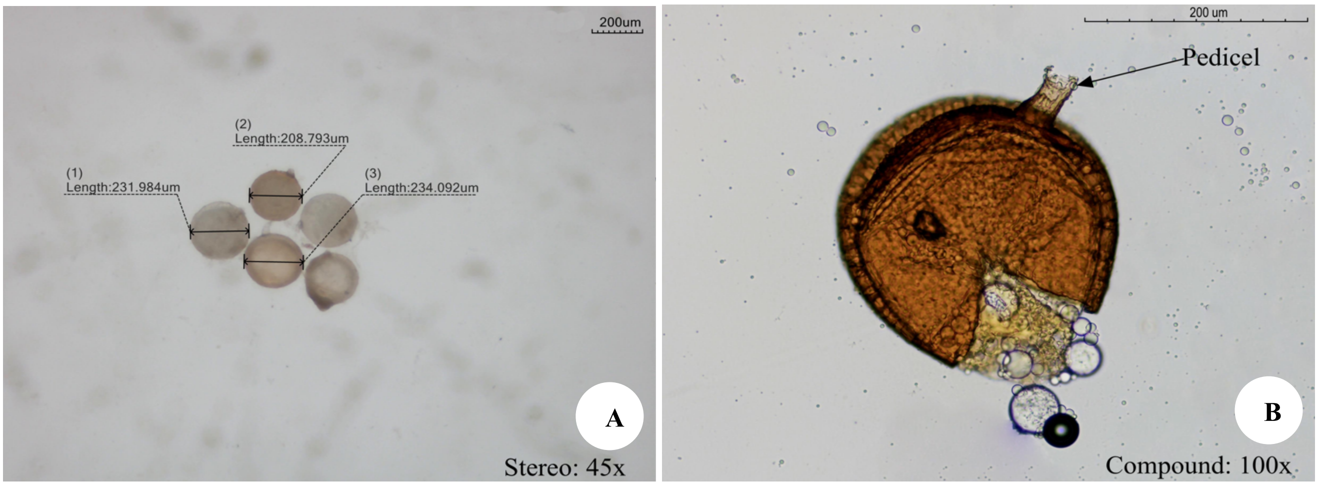

Figure 2. Spores of isolate SP1. A. Intact spores showing a truncated pedicel attachment under brightfield microscopy, B. Positive reaction (rust-brown) in Melzer's reagent

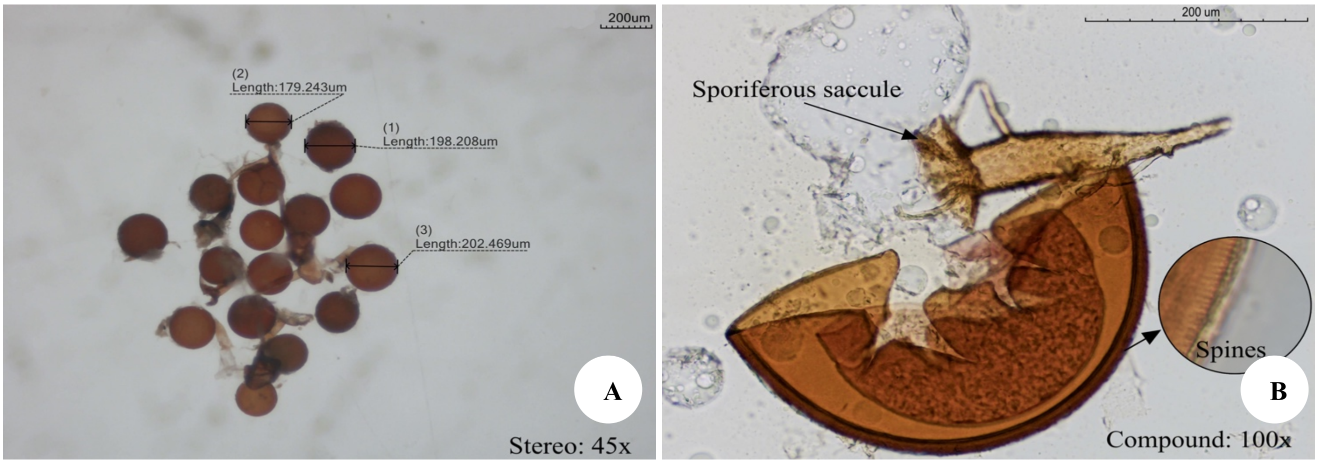

Figure 3. Morphological Spores of isolate SP2. A. Spores showing a sporiferous saccule and spine-like ornamentation under brightfield microscopy, B. Positive reaction in Melzer's reagent

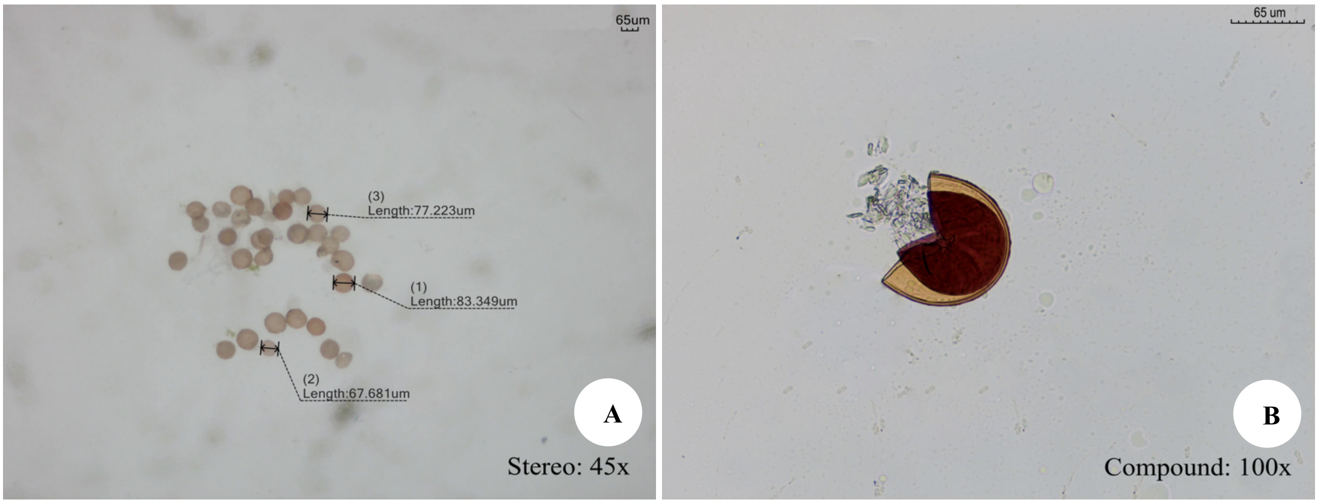

Figure 4. Spores of isolate SP3. A. Bright, small spores lacking distinct auxiliary structures under brightfield microscopy, B. Positive reaction in Melzer's reagent

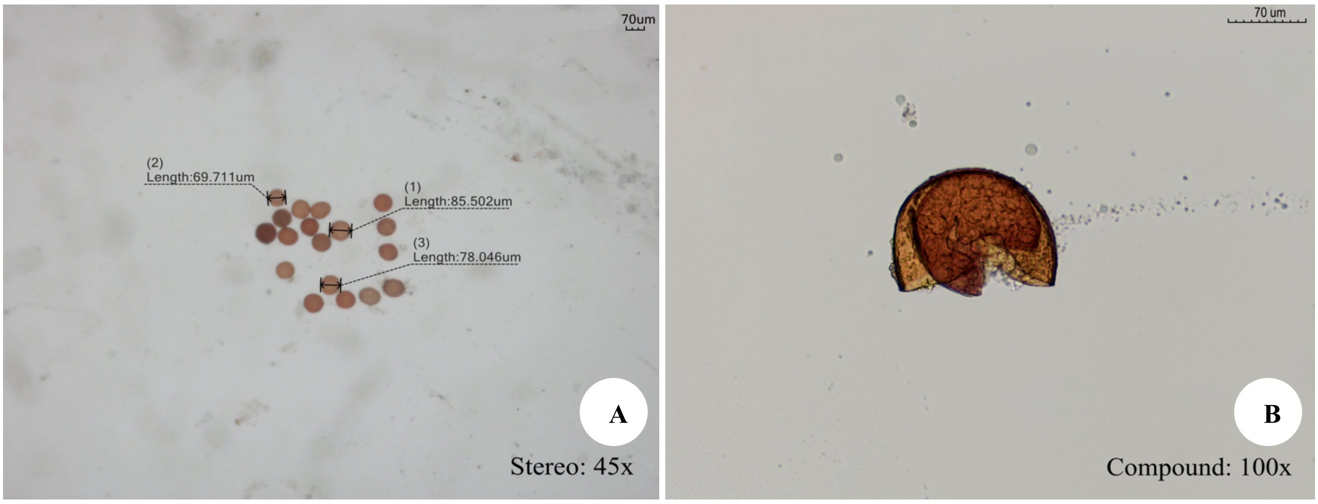

Figure 5. Spores of isolate SP4. A. Cream-colored spores under brightfield microscopy, B. Strong dextrinoid reaction (reddish-brown) in Melzer's reagent

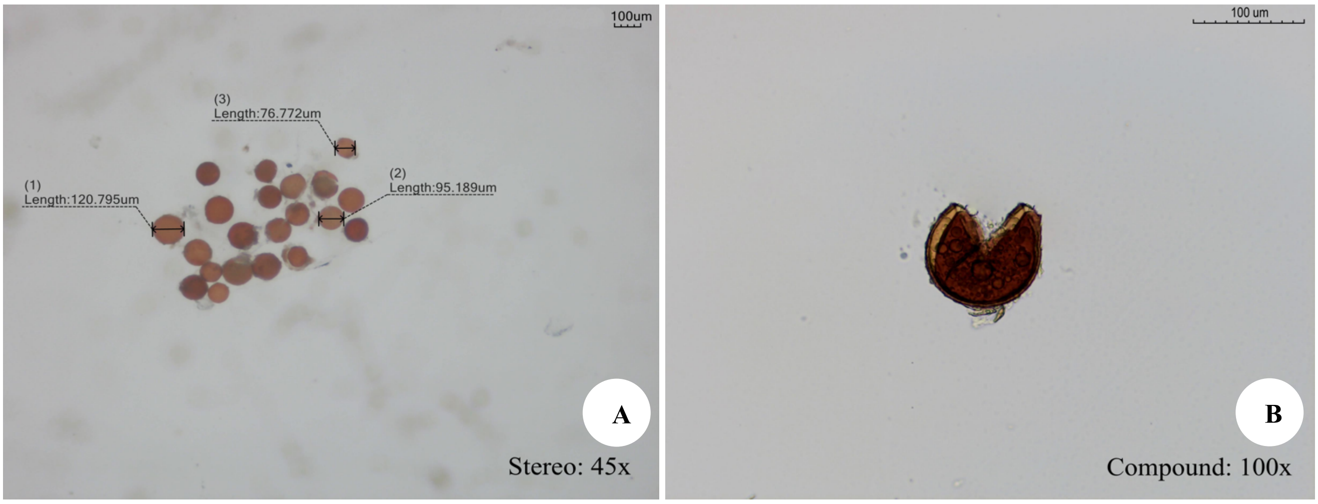

Figure 6. Spores of isolate SP5. A. Darker, larger orange spores under brightfield microscopy, B. Positive reaction in Melzer's reagent

Table 3. Identification results of DNA samples (SP1-SP5) based on BLAST analysis

Sample	Isolate accession number	Molecular identification (BLAST)	Percent identity (%)	Query coverage (%)	Closest match accession number (BLAST)
SP1	PZ225325	*Archaeospora leptoticha* (*Ambispora leptoticha*)*	100.00	99.71	AB047306.1
SP2	PZ225326	*Acaulospora* cf. *spinosa*	95.00	99.23	JX461237.1
SP3	PZ225327	*Glomeromycotina* sp.	100.00	99.44	MG829323.1
SP4	PZ225328	*Entrophospora colombiana* (*Acaulospora colombiana*)**	100.00	99.87	AB220170.1
SP5	PZ225329	*Glomeromycotina* sp.	100.00	99.62	MG829323.1

Note:*: The name *Ambispora leptoticha* reflects the taxonomic reclassification from its former name, *Archaeospora leptoticha* (Bills and Morton 2015). **: The name *Acaulospora colombiana* reflects the taxonomic reclassification from its former names, *Entrophospora colombiana* and *Kuklospora colombiana* (Kaonongbua et al. 2010)

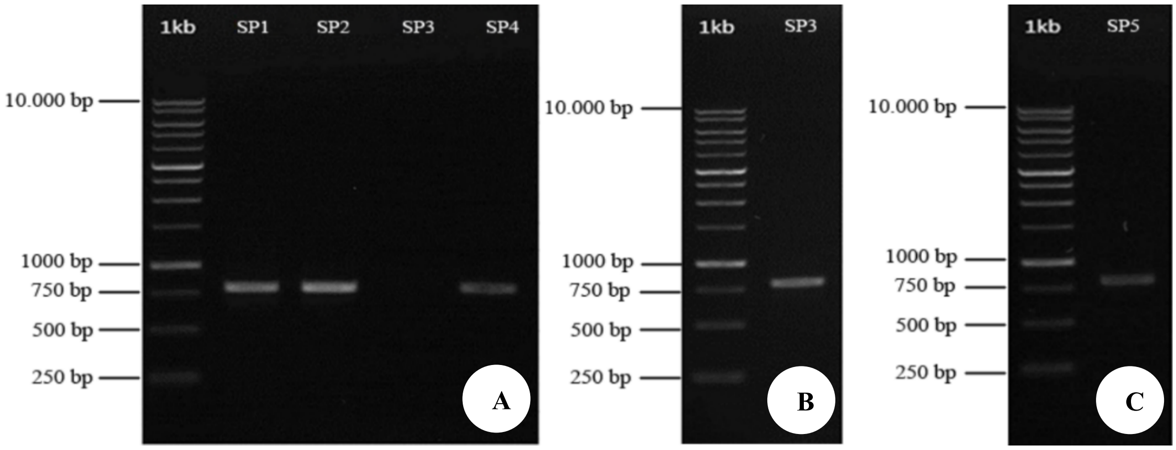

Figure 7. Visualization of electrophoresis results of PCR products (~800 bp) matching the expected size for AML1/AML2 primers. A. Isolate SP1, SP2, SP3 (no band), and SP4, B. Isolate SP3 (repeated amplification), C. Isolate SP5. The 1kb DNA ladder was used as a molecular size marker

Discussion

The accurate identification of arbuscular mycorrhizal fungi is a fundamental prerequisite for understanding their ecological roles and potential agronomic applications. In this study, the use of the SSU rRNA marker proved highly effective for the initial molecular identification of indigenous AMF from the black pepper rhizosphere. As supported by recent literature, the SSU rRNA gene provides a stable phylogenetic backbone for the phylum *Glomeromycota* with lower intragenomic variability than the Internal Transcribed Spacer (ITS) region (Thiéry et al. 2016; Kolaříková et al. 2021). The robust AML1/AML2 primer pair successfully amplified ~800 bp fragments from all five isolates, thereby minimizing non-target amplification from host plants or saprotrophic fungi, and ensuring that the sequences obtained were exclusively of AMF origin (Lee et al. 2008; Rini et al. 2021).

Phylogenetic analysis, supported by morphological data, revealed a community comprising distinct evolutionary lineages. Isolate SP2 was confidently identified as *Acaulospora spinosa* based on morphological characteristics, a finding strongly supported by the presence of a sporiferous saccule and specific spine-like ornamentation on the outer wall (Stürmer and Morton 1999; da Silva et al. 2022). However, isolate SP2 is tentatively assigned as *Acaulospora* cf. *spinosa* in this study. Although its morphological features are consistent with *A. spinosa*, its SSU rRNA sequence showed 95% identity. In conserved ribosomal regions, such as the SSU, 95% identity may indicate a closely related but potentially distinct taxon rather than a definitive species-level match. By using the 'cf.' designation, we acknowledge the single-marker limitation and provide a more cautious taxonomic assignment until further multi-locus data (SSU+ITS+LSU) can be obtained to resolve its fine-scale identity. This molecular confirmation is crucial because morphological plasticity in field-collected spores often leads to misidentification if relying on microscopy alone.

A significant taxonomic finding was the identification of isolate SP1 as *Ambispora leptoticha*. This species was formerly classified as *Archaeospora leptoticha* but was reclassified based on multi-gene analyses (Bills and Morton 2015). Our study confirms this reclassification through molecular phylogeny and its characteristic rust-brown reaction to Melzer's reagent. To our knowledge, this is the first report and molecular validation of this ancient lineage in Indonesian black pepper plantations. Isolate SP4 was initially identified as *Entrophospora colombiana*, supported by its strong dextrinoid reaction (Stürmer and Morton 1999).

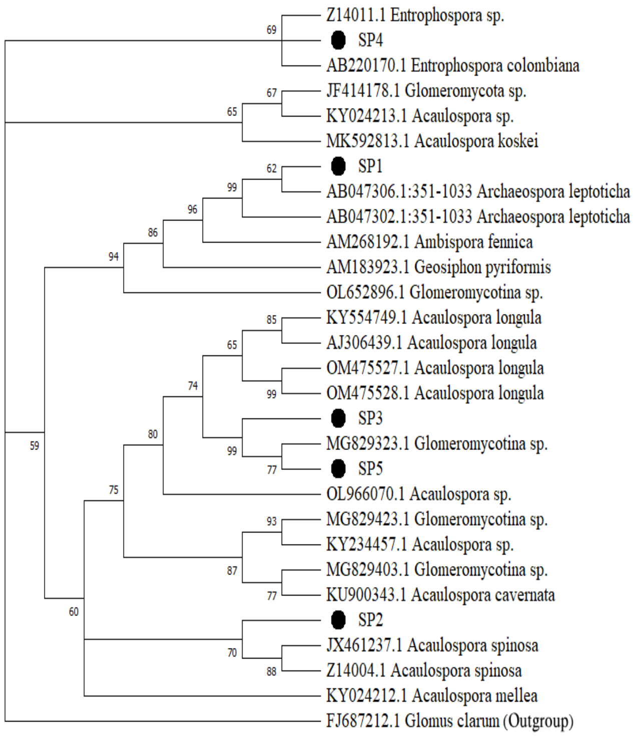

Figure 8. Phylogenetic tree describing the relationships of the five indigenous AMF isolates (SP1-SP5) inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Tamura 3-parameter model with Gamma distribution (T92+G). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The analysis involved SSU rRNA gene sequences from the isolates and reference sequences retrieved from GenBank; accession numbers are listed directly preceding the species names in the tree labels. *Glomus clarum* was used as the outgroup to root the tree

However, recent taxonomic revisions indicate that this species should be reclassified as *Acaulospora colombiana*. Originally described as *E. colombiana* based on its entrophosporoid spore formation (Schenck et al. 1984; Stürmer and Morton 1999), it was later transferred to the genus *Kuklospora* (Sieverding and Oehl 2012). Subsequent molecular and ontogenetic studies demonstrated that the entrophosporoid mode of spore formation is the result of convergent evolution, leading to its official reclassification as *A. colombiana* (Kaonongbua et al. 2010). Although the observed spore size was smaller than the typical ranges—likely indicating an early developmental stage or environmental stress (da Silva et al. 2025)—its phylogenetic position clustered closely with reference sequences for this taxon.

The composition of the AMF community in this study, now characterized by the absolute dominance of the *Acaulosporaceae* lineage (comprising both *A.* cf. *spinosa* and the reclassified *A. colombiana*) and the apparent absence of *Glomus*, reflects potential ecological filtering driven by the unique edaphic conditions of the study site (Vázquez-Santos et al. 2025). The research site presents a paradoxical condition: the soil is extremely acidic (pH 4.13) yet contains remarkably high levels of available phosphorus (77.17 ppm) (Yao et al. 2018; Huang et al. 2023). We hypothesize that this specific combination (high P and low pH) may act as a "double filter" that excludes Glomeraceae (Pan et al. 2020; Amir et al. 2021). To elaborate on this "double filter" hypothesis, it is important to consider the physiological mechanisms mediating plant-fungal interactions. When soil phosphorus is abundant, host plants typically reduce the exudation of strigolactones—key signaling molecules that initiate AMF hyphal branching and root colonization (Yoneyama et al. 2020; Wang et al. 2021). This chemical downregulation disproportionately affects highly nutrient-dependent *Glomus* species, rendering them less competitive in the rhizosphere (Liu et al. 2016; Fang et al. 2024). Concurrently, the high aluminum toxicity characteristic of pH 4.13 soils demands specific fungal survival strategies. *Acaulospora* species are renowned "stress-tolerators" capable of producing substantial amounts of Glomalin-Related Soil Proteins (GRSP) and possess efficient vacuolar sequestration mechanisms that immobilize toxic Al^{3+} ions (Aguilera et al. 2017; Tiwari et al. 2020; Alotaibi et al. 2021;). Based on previous studies, it is plausible that under high P and high aluminum toxicity conditions, black pepper may favor AMF taxa that confer stress tolerance over primarily nutrient-acquisitive taxa. However, our study did not directly assess plant responses, root colonization patterns, or aluminum tolerance; therefore, this functional shift hypothesis requires dedicated physiological experiments to be demonstrably proven.

The recovery of *Ambispora* and *Acaulospora* may also be linked to local land management practices. According to AMF life-history strategies, these genera are often classified as 'K-strategists' that prioritize long-term survival and build extensive, durable hyphal networks, making them highly vulnerable to mechanical soil disruption (Ijdo et al. 2010; Bowles et al. 2017; Cahyaningtyas and Ezawa 2023). In contrast, *Glomus* species behave as opportunistic 'r-strategists' that tolerate disturbance (Oehl et al. 2009). Our field records indicate that the plantation is managed with minimal tillage, which may preserve the extraradical mycelial networks and create a stable subterranean niche for these disturbance-sensitive fungi to persist (Gu et al. 2020). However, while our field records indicate minimal tillage, dedicated comparative studies would be necessary to test this mechanism. Another critical factor shaping the observed AMF community profile is the methodology employed for spore propagation. In this study, trap cultures utilizing *Zea mays* (maize) were maintained for three months to induce sporulation. While trap culturing is a standard and necessary procedure, it introduces an inherent selective bias (Błaszkowski et al. 2020). The host plant identity significantly influences which AMF species thrive; thus, the use of a generic grass host like maize might preferentially amplify certain fast-sporulating taxa while failing to capture the full diversity of slow-growing native species strictly adapted to the woody root system of *Piper nigrum* (Willis et al. 2013; Symanczik et al. 2014). Furthermore, a three-month culture period may be insufficient long for certain late-sporulating K-strategists to complete their life cycles.

While the identification of stress-tolerant taxa such as *A.* cf. *spinosa* and *A. colombiana* highlights their potential for developing site-specific bioinoculants in acidic drylands (He et al. 2022; Kebede et al. 2023), we emphasize that this study primarily provides a foundational molecular baseline inventory of candidate species. The actual functional efficacy of these indigenous consortia—specifically regarding their impact on black pepper yield, phosphorus uptake, aluminum tolerance, and disease resistance—has not yet been tested in our current study. Therefore, conducting comprehensive in vivo greenhouse and field trials must be the primary focus of future research before any practical field application can be recommended (Kumar et al. 2025; Lethielleux-Juge 2025).

Furthermore, the successful molecular identification of these indigenous isolates underscores the critical importance of building localized taxonomic databases. In the current era of mycorrhizal research, broad-scale taxonomic identification is rapidly transitioning from traditional spore-based Sanger sequencing to High-Throughput Environmental metabarcoding (HTS) directly from roots or bulk soil (Öpik et al. 2014; Tedersoo et al. 2021). While HTS provides a comprehensive snapshot of the entire fungal community—including non-sporulating or 'dark' taxa that evade trap culturing—its taxonomic accuracy is fundamentally constrained by the quality of public reference databases (Nilsson et al. 2019). Currently, many environmental sequences obtained from tropical regions remain classified merely as "uncultured *Glomeromycota*" due to the lack of morphologically and molecularly validated voucher specimens. Therefore, the SSU sequences generated in this study, particularly for ancient and poorly represented taxa such as *A. leptoticha*, serve as invaluable, localized reference barcodes. By depositing these precisely identified sequences into public repositories, this study bridges the gap between classical spore-based taxonomy and modern environmental metabarcoding. Future ecological surveys utilizing genomic tools in Indonesian plantations will be able to map environmental reads back to these verified indigenous spores, enabling a much higher-resolution understanding of AMF biogeography in marginal lands.

Finally, we explicitly acknowledge the methodological limitations of our study. Our conclusions are constrained by limited spatial sampling (a single site) and a small number of isolates, meaning the patterns observed may not fully represent the broader West Lampung region. Furthermore, while the SSU rRNA marker successfully identified key lineages, it lacked sufficient variability to distinguish closely related species within the *Glomeromycotina*, as evidenced by the unresolved status of isolates SP3 and SP5 (Thiéry et al. 2016; Perez-Lamarque et al. 2022). This phenomenon highlights a well-documented challenge in AMF taxonomy: the molecular-morphological discrepancy (Krüger et al. 2012). AMF spores are inherently coenocytic, harboring hundreds to thousands of nuclei that can exhibit significant genetic polymorphism even within a single spore (Corradi and Brachmann 2017). Despite SP3 and SP5 displaying distinct morphological traits—such as variations in size and pigment accumulation likely driven by developmental age or environmental plasticity (Kokkoris et al. 2024)—their SSU sequences were identical. This suggests either that these isolates represent distinct morphological variants of the same species or that the SSU region is simply too conserved to resolve their species-level divergence. Future studies must employ multi-locus approaches, combining SSU with ITS or LSU regions, or utilize genomic tools to achieve finer taxonomic delimitation.

In conclusion, five indigenous AMF isolates from the black pepper rhizosphere in West Lampung were characterized using a combination of morphological and molecular methods. The SSU rRNA analysis successfully resolved four distinct evolutionary lineages: *Ambispora leptoticha* (SP1), *Acaulospora* cf. *spinosa* (SP2), *Acaulospora colombiana* (SP4), and unclassified *Glomeromycotina* sp. (SP3 and SP5). The findings provide a critical molecular baseline reporting the presence of the *Ambispora* lineage for the first time in this region. Notably, the recovered isolates were dominated by the Acaulosporaceae family, with an apparent absence of *Glomus* in our samples, reflecting the unique context of the extremely acidic, high-phosphorus soils at the study site. To build upon this taxonomic baseline, future research should expand geographic sampling, conduct in vivo functional trials, and utilize multi-locus sequencing to further resolve the biodiversity of indigenous AMF.

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