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Diversity of endophytic fungi isolated from root of *Paphiopedilum javanicum*

ARI PITOYO[1,2], SOLICHATUN[2], ARTINI PANGASTUTI[2], SUGIYARTO[2,♥]
[1]Doctoral Program in Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami No. 36A, Surakarta 57126, Central Java, Indonesia. Tel.: +62-271-669376, Fax.: +62-271-663375 ♥email: sugiyarto67@staff.uns.ac.id

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Abstract. *Pitoyo A, Solichatun, Pangastuti A, Sugiyarto. 2026. Diversity of endophytic fungi isolated from root of* Paphiopedilum javanicum. *Biodiversitas 27 (3): d270304. https://doi.org/10.13057/biodiv/d270304.* Understanding the intimate relationship between orchids and their fungal communities is essential for effective conservation. To address this, we investigated the root anatomy and fungal associates of *Paphiopedilum javanicum*, an endangered slipper orchid native to Java. Root samples comprising 75 segments collected from five montane habitats were examined microscopically and subjected to fungal isolation. The recovered isolates were identified through ITS sequencing, and their taxonomic placement was confirmed by phylogenetic analysis using the Neighbor-Joining method. Microscopy revealed peloton structures in cortical cells, with colonization levels varying among roots and tending to be patchy rather than systemic. Direct peloton isolation yielded no viable fungi, while root-segment and seed-baiting methods successfully isolated 23 fungi. ITS analyses revealed that most isolates belonged to Ascomycota (15 species in 10 genera), including *Fusarium*, *Xylaria*, and *Nigrospora*. Basidiomycota represented 7 species in 6 genera, among them two *Ceratobasidium* isolates recognized as orchid mycorrhizal fungi, along with lignicolous taxa such as *Phanerochaete*, *Schizophyllum*, *Cubamyces*, and *Polyporus*. A single Mucoromycota isolate (*Umbelopsis*) was also identified. These findings demonstrate that *P. javanicum* roots host a taxonomically diverse fungal assemblage comprising both putative mycorrhizal symbionts and a broad suite of opportunistic endophytes and saprotrophs. The coexistence of these guilds suggests ecological flexibility in fungal associations. This study provides the first integrated anatomical and molecular characterization of root-associated fungi in *P. javanicum* from Indonesia and establishes baseline data to inform symbiotic propagation and conservation strategies.

Keywords: *Ceratobasidium*, fungal diversity, orchid conservation, orchid mycorrhiza, *Paphiopedilum javanicum*

INTRODUCTION

Orchidaceae is the second most diverse and widespread flowering plant family after Asteraceae, with more than 30.000 species grouped in 760 genera and distributed worldwide (https://www.catalogueoflife.org/?taxonKey=DPL). Although species numbers remain debated due to ongoing discoveries and taxonomic revisions (Chase et al. 2015; Chase et al. 2016; Wang et al. 2024); most orchids are concentrated in tropical regions. Approximately 70% orchid are epiphytic (Zhang et al. 2025), while about 20% are terrestrial, inhabiting shaded forest floor, where they rely on mycorrhizal fungi for nutrient and carbon acquisition. This association enables persistence in low-light environments where photosynthesis alone provides insufficient energy for survival (McCormick et al. 2004; Xing et al. 2019; Hartvig et al. 2024).

Orchids are a major focus of global biodiversity conservation due to their extraordinary diversity and vulnerability to overexploitation and habitat loss. Up to 40% of assessed species are classified as Vulnerable (VU), Endangered (EN), or Near Threatened (NT), although this estimate is based on only 2439 evaluated species and does not represent the ~30,000 orchids worldwide (https://www.iucnredlist.org/search?query=orchidandsearchType=species). Despite limited assessments, projections indicate that many orchids face significant risks under current global conditions (Kolanowska 2023), while their reliance on specialized fungi and pollinators increases sensitivity to habitat disturbance (Štípková et al. 2020). Furthermore, overcollection, unsustainable forest cutting, and land conversion practices further accelerate population decline (Fay 2018; Wraith and Pickering 2018).

Paphiopedilum and its close relatives within the slipper orchid subfamily (Cypripedioideae) represent one of the most threatened groups of orchids globally. Many species in this lineage are highly sought after in horticulture and are simultaneously restricted in distribution (Fay et al. 2025). As a result, numerous species are listed in threatened categories of the IUCN Red List, and the entire genus is included under CITES Appendix I (Rankou 2015). Among these, *Paphiopedilum javanicum* (Reinw. ex Lindl.) Pfitzer, native to Java and adjacent islands such as Sumatra and the Lesser Sunda Islands, faces mounting pressures that threaten its long-term persistence. Although it grows in highland forests between 900 and 2,000 m above sea level (Romadlon et al. 2021) its habitats are increasingly constrained by human population growth and climate change. In Java, its populations are now limited to mountain ranges such as Ungaran (Kurniawan et al. 2021), Dieng, Lawu, Merbabu, Sumbing, Sindoro, Slamet, Prau, and Kawi, along with protected areas like Halimun Salak and Gunung Gede Pangrango (Comber 1990). This restricted distribution, combined with ongoing habitat pressure, makes the species especially vulnerable.

Despite the recognized conservation importance of *Paphiopedilum*, research on its fungal symbionts remains scarce, particularly in Indonesia, which is a center of diversity for the genus. Addressing this knowledge gap is crucial for developing conservation strategies that account for the biological complexity underlying the survival of *P. javanicum*. Like many orchids, *Paphiopedilum* depends on fungi for seed germination, yet these associations can be highly species-specific, making the conservation of *P. javanicum* inseparable from that of its fungal partners (Kaur et al. 2019; Reiter et al. 2020; Hartvig et al. 2024). To address this, our study investigates the association between the endangered *P. javanicum* and its fungi through root anatomy, fungal colonization, and the isolation and identification of fungal partners. This work builds on previous conservation initiatives for the species, which have focused on habitat characterization (Romadlon et al. 2021) and the development of ex-situ programs in botanical gardens.

As in many orchids, *Paphiopedilum* species are expected to associate with *Rhizoctonia*-like fungi that play key roles during seed germination. However, adult orchids frequently harbor diverse root-associated fungal communities comprising both mycorrhizal partners and non-mycorrhizal endophytes. Given the limited information available for Indonesian *Paphiopedilum* species and the documented variability in orchid-fungus specificity, we hypothesize that *P. javanicum* associates with classical *Rhizoctonia*-like mycorrhizal fungi (e.g., *Tulasnella* and *Ceratobasidium*) while also hosting a broader assemblage of endophytic and saprotrophic fungi within its roots. We further predict that the culturable fungal community will reflect this heterogeneity, providing insight into the balance between mycorrhizal specificity and ecological flexibility in *P. javanicum*, with implications for conservation and symbiotic propagation.

MATERIALS AND METHODS

Study sites and collection procedures

Root samples of *P. javanicum* were collected from four natural populations across geographically distinct montane habitats in Java, Indonesia. The sampling sites included Mount Merbabu, Mount Lawu, Mount Sindoro (Central Java), Mount Kawi (East Java), and Mount Sumbul (West Java). At each site, root fragments were pooled from 3-7 individual plants growing in proximity to ensure ecological representativeness while minimizing damage to wild populations. Fresh roots were stored in sterile tubes, kept cool during transport, and processed within 24 hours in the laboratory.

Root microscopic examination

Root anatomy and fungal colonization were assessed using both fresh and paraffin-embedded sections. For fresh sections, root cortex slices were hand-cut with a razor blade, stained with lactophenol cotton blue or chlorazol black E to visualize hyphae and pelotons, and mounted in glycerol for light microscopy. For paraffin sections, roots were fixed in FAA, dehydrated in graded ethanol, cleared with xylene, embedded in paraffin, sectioned at 8-12 µm, and mounted on slides. Sections were stained with safranin-fast green, dehydrated, and permanently mounted for detailed anatomical analysis.

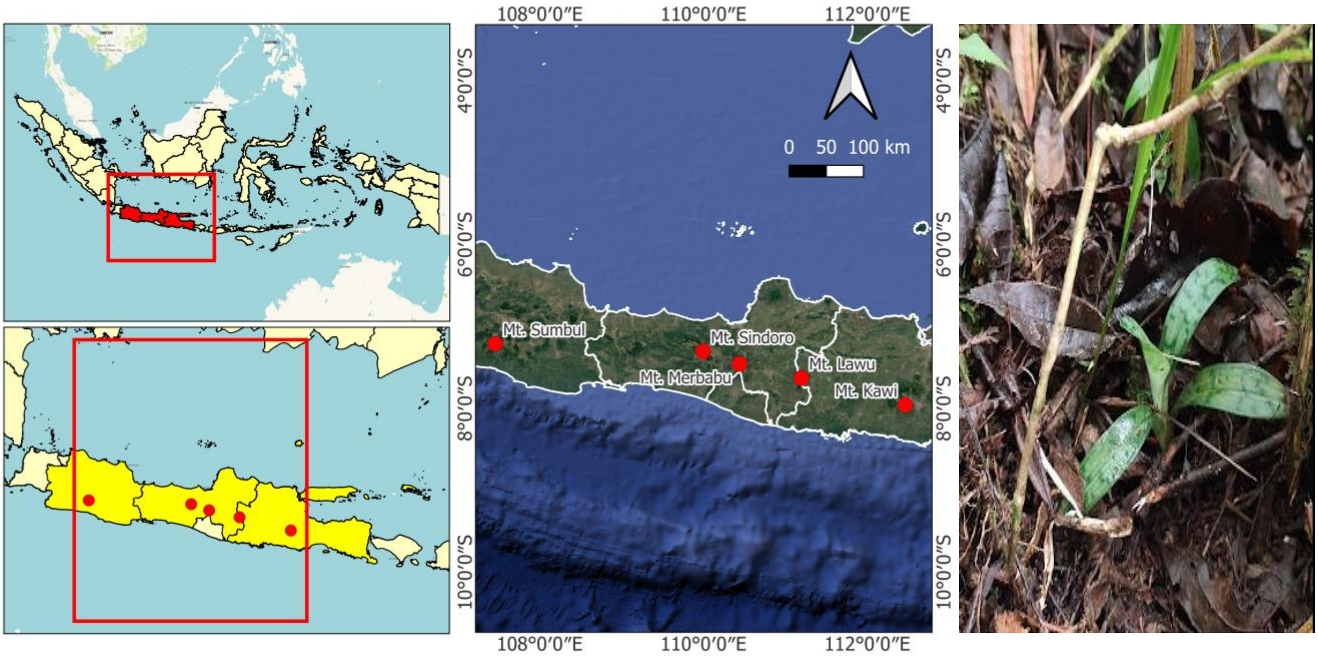

Figure 1. Sampling sites of *Paphiopedilum javanicum* in Java (left) and a seedling in its natural habitat (right)

Isolation of endophytic fungi

Endophytic fungal isolation

Endophytic fungi were isolated using a modified method based on Tian et al. (2022). Root samples were collected from three *P. javanicum* individuals at each of four sites (one root per plant). Each root was sectioned into five segments, yielding 75 segments in total. Roots were rinsed with sterile distilled water, and root hairs and velamen were aseptically removed. Segments with brownish peloton zones were selected, rinsed three times, surface-sterilized in 70% ethanol (30 s) and 10% commercial bleach (~6% NaOCl, 5 min), then washed five times with sterile water. Surface sterility was verified by plating the final rinse water and root imprints onto PDA, and only sterile samples were cultured. Sterilized segments were placed on Potato Dextrose Agar (PDA) for fungal isolation. For peloton isolation, 2-mm root pieces were dissected, pelotons gently released, and incubated in sterile distilled water at 24°C in the dark for 24 h. Germinating pelotons were observed microscopically and transferred to 1 cm^2 PDA blocks. Emerging hyphae were sub-cultured 4-5 times to obtain pure isolates, which were stored at 4°C. This procedure yielded 21 fungal isolates from 75 root segments.

In addition, ex-situ seed baiting was performed by sowing orchid seeds directly onto culture media containing freshly prepared root segments. After incubation, seeds colonized by fungi were collected, surface-sterilized, and transferred to isolation media. This modification avoids field burial and poaching of seeds, while maintaining the ability to recover root-associated endophytic fungi relevant to orchid symbiosis, this procedure results in 2 fungal isolates.

Morphological characteristics of colonies and mycelia

On PDA plates, the purified fungal strains were plated and incubated at 24°C. The colony morphological characteristics of each strain were observed and recorded on PDA medium daily, including colony morphology and mycelium cell structure. All the purified fungal strains were inoculated in the middle of the PDA plates, then a sterile coverslip was placed into the medium at a 45-degree angle using forceps. When the hyphae grew to about 2/5 of the cover glass, the cover glass was taken out, and the structural characteristics of the hyphae were observed under an optical microscope.

Molecular identification of endophytic fungi of Paphiopedilum javanicum

Genomic DNA was extracted from fungal isolates using the Plant Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer’s protocol with minor modifications. The ITS region was amplified using ITS1 and ITS4 primers in standard PCR reactions. PCR products were visualized on 1.5% agarose gels, purified, and sequenced by an external provider (Apical Scientific Sdn. Bhd.). Sequences were identified via BLAST searches against the NCBI GenBank database. For visualization, sequences were aligned in MEGA X (ClustalW), and a Neighbor-Joining Phylogenetic tree was constructed to illustrate the taxonomic placement and relationships of the isolates with reference taxa.

RESULTS AND DISCUSSION

Root anatomy of *Paphiopedilum javanicum* and fungal colonization

The roots of *P. javanicum* display typical orchid anatomy, but peloton colonization is highly heterogeneous and localized within the cortex, rather than continuous. Transverse sections reveal varying peloton densities among and within roots, with colonization restricted to discrete cortical patches (Figures 2.A-2.C). Notably, pelotons are abundant in some cortical regions but absent in adjacent areas of the same root, highlighting a dynamic spatial pattern of fungal colonization. At higher magnification, pelotons are confined to the cortical parenchyma, particularly the inner cortex. Although hyphal threads occur in the velamen and root hairs, these tissues do not form pelotons (Figures 2.B-2.C). No fungal structures were detected in the endodermis or stele, confirming colonization is limited to the cortex (Figures 2.D-2.E).

Fungal colonization

Fungal colonization in *P. javanicum* roots was patchy and localized, occurring mainly in the velamen, root hairs, and outer cortex, with minimal presence in the inner cortex or stele (Figures 3.A-3.C). Notably, different peloton types were observed at varying developmental stages. Loosely packed pelotons within cortical cells (Figure 2.D) appeared more viable and indicative of active fungal symbiosis, while compact or degraded pelotons (Figure 3.E) likely represented maturation or breakdown stages. The coexistence of these peloton forms, particularly the excised, loosely structured pelotons (Figure 3.F). The simultaneous occurrence of these peloton forms indicates structural variation in fungal colonization within individual roots.

The coexistence of peloton developmental stages suggests a dynamic colonization process, potentially reflecting cycles of fungal entry, degradation, and renewal typical of orchid mycorrhizal interactions. This localized and dynamic pattern of mycorrhizal association implies that *P. javanicum* depends on consistent access to compatible fungi in the rhizosphere. Thus, the conservation of this species requires safeguarding both its habitat and the diversity of soil fungal communities critical for successful root symbiosis and population sustainability.

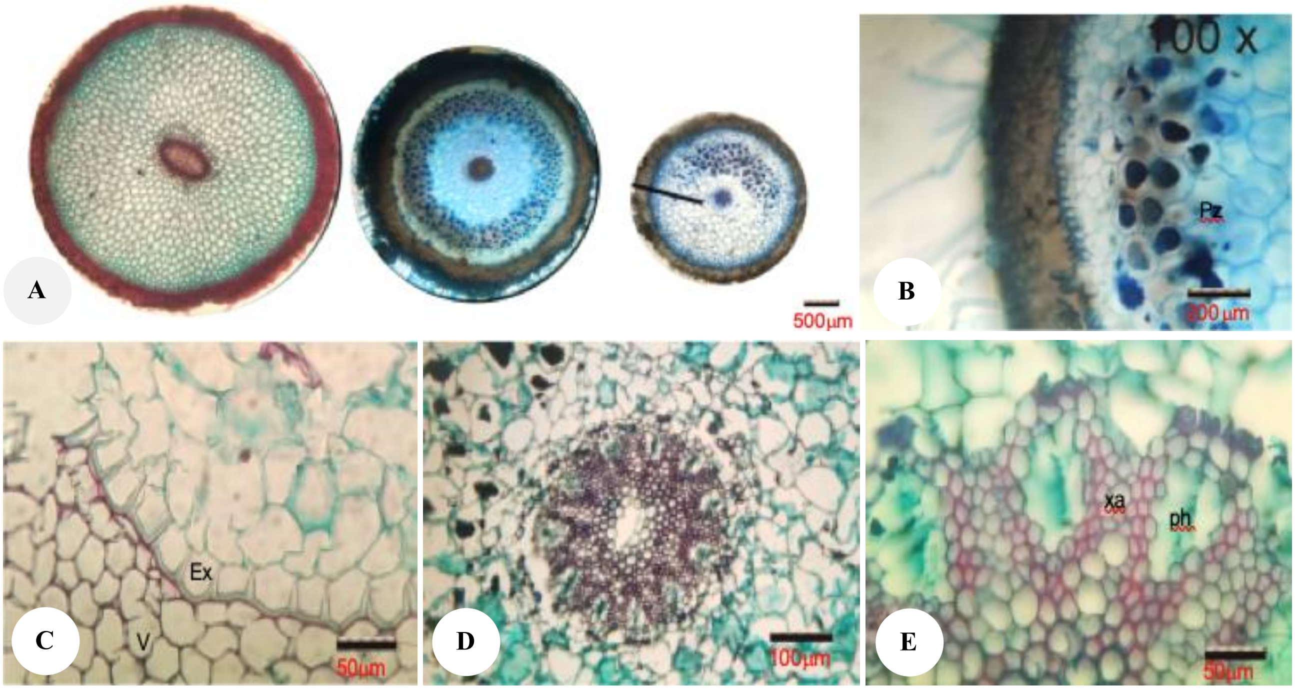

Figure 2. Morphology and anatomy of *Paphiopedilum javanicum* roots: A. Cross sections of orchid roots showing variation in root diameter and fungal colonization, B. Partial root sections depicting the occurrence and structural diversity of fungal pelotons within cortical tissues, showing varying types of fungal pelotons, C. Exodermis-velamenzone, D. Cortex and stele region of the root, F. Velamen tissue. Abbreviations: pz: Peloton zone, v: Velamen cells, ex: Exodermis, en: Endodermis, xa: Xylem arch, ph: Phloem arch

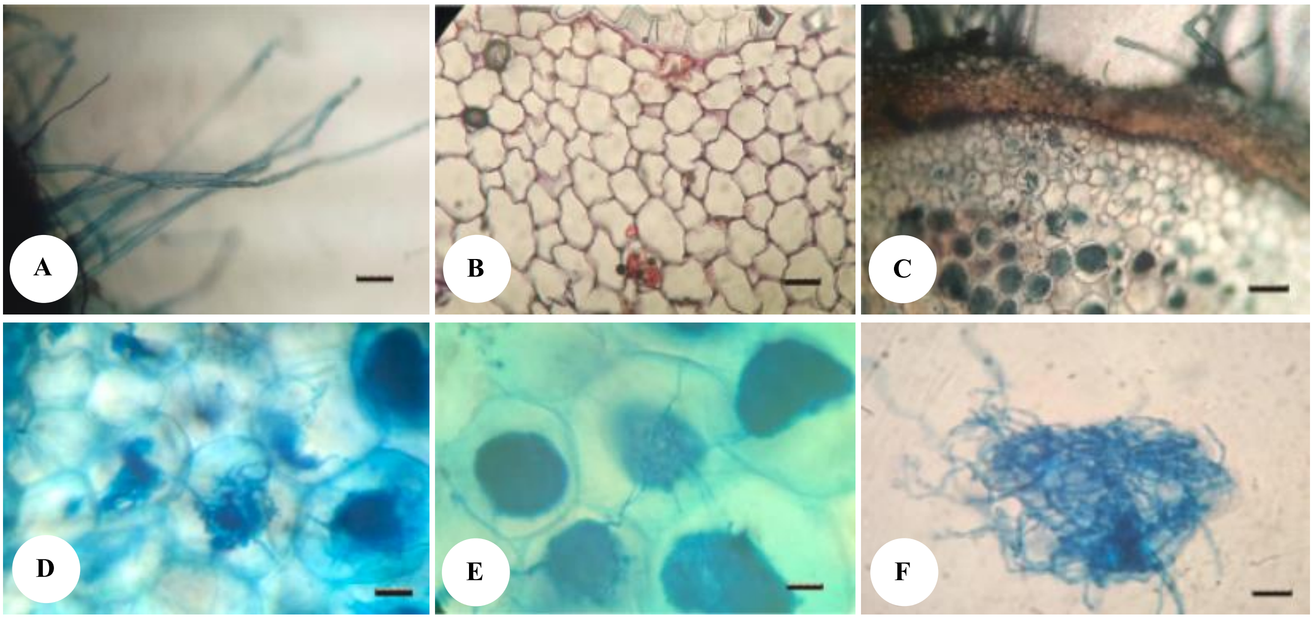

Figure 3. Microscopic features of fungal colonization in orchid roots: A. Root hair colonized by hyphae, B. Velamen containing fungal hyphae, C. Outer cortex beneath exodermis with pelotons, D. Loosely coiled peloton in cortical cell, E. Degraded compact pelotons in cortical parenchyma, F. Single excised peloton. Scale bar: 50 µm

Culturable fungal isolates

In this study, both peloton culture and root-segment isolation were initiated by surface sterilization of the root tissues to remove potential epiphytic contaminants. An ex-situ seed baiting technique was also applied, in which orchid seeds were sown on PDA medium together with root segments for two weeks, after which fungi were isolated from the colonized seeds. While seed baiting and root-segment approaches yielded several isolates, peloton culture did not result in any fungal recovery, most likely because the pelotons had already been digested or remained highly dependent on the host root. To avoid over-interpretation of surface flora, we deliberately excluded numerous isolates of *Aspergillus* and several *Penicillium* strains that consistently appeared on the outer velamen, suggesting that they are more likely surface-associated saprophytes or opportunistic colonizers rather than genuine endophytic or mycorrhizal partners. Therefore, only isolates with potential symbiotic or endophytic relevance were retained for further analysis. In total, 23 pure isolates were successfully recovered from 5 sampling sites. As illustrated in Figure 4, most isolates were assigned to Ascomycota (15 isolates), which encompassed the broadest range of recovered genera, including *Fusarium*, *Arcopilus*, *Trichoderma*, *Penicillium*, and *Geotrichum*. Basidiomycota constituted the second most represented phylum (7 isolates), particularly members of the Agaricales, within which *Rhizoctonia*-like fungi—commonly recognized as orchid mycorrhizal associates—were observed. In contrast, Mucoromycota was represented by a single isolate identified as *Umbelopsis*. The complete set of isolates is presented in Table 1 and further illustrated in Figure 5.

Rhizoctonia-like isolates

Two isolates, CIAK02 and MRAK02 (Figures 5.A-5B), exhibited morphological traits consistent with *Rhizoctonia*-like fungi. Colonies on PDA were fast-growing, surpassing the growth rate of *Fusarium* isolates, and produced whitish to light brown mycelium. Microscopic observations showed right-angle branching with characteristic costrictions, frequent anastomosing hyphae (Figure 6.A), and monilioid cells (Figure 6.B), all diagnostic features of the *Rhizoctonia* complex.

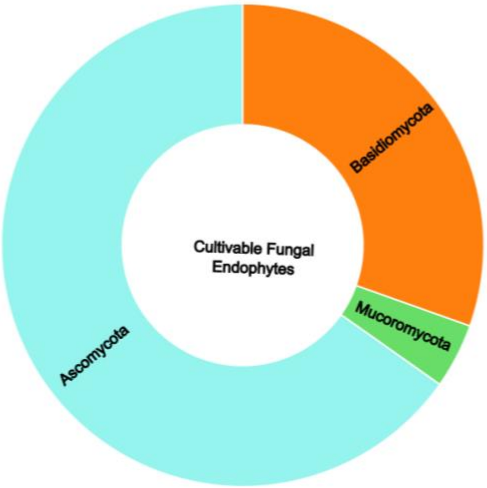

Figure 4. Sunburst chart showing the distribution of cultivable fungal endophytes across phyla

Table 1. Fungal isolates were obtained from *Paphiopedilum javanicum* roots collected from five mountain localities in Java (Mt. Kawi, Mt. Lawu, Mt. Merbabu, Mt. Sindoro, and Mt. Sumbul)

Isolate code	Proposed name	Phylum	Accession	% Identity	% query cover	E-value
CIAK02	*Ceratobasidium* sp.	Basidiomycota	Mt. Sumbul	100	100	0.0
MRAK02	*Ceratobasidium* sp.	Basidiomycota	Mt. Merbabu	91.47	98	0.0
SNAK02	*Polyporus tricholoma*	Basidiomycota	Mt. Sindoro	100	100	0.0
KWAK07	*Phanerochaete* sp.	Basidiomycota	Mt. Kawi	100	100	0.0
MRAK05	*Schizophyllum commune*	Basidiomycota	Mt. Merbabu	100	100	0.0
KWAK06	*Climacodon dubitativus*	Basidiomycota	Mt. Kawi	99.48	100	0.0
MRAK03	*Cubamyces lactineus*	Basidiomycota	Mt. Merbabu	100	96	0.0
KWAK09	*Umbelopsis* sp.	Mucoromycota	Mt. Kawi	100	100	0.0
KWAK01	*Biscogniauxia* sp.	Ascomycota	Mt. Kawi	99.53	99	0.0
KWAK02	*Hypoxylon* sp.	Ascomycota	Mt. Kawi	99.49	100	0.0
KWAK03	*Penicillium brefeldianum*	Ascomycota	Mt. Kawi	100	100	0.0
KWAK04	*Geotrichum* sp.	Ascomycota	Mt. Kawi	99.69	100	7e-1630
KWAK05	*Nigrospora sphaerica*	Ascomycota	Mt. Kawi	100	100	0.0
CIAK01	*Botryosphaeria* sp.	Ascomycota	Mt. Sumbul	99.62	100	0.0
MRAK04	*Arcopilus* sp.	Ascomycota	Mt. Merbabu	100	100	0.0
SNAK03	*Penicillium* sp.	Ascomycota	Sindoro	89.21	74	3e-115
LWAK01	*Fusarium* sp.	Ascomycota	Mt. Lawu	100	100	0.0
LWSB02	*Fusarium oxysporum*	Ascomycota	Mt. Lawu	100	100	0.0
LWAK02	*Peziza* sp.	Ascomycota	Mt. Lawu	95.8	100	0.0
KWAK08	*Peziza* sp.	Ascomycota	Mt. Kawi	95.8	100	0.0
SNAK01	*Trichoderma hamatum*	Ascomycota	Mt. Sindoro	100	100	0.0
MRAK01	*Trichoderma hamatum*	Ascomycota	Mt. Merbabu	100	100	0.0
MRAK07	*Xylaria feejeensis*	Ascomycota	Mt. Merbabu	99.81	100	0.0

Note: Identification was based on ITS sequence similarity using BLAST search against the NCBI GenBank database

ITS sequence analysis indicated an affinity with *Ceratobasidium*, one of the main orchid mycorrhizal lineages within the Basidiomycota. Members of this group are well established as orchid mycorrhizal fungi, forming pelotons in cortical cells and enabling seed germination and nutrient exchange. The recovery of these *Rhizoctonia*-like isolates in *P. javanicum* roots highlights their role as core symbionts, contrasting with the more opportunistic endophytic Ascomycota encountered in the same samples. Importantly, these isolates were retrieved both from root segments and through seed-baiting, reinforcing their likely role in both seed germination and mature root colonization—hallmarks of true orchid mycorrhizal fungi. Previous work on *Paphiopedilum spicerianum* also reported the presence of *Ceratobasidum* isolates from root samples collected during both dry and wet seasons (Han et al. 2016). This match further underscores the ecological significance of *Rhizoctonia*-like fungi in *Paphiopedilum* symbioses. However, many studies have reported that members of the genus *Tulasnella* are more frequently detected as mycorrhizal partners in *Paphiopedilum* (Nontachaiyapoom et al. 2010; Tian et al. 2022), suggesting that multiple *Rhizoctonia*-like fungal lineages contribute to orchid-fungus associations.

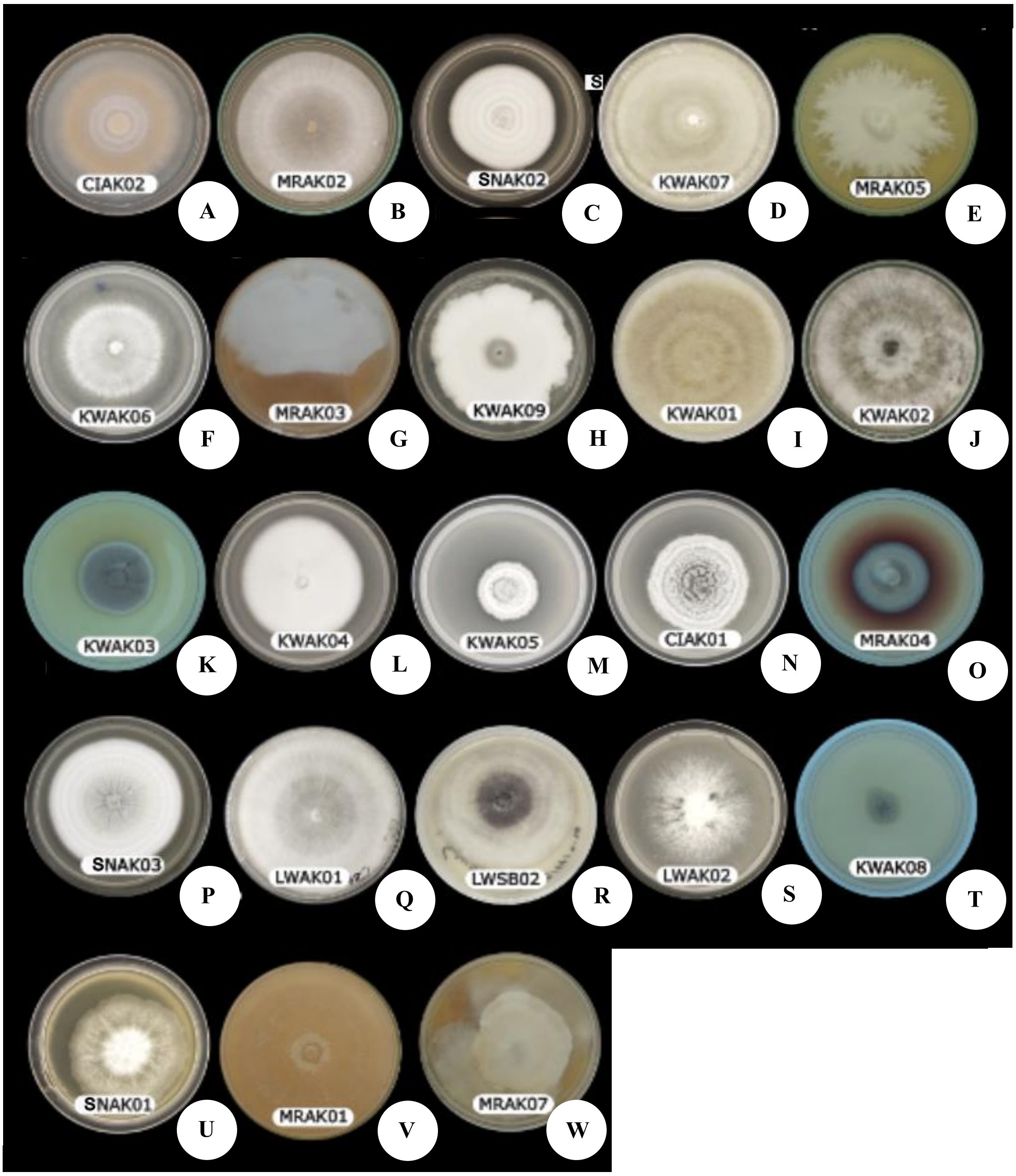

Figure 5. Representative colony morphologies of root-associated fungal isolates from *Paphiopedilum javanicum* grown on PDA medium. A-B: *Rhizoctonia*-like isolates, C-G: Non-*Rhizoctonia* Basidiomycota, H. Mucoromycota, I-W. non-*Rhizoctonia* Ascomycota

Non *Rhizoctonia* Basidiomycota

Beyond the recovery of *Ceratobasidium* isolates representing potential orchid mycorrhizal partners, we consistently obtained a diverse assemblage of other basidiomycota from the roots of *P. javanicum*. Phylogenetic analyses placed these taxa outside the recognized orchid mycorrhizal lineages (Tulasnellaceae, Ceratobasidiaceae, and Sebacinales), instead clustering with lignicolous white-rot fungi—for example, isolate KWAK07 with *Phanerochaete*, KWAK06 with *Climacodon*, MRAK03 with *Cubamyces*, SNAK02 with *Polyporus*, and MRAK05 with *Schizophyllum*. These fungi belong to diverse lineages of wood-decaying Basidiomycota.

In culture, their macroscopic growth forms were variable and often failed to produce diagnostic reproductive structures, making morphological identification challenging. Previous studies have similarly reported that isolates from these genera frequently remain sterile under laboratory conditions. As white-rot decomposers, these fungi are characterized by strong ligninolytic activity that drives wood decay and nutrient cycling in forest ecosystems. Their repeated recovery from orchid roots suggests that they may persist as opportunistic endophytes or incidental colonizers rather than functioning as true mycorrhizal symbionts. This observation is consistent with reports that basidiomycetous decomposers can occasionally enter orchid root tissues without forming functional pelotons. Ecologically, their role may be indirect: by decomposing litter and woody substrates in orchid habitats, they enrich the rhizosphere with organic byproducts and may influence the competitive dynamics faced by classical orchid mycorrhizal fungi. Moreover, their transient colonization of cortical cells could modulate host defense responses or shift the microbial balance within the velamen and cortex.

Although none of these lineages are currently recognized as classical orchid mycorrhizal fungi, the consistent detection of white-rot taxa in orchid roots warrants attention. Their ability to degrade lignocellulosic substrates positions them as potential contributors to nutrient cycling in forest ecosystems where *Paphiopedilum* occurs. Occasional reports of *Schizophyllum* and other lignicolous fungi behaving as orchid endophytes (Salazar-Cerezo et al. 2018; Bhatti and Thakur 2022) further highlight the fluid boundaries between strict saprotrophy and opportunistic root colonization. In sum, these non-mycorrhizal Basidiomycota likely represent a secondary but ecologically relevant component of the orchid root microbiome, with roles distinct from, yet complementary to, the *Rhizoctonia*-like mycorrhizal fungi.

Non *Rhizoctonia* Ascomycota

A substantial proportion of the isolates obtained from *P. javanicum* roots belonged to the Ascomycota, including *Fusarium*, *Trichoderma*, *Penicillium*, *Arcopilus*, *Xylaria*, *Geotrichum*, *Hypoxylon*, and *Nigrospora*. Although these genera are not traditionally regarded as orchid mycorrhizal fungi, many of them have been repeatedly reported from orchid roots in both tropical and temperate regions. Their recurrent detection across orchid lineages suggests that they represent a stable and widespread component of the orchid mycobiome rather than incidental contaminants.

Across these taxa, several colony characteristics were broadly consistent. Most Ascomycota isolates grew rapidly on PDA, typically forming cottony to floccose colonies with colours ranging from white and pale pink (*Fusarium*) to green (*Trichoderma*) or darkly pigmented (*Xylaria*, *Hypoxylon*). Sporulation was frequent, and diagnostic structures were readily observed: *Fusarium* produced falcate macroconidia and abundant microconidia; *Trichoderma* formed compact conidiation in concentric zones; *Penicillium* generated broom-like penicilli; *Geotrichum* produced arthroconidia; and *Nigrospora* developed characteristic black, globose conidia. By contrast, Xylariaceae members formed initially pale colonies that darkened over time and were often slow to sporulate. None of these isolates formed pelotons within root cortical cells, distinguishing them functionally from the *Rhizoctonia*-like mycorrhizal fungi.

Figure 6. Microscopic features representing common characteristics of orchid mycorrhizal fungi. A. Septate hyphae of MRAK02 with right-angle branching and slight constrictions at the branch points, typical of *Rhizoctonia*-like fungi, B. Chains of monilioid cells (swellings) of CIAK02, a diagnostic feature associated with peloton formation inside orchid cortical cells. Scale bar: 50 µm

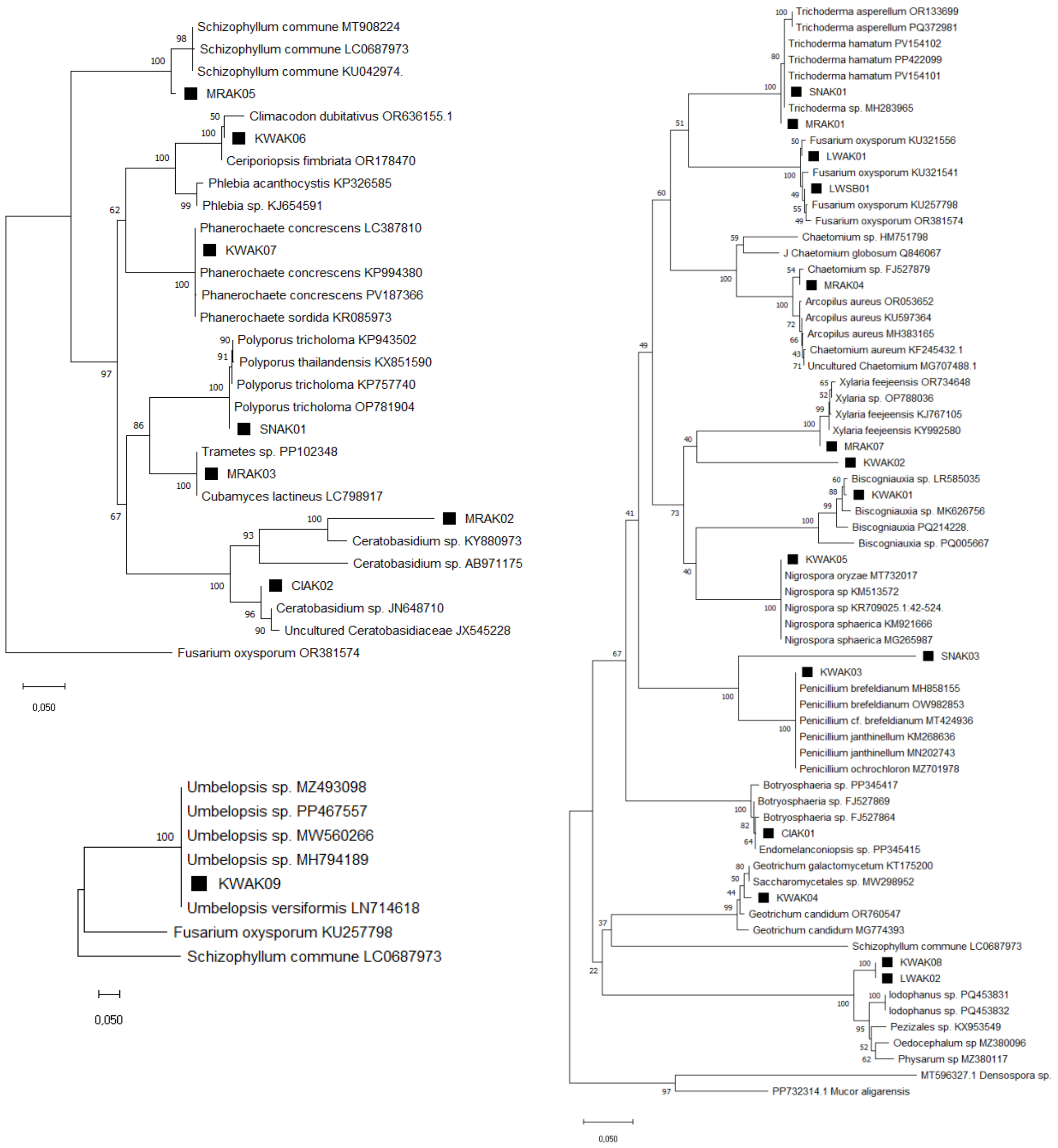

Figure 7. Taxonomic placement of fungal isolates from *Paphiopedilum javanicum* roots and seeds inferred from ITS sequence analysis. Isolates are grouped with reference sequences from GenBank, and a Neighbor-Joining Phylogenetic Tree was constructed with bootstrap support values shown

Functionally, these Ascomycota are best interpreted as opportunistic or facultative endophytes. Several genera — particularly *Trichoderma* and *Penicillium* are known to produce antimicrobial metabolites, mobilize nutrients, or enhance stress tolerance (Shankar Naik 2019). Also, the ecological role of *Fusarium*, however, is more context-dependent, although frequently associated with pathogenic symptoms, including root rot and reduced seedling survival (Benyon et al. 1996; Srivastava et al. 2018), it has also been reported as an asymptomatic endophyte (Sufaati et al. 2016) and, in some cases, as a putative peloton-forming associate in orchid seed germination (Jiang et al. 2019). These contrasting findings highlight the ecological plasticity of *Fusarium*, suggesting that its impact on orchids may shift along a spectrum from pathogenic to mutualistic, depending on factors such as host species, environmental conditions, and fungal strain identity. Xylariaceae are also widely documented orchid endophytes and appear to occupy a persistent niche within orchid roots despite lacking peloton formation (Ma et al. 2022). Thus, although these taxa do not represent classical orchid mycorrhizal fungi, their colonization of *P. javanicum* roots is consistent with a broader pattern observed across orchids globally.

Taken together, these findings reinforce the concept that orchids coexist not only with *Rhizoctonia*-like mycorrhizal partners but also with a diverse assemblage of non-mycorrhizal endophytes that may contribute indirectly to host fitness. Such fungi may influence rhizosphere microbial dynamics, suppress pathogens, or alter nutrient environments, forming part of a flexible symbiotic network that supports orchid survival under variable ecological conditions.

Figure 7 confirms that the fungal community associated with *P. javanicum* is taxonomically diverse, spanning three major fungal phyla. ITS-based phylogeny placed two of our isolates (MRAK02 and CIAK02) within, or immediately adjacent to, the Ceratobasidiaceae. This lineage includes many well-characterized orchid mycorrhizal fungi, supporting the interpretation that these strains represent true *Rhizoctonia*-like mycorrhizal partners rather than incidental colonizers. The remaining Basidiomycota clustered with non-mycorrhizal white-rot taxa such as *Schizophyllum*, *Phanerochaete*, *Polyporus*, and *Climacodon*, indicating that most basidiomycetes recovered here are not part of the classical orchid mycorrhizal guild.

In contrast, the majority of recovered isolates were Ascomycota, including members of the Xylariaceae (*Xylaria*, *Hypoxylon*, *Biscogniauxia*), Chaetomiaceae (*Arcopilus*), Nectriaceae (*Fusarium*), and Trichocomaceae (*Penicillium*). These taxa grouped confidently with named reference strains, indicating reliable placement at the genus level, even though many do not form orchid mycorrhiza. Their strong representation suggests that endophytic and saprotrophic fungi form a major component of the *Paphiopedilum* root microbiome. The placement of one isolate within *Umbelopsis* (Mucoromycota) further highlights the breadth of fungal associations and supports the view that orchid roots host a heterogeneous microbial assemblage extending beyond canonical orchid mycorrhizal fungi.

Taken together, the phylogeny confirms two co-occurring but functionally distinct guilds: (i) *Ceratobasidium*-like mycorrhizal fungi, likely essential for germination and nutrient exchange, and (ii) a diverse suite of opportunistic Ascomycota and non-*Rhizoctonia* Basidiomycota, which may function as endophytes, saprotrophs, or microbial competitors. This pattern mirrors reports from other *Paphiopedilum* species globally, yet the dominance of non-*Rhizoctonia* fungi in our Indonesian populations emphasizes the ecological plasticity and regional variability of *Paphiopedilum*-fungal symbioses.

Discussion

This study provides a comprehensive assessment of root-associated fungal diversity in P. javanicum from several montane habitats in Java, Indonesia—a region where such data remain scarce. Our findings of fungal isolates across fungal phyla mainly Ascomycota (15 isolates), Basidiomycota (7 isolates), and Mucoromycota (1 isolate). These results contribute novel insights into the mycorrhizal and endophytic associations of this threatened orchid, with important implications for both conservation and propagation.

Mycorrhizal partners: Confirmed and putative

Anatomical observations confirmed the presence of pelotons within cortical cells, substantiating the occurrence of mycorrhizal associations in natural populations. Notably, however, efforts to isolate fungi directly from pelotons were unsuccessful, possibly due to non-viable or highly host-dependent fungal structures at the time of sampling. Instead, *Ceratobasidium* isolates—recovered via root-segment and seed-baiting methods—exhibited features consistent with known orchid mycorrhizal fungi. While these isolates are strong candidates for symbiotic germination and propagation trials, their functional capacity as true mycorrhizal partners requires further experimental validation. This pattern of encountering putative, rather than confirmed, mycorrhizal partners echoes reports from other *Paphiopedilum* studies, especially in tropical and subtropical regions, where successful peloton isolation is often challenging and mycorrhizal specificity appears variable.

Roles of opportunistic endophytes

The majority of cultured isolates belonged to Ascomycota (e.g., *Fusarium*, *Penicillium*, *Arcopilus*, *Xylaria*, *Nigrospora*) and included some Basidiomycota (e.g., *Phanerochaete*, *Climacodon*, *Polyporus*, *Schizophyllum*). This broad taxonomic diversity—and the abundance of fast-growing, opportunistic fungi—parallels findings from global *Paphiopedilum* research, where culture-based methods often underrepresent obligately biotrophic mycorrhizal taxa such as *Tulasnella*. The presence of diverse endophytes and saprotrophs supports the view that the *P. javanicum* root mycobiome is not restricted to classical orchid mycorrhizal fungi, but instead represents a mosaic of taxa with potential roles in substrate modification, defense, or latent symbiosis. Such complexity suggests functional redundancy and ecological flexibility in fungal associations, which may buffer plants against environmental fluctuations—a pattern increasingly recognized in tropical orchid systems.

Conservation and propagation implications

Our results highlight both opportunities and challenges for conservation. The detection of *Ceratobasidium* isolates as putative mycorrhizal partners is promising for developing symbiotic germination protocols and ex situ propagation strategies for *P. javanicum*. At the same time, the rich community of non-canonical endophytes may serve as a reservoir of beneficial traits that could enhance seedling establishment, stress tolerance, or disease resistance. Comparative evidence from other regions indicates that *Paphiopedilum* species exhibit a spectrum of mycorrhizal specificity, from narrow *Rhizoctonia*-like symbiont dependence (e.g., *Tulasnella* in China and Southeast Asia) to broader associations involving *Ceratobasidium* and other fungi. Our findings suggest that *P. javanicum* may possess similar flexibility in its fungal partnerships, which could be advantageous in fragmented or changing habitats, but also caution against a one-size-fits-all approach to conservation and propagation. Because *Paphiopedilum* survival is inseparably linked to fungal symbionts (Parthibhan and Ramasubbu 2020; Tian et al. 2022) understanding their diversity is essential for designing propagation protocols, guiding reintroduction efforts, and informing habitat management. In doing so, this research not only advances ecological knowledge but also provides a practical foundation for safeguarding some of the most threatened and charismatic orchids in the country.

These considerations are particularly important in the context of accelerating climate change and anthropogenic habitat disturbance, which may disrupt orchid-fungal compatibility by altering fungal availability, microhabitat conditions, and host recruitment dynamics. Within such unstable environments, the recovery of viable fungal isolates remains highly valuable, as culturable symbionts can be experimentally validated and intentionally incorporated into adaptive conservation strategies. Integrating compatible fungal partners into assisted propagation, translocation, and reintroduction programs may therefore enhance establishment success and improve resilience of *P. javanicum* populations under ongoing environmental change.

Future directions

This study provides a baseline characterization of root-associated fungi in *Paphiopedilum javanicum* from Java, revealing diverse fungal partnerships and addressing a major knowledge gap on orchid-fungal symbioses in Indonesia. The findings offer an initial reference for understanding symbiotic interactions in native slipper orchids and may support future research and conservation efforts across the Indonesian archipelago.

However, several limitations must be acknowledged. The study relied primarily on culture-based isolation, which may underrepresent obligately biotrophic or slow-growing mycorrhizal taxa. Direct peloton isolation was unsuccessful, limiting functional confirmation of mycorrhizal status. In addition, ITS sequencing allows reliable genus-level placement but does not establish ecological function. To fully resolve the ecological roles of these fungi—and to optimize strategies for conservation and restoration—future work should integrate culture-based isolation with high-throughput sequencing and functional assays, including symbiotic germination trials. Such approaches will clarify whether *P. javanicum* relies on a narrow set of *Rhizoctonia*-like fungi or can exploit a broader range of root-associated partners, ultimately informing more effective management of this endangered orchid in Indonesia and beyond.

In conclusion, this study revealed that microscopic observations confirmed peloton formation restricted to cortical cells, with colonization occurring in a patchy and spatially heterogeneous pattern. From 75 root segments and complementary seed-baiting, 23 fungal isolates were recovered. ITS-based identification revealed that 15 isolates (≈65%) belonged to Ascomycota, 7 isolates (≈30%) to Basidiomycota, and 1 isolate (≈4%) to Mucoromycota. Among the Basidiomycota, two isolates were identified as *Ceratobasidium* spp., consistent with *Rhizoctonia*-like orchid mycorrhizal fungi. The remaining basidiomycetes clustered with lignicolous white-rot taxa, while the majority of isolates comprised opportunistic or facultative endophytes such as *Fusarium*, *Trichoderma*, *Penicillium*, and Xylariaceae members. These results demonstrate that *P. javanicum* roots harbor a diverse fungal community composed of both putative mycorrhizal symbionts and non-mycorrhizal endophytes, indicating ecological complexity and potential flexibility in fungal associations.

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