CTU Journal of Innovation and Sustainable Development
ISSN 2588-1418 | e-ISSN 2815-6412

DOI:10.22144/ctujoisd.2026.037

Identification of *Exserohilum rostratum* causing leaf spot on *Dendrobium amabile* and *Dendrobium clavatum*

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Article info.

Received 30 Jan 2026
Revised 18 May 2026
Accepted 25 Jun 2026

Keywords

Dendrobium amabile, Dendrobium clavatum, Exserohilum rostratum, leaf spots, multi-locus, native orchid

ABSTRACT

Fungal diseases are among the most persistent and underestimated threats to orchid cultivation, often causing severe decline if not detected early. In this study, leaf spot disease was recorded on two native orchid species, Dendrobium amabile and D. clavatum. To identify the causal agent, symptomatic leaves were collected and subjected to fungal isolation, pathogenicity testing, morphological characterization, and multi-locus sequence analysis. Artificial inoculation reproduced typical leaf spot symptoms, confirming pathogenicity. Morphological traits of colonies and conidia indicated affiliation with the genus Exserohilum. Molecular analyses of the ITS, LSU, TEF1, and ACT gene regions further resolved the isolates as E. rostratum, with high sequence similarity among the representative strains. Minor morphological variation was noted, particularly in conidial dimensions, but overall consistency supported species-level identification. This study establishes a new host association between E. rostratum and D. amabile and D. clavatum and demonstrates the effectiveness of combining morphological and molecular approaches for precise fungal identification. The generated multilocus dataset provides a useful reference for future studies on orchid pathogens and fungal taxonomy.

1. INTRODUCTION

The Orchidaceae family is among the most diverse and economically valuable plant groups. These plants are important for both biodiversity conservation and the ornamental horticulture industry. Global conservation analyses by Vitt et al. (2023) highlight the need to prioritize regions with high evolutionary distinctiveness and taxa with insufficient data. Viet Nam is recognized as a key global biodiversity hotspot, known for its substantial endemism and species richness. In a recent detailed account of Viet Nam's orchid diversity, Averyanov et al. (2025) documented 1390 species across 188 genera, with the genus *Dendrobium* alone comprising 139 species. However, consistent with global trends identified by Vitt et al. (2023), these populations face severe extinction risks that are exacerbated by incomplete taxonomic and phylogenetic data. Sixty-eight orchid species in Viet Nam are currently designated as rare, endangered, and precious and require immediate conservation actions (Ministry of Science and Technology, 2007). Beyond conservation challenges, orchid populations are increasingly threatened by fungal diseases, which can reduce plant health and survival in both natural habitats and cultivated collections.

1.1. Leaf spot disease of *Dendrobium* orchids

Leaf diseases are a major constraint on the growth and productivity of orchids. Since July 2023, leaf spots have been observed on *Dendrobium amabile* (Kiều tím) and *Dendrobium clavatum* (Hoàng thảo Kim Thoa) in a native orchid collection in Ho Chi Minh City. *D. amabile* is classified as an Endangered species (EN), prioritized for conservation in Viet Nam (Ministry of Science and Technology of Viet Nam, 2007). Initial symptoms appeared as small black spots on the leaves, which gradually developed into irregular, brown lesions with distinct black margins. Although the disease was not immediately lethal, its symptoms rapidly spread to other Dendrobium plants in the collection, and isolation measures became necessary.

1.2. Pathogen identification

According to Agrios (2005), plant pathogens encompass viruses, bacteria, fungi, nematodes, and parasitic plants, with fungi being the most frequent causal agents of foliar diseases. In Viet Nam, several fungal species causing leaf spot on *Dendrobium* orchids have been reported, including *Phyllosticta capitalensis*, *Curvularia intermedia*, *Nigrospora oryzae*, and *Pestalotiopsis* sp. (Huynh et al., 2025). However, the morphology of the lesions observed on *D. amabile* and *D. clavatum* differs from the spots previously reported on *Dendrobium* species.

Accurate fungal identification requires both morphological analysis with molecular techniques for taxonomic consistency, as emphasized by Crous et al. (2009). In addition to morphological data, we performed multi-locus sequence analysis of the causal agent. The analyzed gene regions include the ITS (Internal Transcribed Spacer), TEF1 (translation elongation factor 1-alpha), LSU (Large Subunit), and ACT (Actin gene). Therefore, the objective of this study was to identify the causal agent of leaf spot disease affecting *D. amabile* and *D. clavatum* using morphological characterization, pathogenicity testing, and multilocus phylogenetic analysis.

2. MATERIALS AND METHOD

2.1. Sample collection and fungal isolation

Leaves of *D. amabile* and *D. clavatum* showing typical brown spot symptoms were collected from a native orchid collection garden in Ho Chi Minh City, Viet Nam (10°55'08.2" N 106°40'05.2" E). Sampling was conducted on three occasions during July, August, and September 2023. Within the collection, which comprised more than 20 *Dendrobium* cultivars, characteristic brown spot symptoms were observed exclusively on *D. amabile* and *D. clavatum*. Disease incidence was high, with all 11 D. clavatum plants and 17 of 21 D. amabile plants showing symptoms. In total, 20 symptomatic leaves (10 from each species) were collected for fungal isolation. The collected leaves were surface dried and stored in sterile ziplock bags containing silica gel desiccants. All samples were transported to the laboratory and processed for fungal isolation within 5 hours.

Fungal isolation was conducted according to the protocol of (Huynh et al., 2025) with minor modifications. Briefly, leaf surfaces were disinfected with 70% Ethanol for 90 seconds and 1% Sodium Hypochlorite solution for 90 seconds, followed by rinsing three times with sterilized water. The treated tissues were then plated onto 1.5% water agar (WA; 242 M, TMMEDIA) and incubated at 28 ± 2°C in the dark to induce hyphal growth. After 48–72 hours, actively growing hyphal tips (2 mm) were excised from the colony margins and transferred to 1.5% potato dextrose agar (PDA; GM096, HIMEDIA) for purification and further morphological and molecular characterization. Cultures were maintained at 28 ± 2°C in the dark for further study.

2.2. Pathogenicity test

Pathogenicity was tested on healthy, detached leaves of Dendrobium using the method of Huynh et al. (2025), with modifications. Briefly, leaves were surface sterilized in 70% Ethanol for 3 min, rinsed three times with sterile water, and dried on sterile paper towels. Each fungal isolate was inoculated onto four leaves, with three wound sites per leaf, each approximately 0.7 mm in diameter. Inoculated leaves were placed in sterile containers lined with moist paper to maintain high humidity and incubated at 28°C under a 12-hour light/dark cycle. Conidial suspensions were prepared from 7-day-old cultures grown on PDA by washing sporulating colonies with sterile distilled water and filtering through sterile gauze to remove mycelial fragments, then adjusted to 10^6–10^8 spores/mL. For inoculation, 10 µL of suspension was applied to each wound site. A conidial suspension was applied to each site, while sterile water served as the control. After seven days, pathogenicity was assessed by tissue discoloration. Fungal isolation from symptomatic tissues was performed to confirm Koch's postulates by comparing the morphology of

the re-isolated fungi with that of the original inocula and by matching the induced symptoms with those observed under natural field conditions. Pathogenicity assays were conducted twice.

2.3. Morphological observations

Morphological characterization involved both macroscopic and microscopic observations. Micromorphological structures were measured on PDA cultures incubated for 7 – 10 days (based on at least 40 replicates) and photographed using a compound microscope (L1100A, Shinea). To observe appressoria, mycelia were transferred to 1 × 1 cm WA blocks on glass slides and incubated in a moist chamber at 28°C in darkness, as described by Yang et al. (2009) with minor modifications. Taxonomic identification was performed in accordance with the morphological descriptions provided by Phillips et al. (2013).

2.4. DNA extraction, PCR amplification, and phylogenetic analysis

Genomic DNA was extracted from fungal cultures grown on PDA for seven days using the Phenol–Chloroform method described by Nakayashiki et al. (1999). PCR amplification targeted four loci (ITS, TEF1, LSU, and ACT) with primer pairs ITS1/ITS4, EF1-1080/EF1-1620, LR0R/LR5, and Act-512/Act-783, respectively. Thermal cycling conditions followed Hernández-Restrepo et al. (2018). PCR products were examined by electrophoresis on 1.5 percent agarose gels, visualized in a gel viewing chamber, purified using a PrimeWay Gel Extraction and PCR Purification Kit (1st BASE), and sequenced bidirectionally.

Forward and reverse sequences were assembled in Geneious Prime (version 11.0.20.1+1). Consensus sequences were compared with reference sequences in the NCBI database using BLAST (version 2.15.0). For reliability, low-quality reads were trimmed, and ambiguous bases were manually corrected before alignment. Sequences obtained here and reference sequences from GenBank were aligned using multiple alignment. Phylogenetic trees were constructed using the maximum likelihood method implemented in MEGA (version 12.1) with the GTR+G substitution model. An appropriate outgroup was included to root the tree, and branch support was evaluated with 1000 bootstrap replicates.

3. RESULTS AND DISCUSSION

3.1. Symptoms and isolation of *Exserohilum* sp.

Leaf spot symptoms on *Dendrobium* were characterized by small brown lesions with distinct black margins on both leaf surfaces. The lesions were observed on both young and mature leaves and occasionally on stems (Figure 1A). A total of 20 symptomatic leaves were collected, including 10 leaves of *D. amabile* and 10 leaves of *D. clavatum*, from which several fungal isolates were obtained. Seventeen isolates (10 from *D. clavatum* and 7 from *D. amabile*) produced dark colonies, and their conidia were elongate, brown, and septate. Based on conidial morphology, these isolates were tentatively identified as belonging to the genus *Exserohilum*, according to the taxonomic key of Sivanesan (1987).

3.2. Pathogenicity of *Exserohilum* sp.

Six isolates of *Exserohilum* were used in the pathogenicity test, including three from *D. clavatum* and three from *D. amabile.* The results showed that inoculated leaves developed tissue discoloration and lesion formation, with lesions reaching up to 2 mm in diameter after 7 days of infection, similar to the natural leaf spot symptoms observed in the field (lesions under natural conditions ranged from < 1 mm to 2.5 mm) (Figure 1B). Control leaves remained symptomless. The same fungus was successfully re-isolated from the inoculated tissues, thus fulfilling Koch's postulates and confirming *Exserohilum* sp. as a pathogenic agent associated with leaf spot disease on *Dendrobium* orchids.

3.3. Morphological characteristics of *Exserohilum* sp.

All isolates produced dark colonies and abundant conidia on PDA. Seventeen isolates were examined, with detailed descriptions provided for two representatives, 1-Dam (from *D. amabile*) and 1-Dcl (from *D. clavatum*), to capture the observed morphological diversity. Both isolates showed similar growth rates, averaging 9.0 mm/day on PDA, 8.6 mm/day on OA, and 9.2 mm/day on SNA. Colony coloration varied with medium: dark brown to olivaceous on PDA, white to pale brown on OA, and hyaline with sparse aerial mycelium on SNA.

Microscopic examination revealed geniculate conidiophores producing conidia terminally. Conidia were brown, septate, and exhibited a conspicuously protruding, truncated hilum, a diagnostic feature of *Exserohilum.* Isolate 1-Dcl

produced conidia ranging from 20.3 – 78.8 × 9.6 – 24.4 µm (n = 50), with 2 – 7 septa, ellipsoidal to rostrate in shape. Meanwhile, isolate 1-Dam produced longer conidia measuring 30.4 – 170.9 × 7.0 – 22.9 µm (n = 50), with a narrower basal end and 2 – 14 septa. Chlamydospores were observed on SNA and OA, appearing globose and hyaline. Both isolates also formed appressoria that were globose to clavate, similar to the bulbous structures typical of attachment organs reported in *Exserohilum* species. These morphological details are illustrated in Figure 2A and Figure 2B.

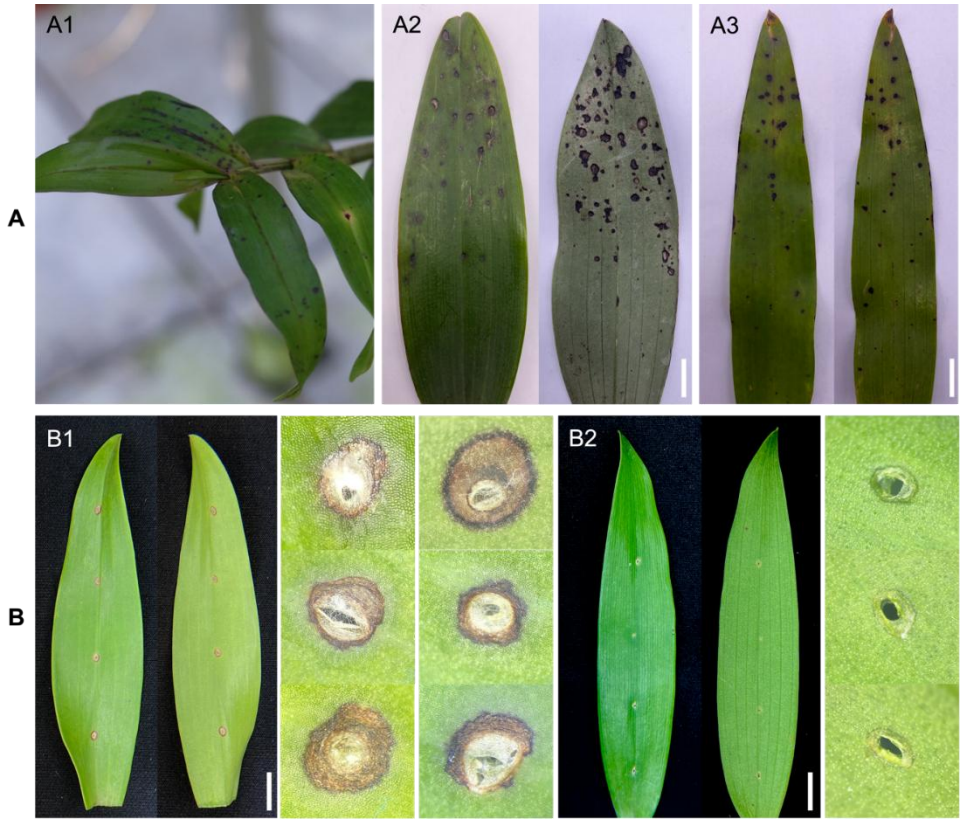


Figure 1. Leaf spot disease on Dendrobium orchids caused by *Exserohilum* sp.

(A) Leaf spot symptoms observed in the field: A1. Scattered lesions on orchid leaves, A2. Lesions on D. amabile (left: adaxial; right: abaxial), A3. lesions on D. clavatum. (B) Pathogenicity test on detached leaves: B1. Brown necrotic lesions with dark margins after inoculation, B2. Control leaves showed no discoloration. Scale bars = 1 cm.

The variation in conidial dimensions and septation observed among the orchid isolates of *Exserohilum* species indicates considerable intraspecific morphological diversity within the genus. Similar variability has been reported in taxonomic studies, where conidial size ranges often overlap with related taxa. Therefore, identification based solely on morphology is unreliable (Sivanesan, 1987). In fact, Hernández-Restrepo et al. (2018) documented conidia of *E. rostratum* measuring 40–160 × 8–20 µm with 5–14 septa, which match the dimensions observed in our isolates. Nevertheless, the presence of a protruding truncated hilum remains a stable diagnostic character for *Exserohilum*. Therefore, although the orchid isolates fit the morphological description of Exserohilum, their variability necessitates the use of molecular data for accurate species identification.

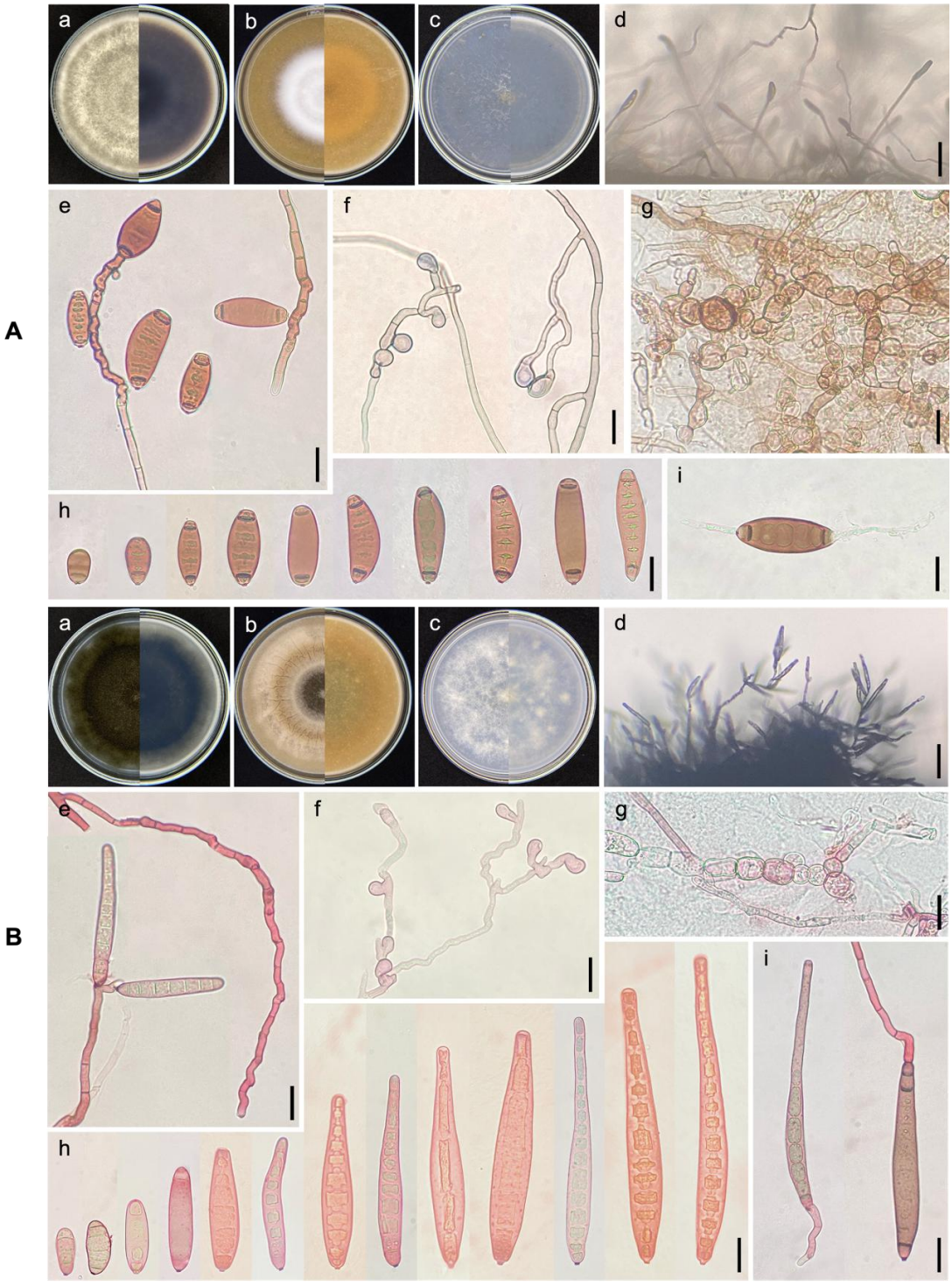
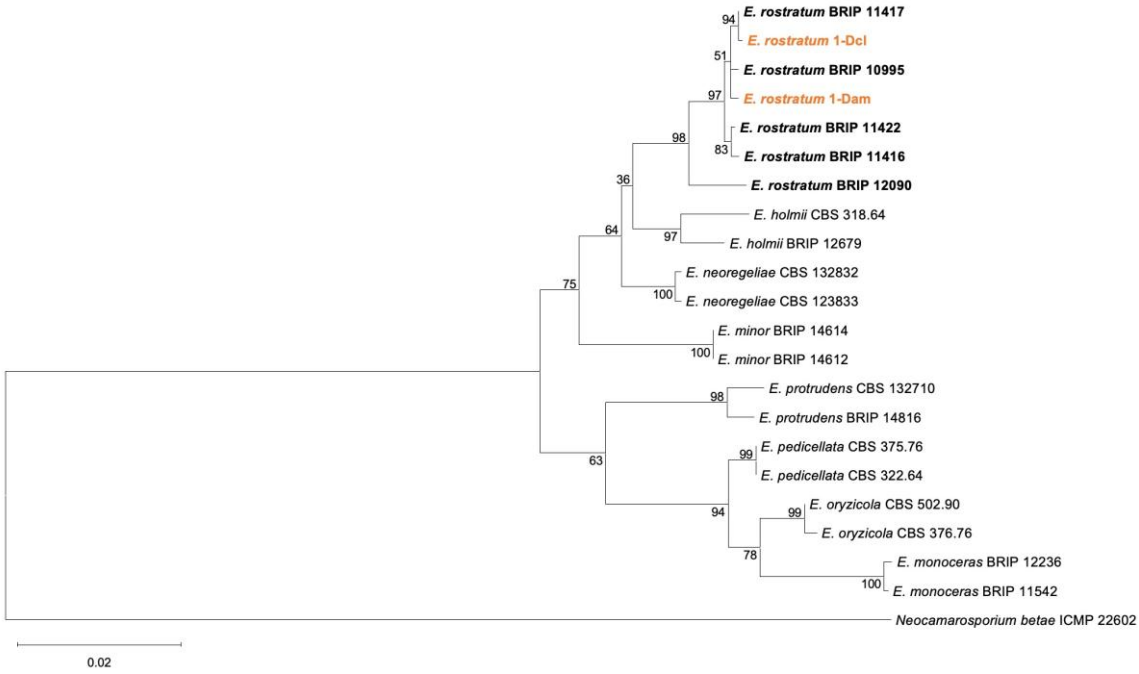


Figure 2. Morphological characteristics of *Exserohilum* sp. from *Dendrobium* orchids

(A)Isolate 1-Dcl from Dendrobium clavatum, (B) isolate 1-Dam from Dendrobium amabile
a. Colony on PDA (left: upper surface; right: reverse), b. Colony on OA, c. Colony on SNA, d-e. Conidiophores, f. Appressoria, g. Chlamydospores, h. Conidia, i. Germinating conidia. Scale bars = 10 µm.

A



B

	ITS	TEF1	LSU						ACT									
	36	870	1343	1459	1499	1543	1616	1724	1897	1904	1924	1925	1932	1934	1944	1956	1994	2078
1-Dam	A	C	C	T	C	C	C	C	C	G	A	T	Y	-	T	C	B	G
1-Dcl	-	T	C	-	C	C	C	C	T	G	G	C	T	A	T	T	G	G
BRIP 10995	A	C	C	T	T	C	C	T	C	G	G	C	T	-	T	C	G	G
BRIP 11416	A	C	C	T	C	G	T	C	C	A	G	C	T	-	C	C	G	A
BRIP 11417	A	C	C	T	C	C	C	C	T	G	G	C	T	A	T	T	G	G
BRIP 11422	A	C	T	T	C	G	C	C	C	A	G	C	T	-	C	C	G	G

Figure 3. Multi-locus phylogeny analysis and sequence comparison of *E. rostratum* from *Dendrobium*

(A) Phylogenetic tree based on concatenated ITS, TEF1, LSU, and ACT sequences of isolates 1-Dam and 1-Dcl with Exserohilum spp. Bootstrap values are indicated at branch nodes. Neocamarosporium betae was used as the outgroup. (B) Nucleotide sequence differences in ITS, LSU, TEF1, and ACT loci between E. rostratum isolates from Dendrobium and reference sequences from GenBank. UPAC codes are used: Y = C or T; B = C, G, or T.

3.4. Molecular characteristics of *E. rostratum*

Representative isolates 1-Dam and 1-Dcl were analyzed at four loci. The amplified fragments corresponded to the expected sizes, approximately 700 bp for ITS and TEF1, 1000 bp for LSU, and 300 bp for ACT. BLAST searches in the NCBI database showed high similarity (≥ 98.3%) to *E. rostratum* reference sequences (Table 1). The sequences of both isolates have been deposited in GenBank, with accession numbers as follows: isolate 1-Dam (PZ456912 for ITS, PZ456932 for LSU, PZ463011 for TEF1, and PZ481741 for ACT) and isolate 1-Dcl (PZ456913 for ITS, PZ462788 for LSU, PZ481740 for TEF1, and PZ481742 for ACT).

Phylogenetic reconstruction using the maximum-likelihood method consistently grouped both isolates within the E. rostratum clade, as supported by strong bootstrap values (Figure 3A). The isolates clustered tightly with reference strains of *E. rostratum* in a well-supported monophyletic group, distinct from closely related taxa such as *E. holmii* and *E. neoregeliae*. Alignment of the concatenated

sequences revealed 18 polymorphic sites across 2148 nucleotides. Variation was limited in ITS and TEF1, each showing a single polymorphic site, whereas LSU and ACT exhibited greater diversity, with six and ten sites, respectively (Figure 3B).

Table 1. BLAST similarity (%) of isolates 1-Dam and 1-Dcl with *E. rostratum* on GenBank

Isolate	Locus	Similarity to *E. rostratum*	
		% Identity	Accession
1-Dam	ITS	100.0	PX232758
	TEF1	99.8	OQ857405
	LSU	100.0	MH877985
	ACT	98.3	LT837664
1-Dcl	ITS	99.7	PX232758
	TEF1	99.5	OQ857405
	LSU	100.0	MH877985
	ACT	98.5	LT837664

These findings confirm that, despite the morphological heterogeneity observed among isolates, all belong to *E. rostratum*. The concentration of polymorphisms in LSU and ACT is notable, given that LSU is usually considered a conserved locus. Such variation may result from microevolutionary processes or host-linked adaptation, as shown in previous phylogenetic revisions that emphasize the importance of multi-locus datasets for accurate species delimitation (Hernández-Restrepo et al., 2018). Further comparative analyses will be necessary to determine whether LSU variation indicates genuine phylogenetic signal or methodological noise.

To our knowledge, this is the first report of *E. rostratum* as a fungal pathogen causing leaf spot in *Dendrobium* orchids, including native species such as *D. amabile* and *D. clavatum*. Although *E. rostratum* has long been recognized as a causal agent of leaf spot and blight in staple crops like rice (*Oryza sativa*) and maize (*Zea mays*), its occurrence on orchids is a novel host record relevant to orchid pathology and conservation (Boonkorn et al., 2024; Wang et al., 2025). The ability of *E. rostratum* to produce both infection structures (appressoria) and survival propagules (chlamydospores) suggests that the fungus may persist in orchid collections. Further studies are required to determine whether this adaptive capacity also enables infection of intact orchid leaves under natural, unwounded conditions. By documenting *E. rostratum* as a novel pathogen of native *Dendrobium*, these findings support integrating plant pathology into conservation strategies. Continuous monitoring and effective disease management are needed to protect vulnerable orchid populations from disease threats, in addition to habitat and taxonomic challenges.

4. CONCLUSION

Combined molecular and morphological analyses confirmed *E. rostratum* as the causal agent of leaf spots on *D. amabile* and *D. clavatum*. Beyond establishing this novel host association, this study also provides morphological and multilocus data on *E. rostratum* from orchid hosts, which may be useful for further work on orchid pathology, fungal diversity, and disease management.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

ACKNOWLEDGMENT

We sincerely thank former students of the Plant Pathology Laboratory for generously supporting this research by providing essential equipment. We are also grateful to Thach Ba Thien and Huynh Nguyen Thuy Duong for their assistance with material preparation and laboratory support.

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