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Paramyrothecium amorphophalli sp. nov., a causal agent of leaf blight on elephant foot yam in northern Thailand

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Abstract

Paramyrothecium was introduced in 2016 containing 20 species and many species have been reported as plant pathogens, causing leaf blight and leaf spots on different plant species. This study revealed a new species *Paramyrothecium amorphophalli* from symptomatic leaves of elephant foot yam (*Amorphophallus* sp.), collected from Chiang Rai, Thailand based on distinct morphological characteristics and phylogenetic analyses. The isolate formed a distinct lineage from *Paramyrothecium eichhorniae* and *P. vignicola* based on maximum likelihood, maximum parsimony and Bayesian posterior probability analyses of ITS, *cmdA*, *rpb2* and *tub2* sequence data. Furthermore, pathogenicity assays confirmed that *P. amorphophalli* is pathogenic to the original host, causing leaf blight.

Keywords – 1 new species – multi-locus phylogeny – plant pathogen – taxonomy – yam

Introduction

Elephant foot yam is a tropical tuber crop that belongs to *Amorphophallus.* It is a significant edible tuberous crop extensively cultivated in tropical and subtropical regions, especially in China and India (John et al. 2014, Prasad et al. 2017). Konjac Glucomannan (KGM) extracted from the tuber of *Amorphophallus konjac*, is used as a medicinal ingredient and nutritional supplement (Yao-ling et al. 2013). Among the significant health advantages of KGMs are those related to lowering cholesterol, regulating triglyceride levels, enhancing sugar levels, and promoting human immunological and intestinal functions (Yao-ling et al. 2013). Among plant pathogens, fungi are considered devastating agents responsible for economic losses in the agriculture circle and threatening food security (Hyde et al. 2018, Gomdola et al. 2022, Withee et al. 2022). Prasad et al. (2017) reported *Colletotrichum siamense* from India causing anthracnose on elephant foot yam. *Sclerotium rolfsii* is a devastating soil-borne disease which causes collar rot on this plant (Sahoo et al. 2016). As the correct identification is the first and vital step in disease management, it becomes imperative to consider a proper taxonomic identification and understanding of fungi to undertake appropriate strategies for managing plant diseases (Bhunjun et al. 2021, Jayawardena et al. 2021).

Paramyrothecium (*Stachybotryaceae, Hypocreales, Sordariomycetes*) (Wijayawardene et al. 2022) was introduced to accommodate taxa earlier included in *Myrothecium sensu lato* (Lombard et al. 2016). According to morphological characteristics and molecular analyses, Lombard et al.

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(2016) showed that this group of fungi is distinguished from other *Myrothecium* species and subsequently established *Paramyrothecium* (Lombard et al. 2016). *Paramyrothecium* is a plant pathogenic genus and among the 20 species reported in this genus (MycoBank 2023), *Paramyrothecium foliicola* and *P. roridum* have been widely isolated from different plant hosts as leaf spot causal agents (Chen et al. 2016, Ben et al. 2017, Withee et al. 2022). *Paramyrothecium foliicola* has been reported on a variety of hosts, including melon (Sabahi et al. 2022), tomato (Huo et al. 2022) and eggplant (Aumentado & Balendres 2022). It has also been isolated from cucumber as the causal agent of stem canker in seedlings (Huo et al. 2021). *Paramyrothecium foliicola* was also discovered in Iran as the causative agent of apple tree canker (Azizi et al. 2020). *Paramyrothecium roridum* as well as *P. breviseta* have been reported from coffee, causing leaf spots and stem rot (Wu et al. 2021, Huamán-Pilco et al. 2022). Wang et al. (2021) isolated *P. roridum* from the false dragonhead plant (*Physostegia virginiana*). Mulberry has also reportedly been infected by *P. roridum* (Pappachan et al. 2019). Moreover, the latter was isolated from muskmelon as a crown canker-causing agent (Chen et al. 2018).

Paramyrothecium eichhorniae and *P. vignicola*, isolated from *Eichhornia crassipes* and *Vigna* sp. respectively, are the latest new species additions to *Paramyrothecium* (Pinruan et al. 2022, Withee et al. 2022). Both species have been reported from northern Thailand. During microfungal investigations in the same part of the country, symptomatic leaves of elephant foot yam, resulting from a fungal attack, were collected. The fungus was isolated and subjected to morpho-molecular analyses. The present study aims to introduce the new species *Paramyrothecium amorphophalli* and confirm its pathogenicity on elephant foot yam.

Materials & Methods

Sample collection and fungal isolation

Symptomatic leaves with leaf spots were collected from living *Amorphophallus* sp. in Doi Pui Sai Khao, Phan District, Chiang Rai, Thailand, in September 2021. Fungal isolation and purification were conducted using the single-spore isolation method (Senanayake et al. 2020). The pure culture was maintained in slant cryogenic tubes and deposited in the culture collection of Mae Fah Luang University and the dried leaf with fungal fruiting bodies was deposited in the Mae Fah Luang University herbarium. Moreover, the isolate was submitted to the Index Fungorum (IF558845), Faces of Fungi (FoF12745) and Greater Mekong Subregion webpage (Chaiwan et al. 2021).

DNA extraction and PCR amplification

Genomic DNA was extracted from fresh mycelia grown on potato dextrose agar (PDA) for 10 days using the DNA Extraction Kit (Omega Bio-tek) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region, β-tubulin (*tub2*), calmodulin (*cmdA*) and RNA polymerase II second largest subunit (*rpb2*) were amplified by ITS5/ITS4 (White et al. 1990), Bt2a/Bt2b (Glass & Donaldson 1995), CAL–CL1/CAL–CL2A (O'Donnell et al. 1998) and 5F2/7–CR (O'Donnell et al. 2007), respectively. The polymerase chain reaction was performed in a total volume of 25 µL, containing 12.5 µL of 2 × Power Taq PCR Master Mix, 1 µL of each primer (20 µM), 1 µL genomic DNA and 9.5 µL deionized water. The PCR procedure was performed using the following conditions: initial denaturation at 94 °C for 4 min; 35 cycles of denaturation for 30 s at 94 °C, annealing at 55 °C (ITS); 57 °C (*tub2*); 51 °C (*cmdA*); 58 °C (*rpb2*) for 60 s, extension at 72 °C for 60 s; and the final extension at 72 °C for 10 min. The PCR reaction system with the total reaction volume was 25 µL PCR mixture. PCR amplification was performed in an Eppendorf (Master Cycler X50s) thermal cycler. PCR products were sequenced by SolGent Co., South Korea.

Phylogenetic analyses

The obtained sequences were searched using BLAST (Table 2) and related sequences were downloaded from GenBank according to BLASTn searches and recently published papers (Pinruan

et al. 2022, Withee et al. 2022) (Table 1). The individual loci were manually adjusted with MAFFT v.7 using the web server (http://mafft.cbrc.jp/alignment/server) (Katoh et al. 2019). BioEdit v.7.0.9.0 was used to manually further adjust the alignment where necessary (Hall 1999). Aligned sequences were automatically trimmed using command-based TrimAl software with the gappyout method. The alignment was converted to phylip and nexus formats using the online tool ALTER (Glez-Peña et al. 2010). *Albifimbria verrucaria* (CBS 328.52) and *A. viridis* (CBS 449.71) were used as the outgroups. The phylogeny was generated by maximum likelihood (ML) analysis using RAxML-HPC2 on XSEDE with bootstrapping of 1000 replicates. The GTR + GAMMA model of nucleotide evolution was used for maximum likelihood analysis. The best-fit evolutionary models for each dataset were evaluated using the Akaike Information Criterion (AIC) using jModeltest 2.1.10 on the CIPRES online platform. The best-fit evolutionary models for *cmdA* and ITS were HKY+I+G, K80+I for *rpb2* and TIM2+I+G for *tub2* (Nylander 2004). The Bayesian posterior probability (BYPP) analysis was performed using a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities in MrBayes on XSEDE (Ronquist et al. 2012). For the BYPP analysis, four MCMC chains were run from random trees for 1,000,000 generations and sampled every 100th generation. The first 25% of the generated trees were discarded as the burn-in and the remaining trees were used for calculating posterior probabilities. Maximum parsimony (MP) analysis was carried out using PAUP XSEDE (Swofford 2002) and gaps were treated as missing data and ambiguously aligned regions were excluded. Trees were inferred using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1,000 random sequence additions. All these analyses were performed on the CIPRES Science Gateway (https://www.phylo.org/portal2) (Miller et al. 2011). The resulting phylograms were visualized in FigTree v. 1.4.0 (Rambaut and Drummond 2012) and annotated in Adobe Illustrator CC 22.0.0 (Adobe Systems, USA). Newly generated sequence data were deposited in GenBank (Table 1).

Pathogenicity assay

Koch's postulates were used to confirm the pathogenicity of the new species on its original host (Bhunjun et al. 2021). Four replicates of both the wounded and non-wounded assays using conidial suspension and mycelial plugs were examined. The leaves of elephant foot yam were first surface sterilized by washing in 70% ethanol for 1 minute, then in 2% sodium hypochlorite for 1 minute, followed by three times washing with sterile distilled water and laminar air drying. A conidial suspension (1×10^6 conidia/mL) was applied to four wounded and four non-wounded leaves while a sterilized water inoculation was used for the control leaves. Mycelial plugs were obtained from colonies grown on PDA (10-day colonies). Control inoculations were performed using uncolonized PDA plugs**.** The inoculated and the control leaves were incubated in a moist chamber at 28 °C with an 80% relative humidity. Koch's postulates were confirmed by re-isolating the fungus from the infected leaves. The re-isolated fungus was identified based on cultural and morphological characteristics.

Table 1 Taxa with their respective GenBank accession numbers used in the phylogenetic analyses.

Taxa	**Strains**	**GenBank accession numbers**			
		ITS	***cmdA***	***tub2***	***rpb2***
Albifimbria verrucaria	CBS 328.52^T	KU845893	KU845875	KU845969	KU845931
A. viridis	CBS 449.71^T	KU845898	KU845879	KU845974	KU845936
Paramyrothecium acadiense	CBS 123.96^T	KU846288	–	KU846405	KU846350
P. amorphophalli	**MFLUCC 22-0085^T**	**OP279643**	**OP434479**	**OP434480**	**OP434481**
P. breviseta	CBS 544.75^T	KU846289	KU846262	KU846406	KU846351
P. cupuliforme	CBS 127789^T	KU846291	KU846264	KU846408	KU846353
P. eichhorniae	TBRC 10637^T	MT973996	MT975319	MT975317	MT977540
P. eichhorniae	KKFC 474	MT973995	MT975318	MT975316	MT977541
P. foeniculicola	CBS 331.51^T	KU846292	–	KU846409	KU846354

Table 1 Continued.

Taxa	Strains	GenBank accession numbers			
		ITS	***cmdA***	***tub2***	***rpb2***
P. foliicola	CBS 113121^{T}	KU846294	KU846266	KU846411	–
P. guiyangense	HGUP 2016-8002^{T}	KY126418	KY196193	KY196201	–
P. humicola	CBS 127295^{T}	KU846295	–	KU846412	KU846356
P. nigrum	CBS 116537^{T}	KU846296	KU846267	KU846413	KU846357
P. pituitipietianum	CBS 146817^{T}	MW175358	MW173100	MW173139	–
P. parvum	CBS 257.35^{T}	KU846298	–	KU846415	KU846359
P. roridum	CBS 357.89^{T}	KU846300	KU846270	KU846417	KU846361
P. roridum	CBS 372.50	KU846301	KU846271	KU846418	KU846362
P. salvadorae	CBS 147074^{T}	MZ064453	–	MZ078277	MZ078210
P. sinense	CGMCC 3.19212^{T}	MH793296	MH885437	MH793313	MH818824
P. tellicola	CBS 478.91^{T}	KU846302	KU846272	KU846419	KU846363
P. terrestris	CBS 564.86^{T}	KU846303	KU846273	KU846420	KU846364
P. verruridum	HGUP 2016-8006^{T}	KY126422	KY196197	KY196205	–
P. vignicola	SDBR-CMU376^{T}	MZ373242	OM810412	ON009015	ON033778
P. vignicola	SDBR-CMU377	MZ373244	OM810413	ON009016	ON033779
P. viridisporum	CBS 873.85^{T}	KU846308	KU846278	KU846425	KU846369

"^{T}" indicates type species and the newly generated sequence is in bold.

Results

Phylogenetic analyses

The phylogram (Fig. 1) was constructed based on a concatenated *cmdA* – ITS – *rpb2* – *tub2* alignment comprising sequence data from 25 taxa and the outgroup taxa (Table 1). The matrix comprised 2322 characters. The concatenated alignment was subjected to ML, MP and BYPP analyses. However, as the tree topology resulting from all three analyses was similar, hence the ML tree is illustrated herein (Fig. 1).

The best-scoring RAxML tree with final optimization had a likelihood value of -10252.351870. The matrix consisted of 690 distinct alignment patterns, with 14.14% gaps and undetermined characters. Estimated base frequencies were as follows: A = 0.232961, C = 0.280953, G = 0.256616, T = 0.229470, with substitution rates AC = 1.287563, AG = 5.379673, AT = 1.231938, CG = 1.290010, CT = 8.976371, GT = 1.000000. The gamma distribution shape parameter = 0.179871 and the tree-length = 1.020557. The maximum parsimonious dataset comprised 1621 conserved characters while 137 characters were variable. The parsimony analysis resulted in two equally parsimonious trees (tree length = 1475, Consistency Index (CI) = 0.647, Retention Index (RI) = 0.752, Rescaled Consistency index (RC) = 0.487 and Homoplasy Index (HI) = 0.353).

Table 2 Blast search results of the GenBank nucleotide database using the ITS, *cmdA*, *rpb2* and *tub2*.

Gene	Taxon	Identities	Gaps
ITS	*Paramyrothecium* sp. (MZ373249)	562/563(99%)	1/563(0%)
cmdA	*Paramyrothecium eichhorniae* (OM810415)	661/673(98%)	1/673(0%)
rpb2	*Paramyrothecium eichhorniae* (ON033785)	959/967(99%)	2/967(0%)
tub2	*Paramyrothecium eichhorniae* (ON033772)	312/316(99%)	0/316(0%)

The phylogenetic tree constructed from the combined dataset showed that the isolated strain (MFLUCC 22-0085) formed a distinct lineage, clustering sister to two strains of *P. eichhorniae* (TBRC 10637 and KKFC 474) (Fig. 1), with high statistical support (100% ML, 97% MP, 0.99 BYPP).

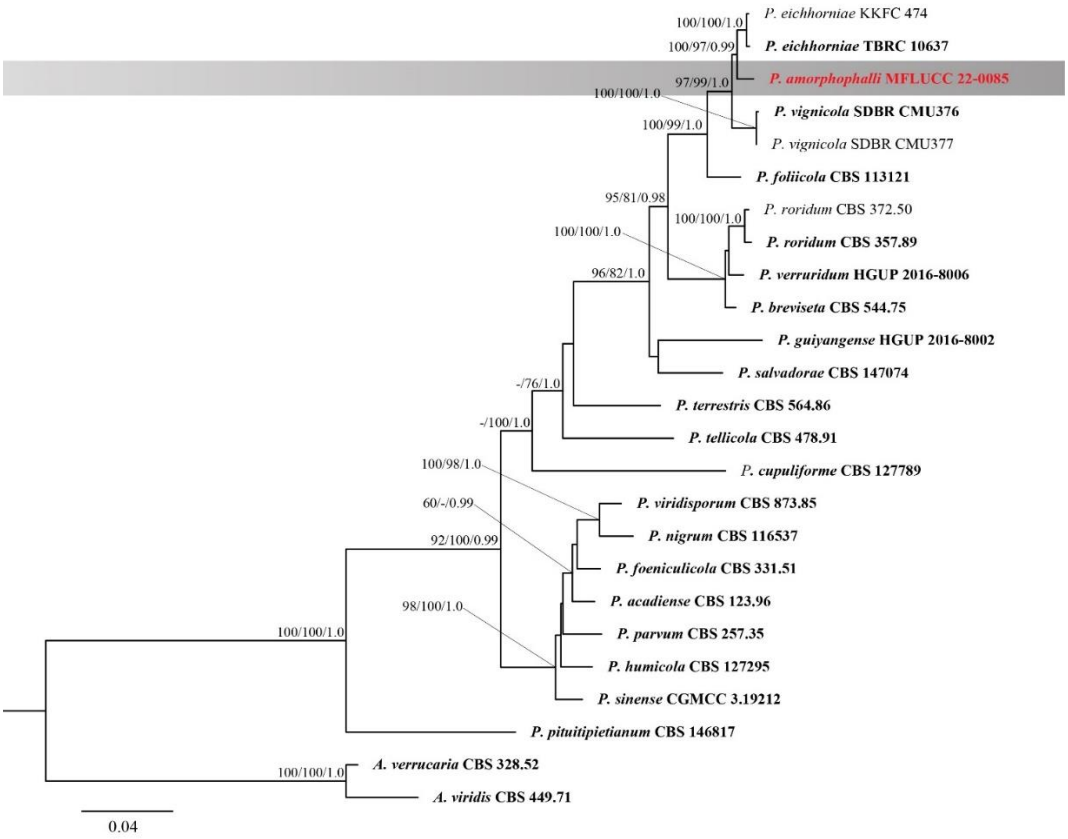

Fig. 1 – Phylogenetic tree generated by maximum likelihood analysis of combined *cmdA* – ITS – *rpb2* – *tub2* sequences. Maximum likelihood (left) and maximum parsimony (middle) bootstrap support values ≥ 60% and Bayesian posterior probabilities (right) ≥ 0.98 are indicated near the nodes. The tree is rooted with *Albifimbria verrucaria* (CBS 328.52) and *A. viridis* (CBS 449.71). The type strains are in bold and the newly generated sequence is highlighted in red.

Taxonomy

Paramyrothecium amorphophalli Armand & Jayawardena. sp. nov. Fig. 2

Index Fungorum number: IF558845; Facesoffungi number: FoF12745

Etymology – The species epithet refers to *Amorphophallus*, the plant genus from which the fungus was isolated.

Holotype – MFLU 22-0145

Pathogenic, causing leaf blight on *Amorphophallus* sp. Sexual morph: Not observed. Asexual morph: *Sporodochia* irregularly shaped, stromatic, superficial, with setose fringe, surrounded by olivaceous to dark green slimy mass of conidia. *Stroma* poorly developed, hyaline to sub-hyaline, made up of *textura angularis*. *Conidiophores* arising from the basal stroma, hyaline or slightly olivaceous, consisting of a stipe and penicillately branched conidiogenous apparatus. *Stipes* 11–20 × 2–2.5 µm, unbranched, hyaline, sometimes olivaceous, septate, smooth; primary branches 7–12 × 1.8–2 µm, aseptate, unbranched, smooth; secondary branches 8–15 × 1.5–2 µm, aseptate, unbranched, smooth, bearing 3–6 conidiogenous cells in a whorl. *Conidiogenous cells* 10.5–14.7 × 1.6–2 µm ($\overline{x}$ = 12 × 2 µm, n = 20), phialidic, cylindrical to subcylindrical, hyaline or olivaceous, smooth. *Conidia* two types: cylindrical conidia 5–6.4 × 1.5–2 µm ($\overline{x}$ = 5 × 1.8 µm, n = 30), abundant, aseptate, hyaline, smooth, rounded at both ends or narrowed at one end; ellipsoidal conidia 6–7 × 3–3.5 µm ($\overline{x}$ = 6.4 × 3.3 µm, n = 4) rarely observed, aseptate, hyaline.

Culture characteristics – Colonies reaching 20 mm in diameter after 7 days (25–28 °C) on PDA, with compact mycelia, white in color, becoming light saffron in the center; *sporodochia* forming superficially on the aerial mycelia, scattered, solitary to gregarious, surrounded by slimy

olivaceous green to dark green conidial masses, reverse on PDA luteous to rosy buff, without exudate into the medium.

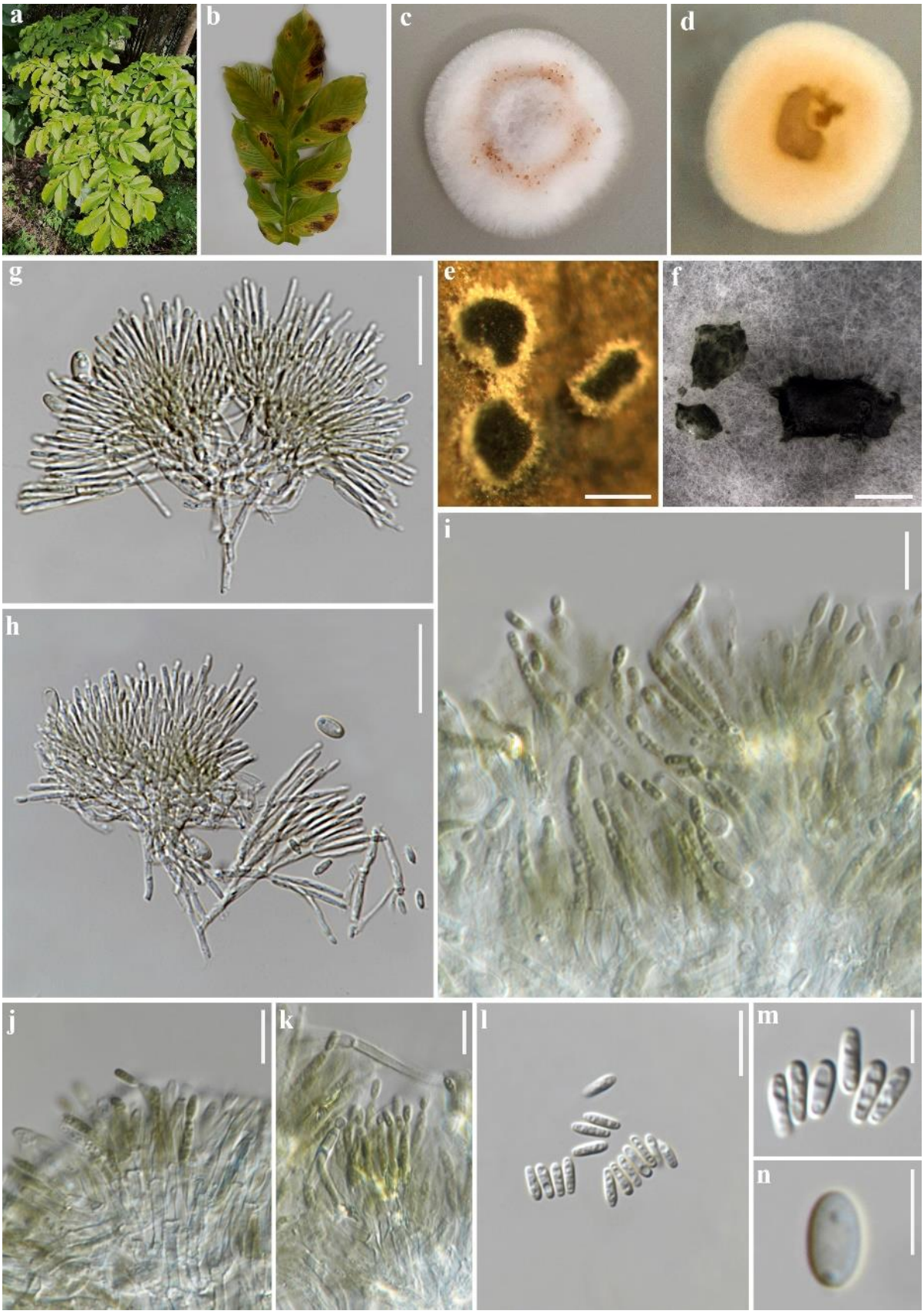

Fig. 2 – *Paramyrothecium amorphophalli* (g, h, n holotype, MFLU22-0145; i-n ex-type, MFLUCC 22-0085). a Host plant. b Symptomatic leaves. c, d Upper and reverse sides of the colony on PDA

(7 days old). e Sporodochia. f Conidial masses on PDA. g, h Conidiogenous cells and conidial attachment. i-k Conidiogenous cells and conidia. l, m Cylindrical conidia. n Ellipsoidal conidium. Scale bars: e, f = 500 µm, g, h = 20 µm, i-l = 10 µm, m, n = 5 µm.

Material examined – Thailand, Doi Pui Sai Khao, Phan District, Chiang Rai, on leaf of *Amorphophallus* sp., 27 September 2021, Alireza Armand, T42 (MFLU 22-0145, holotype); ex-type living culture MFLUCC 22-0085.

Notes – The new species *Paramyrothecium amorphophalli* is phylogenetically close to *P. eichhorniae* (TBRC 10637) and *P. vignicola* (SDBR-CMU376). *Paramyrothecium amorphophalli* is morphologically similar to *P. eichhorniae* in having stromatic, superficial sporodochia with an irregularly shaped outline and surrounding setose fringe, scattered or aggregated on the media (Pinruan et al. 2022). Moreover, the two taxa are similar in producing green to dark green slimy masses of conidia and both have aseptate, hyaline, smooth, cylindrical to ellipsoidal conidia (Pinruan et al. 2022). However, *P. amorphophalli* differs from *P. eichhorniae* in lacking setae on sporodochia and producing thick ellipsoidal conidia with completely rounded ends which are not observed in *P. eichhorniae*. It also produces shorter conidiophores (11–20 µm in *P. amorphophalli* vs. 15–40 µm in *P. eichhorniae*) and grows more slowly on media (20 mm after 7 days in *P. amorphophalli* vs. 90 mm after 14 days in *P. eichhorniae*) (Pinruan et al. 2022). *Paramyrothecium amorphophalli* can be recognized from *P. vignicola* by producing relatively smaller conidia (5–6.4 µm in *P. amorphophalli* vs. 5–7 µm in *P. vignicola*), smaller conidiophores (11–20 m in *P. amorphophalli*, 15–40 µm vs. 40–60 µm in *P. vignicola*) and absence of setae associated with sporodochia (Withee et al. 2022). Following the recommendations of Chethana et al. (2021) and Jayawardena et al. (2021), we used a polyphasic approach to confirm our isolate is a new species.

Pathogenicity assay

Paramyrothecium amorphophalli was isolated from the leaf blight of elephant foot yam which appeared mostly around the leaf margins. The pathogenicity tests have been conducted by both conidial suspension and mycelial plug on wounded leaves showed that the fungus can cause disease on the host leaves (Fig. 3). The wounded leaves inoculated with conidial suspension showed symptoms after 24 hours of incubation, whereas the wounded leaves treated with mycelial plugs showed symptoms 48 hours later. The symptoms continued to spread in the leaves for 7 days, after which most of the leaves started to rot. After 10 days of incubation, sporodochia were observed on the symptomatic areas of the wounded leaves. The symptoms and sporodochia that appeared on the leaves during the pathogenicity test were similar to those symptoms and sporodochia formed on the host leaves in nature (Fig. 3). The re-isolated fungus was identified as the same species according to the morphological characteristics of its sporodichia and conidia. The negative controls (wounded and non-wounded leaves) did not show any symptoms during the test. Interestingly, no symptoms were observed on the non-wounded leaves incubated with conidial suspension or mycelial plugs.

Discussion

A morphological and multi-locus phylogenetic study revealed that *Paramyrothecium amorphophalli* isolated from elephant foot yam is a new species of *Paramyrothecium*. *Paramyrothecium amorphophalli* was found in a separate lineage from *P. eichhorniae* and *P. vignicola*. Based on the morphological characters, it can be distinguished from *P. vignicola* in that it produces slightly smaller conidia (5–6.4 µm in *P. amorphophalli*; 5–7 µm in *P. vignicola*) (Withee et al. 2022). *Paramyrothecium amorphophalli* differs from both *P. eichhorniae* (Pinruan et al. 2022) and *P. vignicola* as observed from the absence of setae associated with sporodochia and producing smaller conidiophores (11–20 µm in *P. amorphophalli*; 15–40 µm in *P. eichhorniae*; 40–60 µm in *P. vignicola*). Moreover, it has a slower growth rate on PDA as compared to *P. eichhorniae*. We found that *P. amorphophalli* (MFLU22-0145) rarely produces robust ellipsoidal conidia that have never been described from *Paramyrothecium*. The ellipsoidal conidia, however, were not observed

in the culture. The base pair differences between *P. amorphophalli* and P. *eichhorniae* (TBRC 10637) for ITS, *cmdA*, *rpb2* and *tub2* were 0.5% (3/583 bp), 2.3% (16/694 bp), 0.5% (4/700 bp) and 1.4% (5/340 bp) respectively.

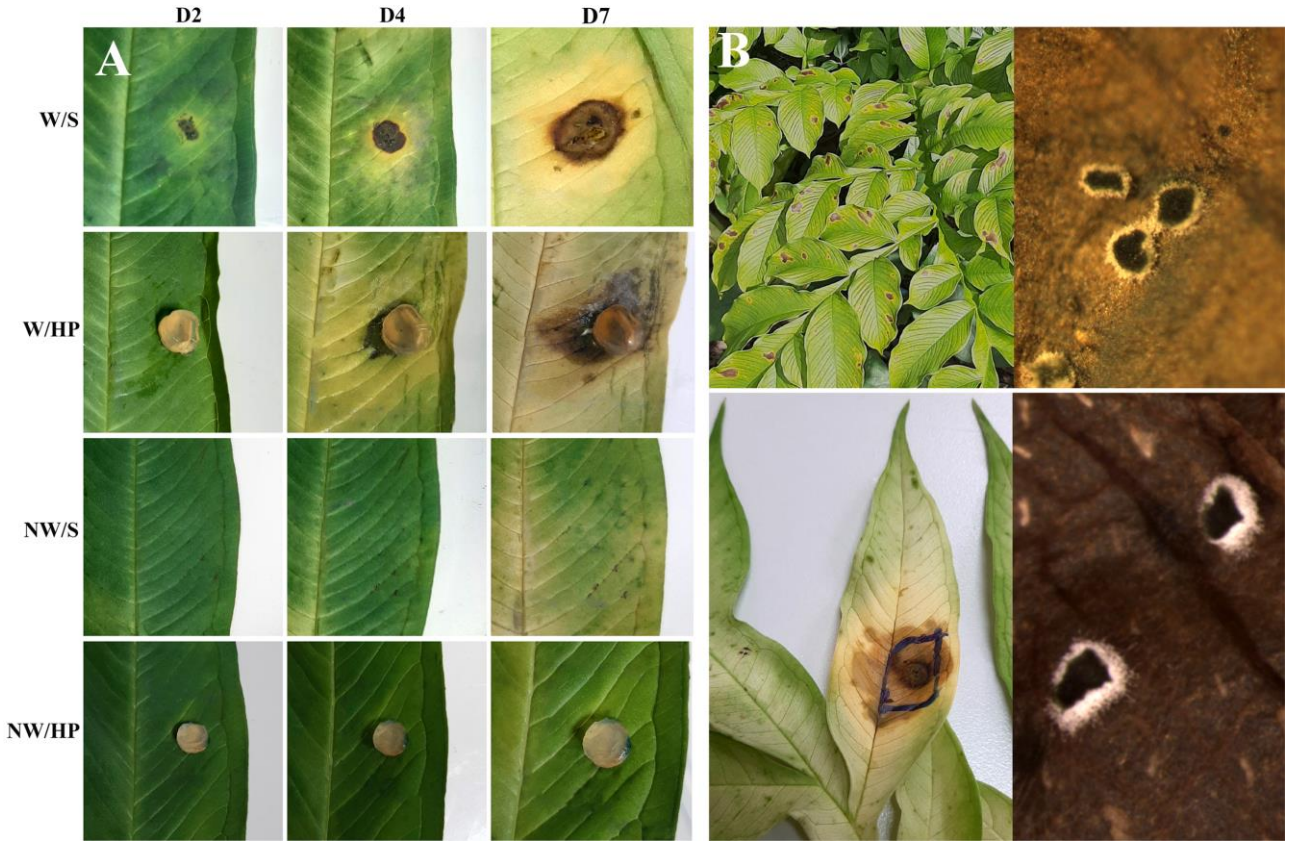

Fig. 3 – A Pathogenicity test results on elephant foot yam's leaves. Symptoms on days 2 (D2), 4 (D4) and 7 (D7) post incubation are shown (W: Wounded, NW: Non-wounded, S: Conidial suspension, HP: Mycelial plug). B Symptoms and sporodochia on the leaves in nature (up); symptoms and sporodochia on the leaves after incubation in the laboratory (bottom).

Pathogenicity tests have shown that *Paramyrothecium amorphophalli* can cause disease in the host from which it was originally isolated. Additionally, the symptoms that appeared throughout the pathogenicity tests conducted under laboratory conditions were similar to the blight symptoms observed on the leaves in nature. Interestingly, the results also showed that *P. amorphophalli* can only infect wounded leaves. It can be hypothesized that the taxon requires wounds to penetrate and infect the host.

Many species of *Myrothecium sensu lato* (including those later transferred to *Paramyrothecium*) were earlier identified based on morphology. Later, taxonomic revision of the genus based on molecular study brought about the establishment of several new genera (Lombard et al. 2016). Since *Paramyrothecium* species have overlapping morphological characteristics, identification based only on morphology is not recommended (Lombard et al. 2016). Additionally, many studies have proven the importance of multi-gene phylogenetic identification, particularly in phytopathogens (Jayawardena et al. 2016, 2019, Phoulivong et al. 2010). Therefore, it becomes imperative to support morphological studies with multi-gene phylogenetic data (Cai et al. 2009, Bhunjun et al. 2021, Hyde et al. 2018). Even though many species of *Paramyrothecium* have been isolated from Thailand, their distribution, host specificity and diversity remained inconclusive (Withee et al. 2022). Hence, extensive sampling from different hosts using a morpho-molecular approach is recommended. The *Myrothecium* group presently comprises 87 species (MycoBank 2023), and many of these species have been synonymized and are scattered into different genera.

Moreover, only a few phylogenetic studies have been carried out that included *Myrothecium* species (Lombard et al. 2016). Recent studies on the *Myrothecium* group showed their high diversity (Liang et al. 2019, Withee et al. 2022) and more species are likely to be found, indicating that these speciose genera and groups of fungi are likely to comprise more new taxa (Bhunjun et al. 2022, Calabon et al. 2022, Senanayake et al. 2022).

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