



Revealing two new records of *Phallus* (Phallaceae) from northern Thailand and optimizing conditions for mushroom mycelial growth

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Abstract

Phallus species are well-recognized for their gasteroid habits. These species are globally distributed across various habitats, ranging from temperate to tropical and subtropical regions. This study introduces two new geographical records of *Phallus fuscoechinovolvatus* T.H. Li, B. Song & T. Li, and *P. lutescens* T.H. Li, T. Li & W.Q. Deng from Thailand. The *Phallus* collections were identified based on morphological features and phylogenetic analyses of combined sequences of the internal transcribed spacer (ITS1–5.8S–ITS2) and partial 28S large subunit (LSU) region. Comprehensive descriptions, illustrations, and a phylogenetic tree to show the proper placement of taxa reported in the study are provided. Additionally, we conducted the report by investigating the optimal for mycelium growth of a selected *Phallus* strain based on the different media, pH, and temperature tests.

Keywords – cultivation – new geographical record – nutritional content – stinkhorn mushroom

Introduction

Phallus is a genus in the family *Phallaceae*, which was established by Junius ex L. with *P. impudicus* L. as a type species (Linn 1753). These fungi are cosmopolitan and commonly called “bamboo mushrooms” and “basket stinkhorn mushrooms,” referring to their morphologically distinctive features (Cabral et al. 2019). Members of this genus are reported from temperate and tropical regions of southern Africa, the Americas, Australia, and Asia, especially in China and Japan (Calonge et al. 2005, Smith 2005, Hosaka 2010, Desjardin & Perry 2015, Cabral et al. 2019). Kirk et al. (2008) estimated that there are 18 species of *Phallus* worldwide. There are 200 records of *Phallus* in the Index Fungorum (<http://www.indexfungorum.org/>; accessed date: May 19, 2024) and 125 records in the Species Fungorum (<https://www.speciesfungorum.org/>; accessed date: May 19, 2024). In Thailand, two *Phallus* species, *P. merulinus* (Berk.) Cooke. and *P. chiangmaiensis* U. Pinruan, S. Sommai & P. Khamsuntorn (Sommai et al. 2021) have been reported.

Phallus consists of divergent clades that present distinctive morphological features throughout their evolutionary histories (Mueller et al. 2001). In 1809, the genus *Dictyophora* Desv. was established with the distinguished presence of an indusium and a perforate pore at the top of the receptacle (Desvaux 1809). Later, several reports illustrated the same species, with or without an indusium, discovered under the genus *Phallus* (Moreno et al. 2013, Cabral et al. 2019). So, the

species of *Dictyophora* (*D. indusiata* Vent.) was verified to be a synonym of *Phallus* (Li et al. 2014, Rebriev et al. 2014, Adamčík et al. 2015, Li et al. 2016, Medeiros et al. 2017, Trierweiler-Pereira et al. 2017, Song et al. 2018, Cabral et al. 2019, Li et al. 2020).

Phallus species have varieties of the colour of fruiting bodies, mainly four colours in the skirt-like net (white, pink to red, yellow, and orange) with short and long skirts (Li et al. 2016) and distinctive fetid odour originating from the gleba. Species of this genus usually develop from the egg stage to long net fruiting bodies (skirt-like net structure) that are unbranched (Li et al. 2016, Cabral et al. 2019, Sommai et al. 2021). Species delimitation relies on crucial morphological characteristics of sponge-like and hollow pseudostipe, the surface structure of the receptacle, the presence of a volva with rhizomorphs, and the existence of an upright to curved shape. Species identification within the genus *Phallus* has been primarily conducted through a combination of morphological characterization and multi-gene phylogenetic analyses, focusing on ITS, LSU, and *atp6* genes (Sommai et al. 2021), with a particular emphasis on ITS and LSU genes (Li et al. 2016, Cabral et al. 2019, Li et al. 2020). Despite these efforts, obtaining reliable DNA samples from mature basidiomes poses significant challenges. The only suitable materials for analysis are the gleba and mycelial strands; however, both are highly susceptible to contamination from foreign DNA (Rebriev et al. 2023). However, scientific investigation regarding the taxonomy and phylogeny study of the genus *Phallus* is limited.

Some *Phallus* species have a long history of culinary and medical use, commonly known as edible medicinal mushrooms (Li et al. 2020). In the thriving economically important mushrooms, *P. indusiatus* was included as one of the economic mushrooms (Liu et al. 2018). Moreover, some species contain bioactive compounds that can be used in pharmaceutical and beauty cosmetic products (Habtemariam 2019). During an investigation on *Phallus* species in Thailand, we introduced two new geographical records based on morphology and molecular data from multi-gene phylogenetic analyses of the internal transcribed spacer region (ITS1–5.8S–ITS2) and the partial 28S large subunit (LSU).

Materials & Methods

Sample collections and morphological study

Three fresh fruiting bodies of *Phallus* were collected from Chiang Rai Province, Thailand. The samples were photographed and transported back to the laboratory, where their macroscopic details from fresh specimens were noted. Pure cultures were obtained by internal tissue isolation. The internal tissue of the volva was isolated into potato dextrose agar (PDA) and incubated at 25 °C for 18 days. They were then air-dried at 38 °C for 36 hours until they were completely dehydrated. The herbarium materials were deposited in the fungarium of Mae Fah Luang University (MFLU).

Macro-characteristics are described following the method described by Largent (1986) and Lodge et al. (2004). Micromorphological examinations were conducted with a Nikon ECLIPSE 80i compound microscope, and photographs were captured using a Canon 750D digital camera attached to the microscope. Details of basidiospores and other microscopic structures were photographed. The basidiospores were described based on the examination of at least 50 basidiospores (Miettinen & Larsson, 2006), and the basidiospore quotient used aligns with the description provided by Tulloss (2005). The tarosoft (R) Image Frame Work program was used for measurements with Adobe Photoshop CS6 Extended Version 10.0 software (Adobe Systems, USA).

DNA extraction, PCR amplification, and sequencing

Dried mushroom fruiting bodies were used in this study. Total DNA was extracted using the OMEGA E.Z.N.A.® Forensic DNA Extraction Kit, following the manufacturer's instructions. The ITS and LSU genes were amplified using ITS5/ITS4 and LR0R/LR5 primers. PCR conditions were adjusted and performed as previously described (Vilgalys & Hester 1990, White et al. 1990). The

verified PCR fragments were purified and sequenced by Biogenomed Co., Ltd., Korea. The obtained nucleotide sequences were deposited in GenBank.

Phylogenetic analyses

The generated sequences were subjected to a nucleotide BLAST search. Related reference sequences were then downloaded from GenBank based on recently published data (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the data are listed in Table 1. Single gene alignments were obtained using MAFFT v.7.036 (<http://mafft.cbrc.jp/alignment/server/>) (Kato et al. 2019) with default settings, and each matrix was trimmed using TrimAl (Capella-Gutiérrez et al. 2009). BioEdit v. 7.0.5.2 was used to visualize the sequences and for manual edits (Hall 1999). Alignments were manually adjusted to maximize sequence similarity, with gaps treated as missing data. Phylogenetic analyses of the single gene and combined gene were analyzed using maximum likelihood (ML) and Bayesian inference posterior probabilities (BYPP). Trees were inferred using a heuristic search with TBR branch swapping and 1,000 random sequence additions. Max trees were unlimited, branches of zero length were collapsed, and all multiple parsimonious trees were saved. The clade stability of the trees from the parsimony analyses was assessed through bootstrap analysis with 1,000 replicates, each using 10 replicates of a random stepwise addition of taxa. Maximum likelihood (ML) analysis was performed using IQ-TREE with bootstrap support obtained from 1000 pseudoreplicates (Nguyen et al. 2014, Chernomor et al. 2016). The Bayesian inference (BI) analysis was performed by the online CIPRES Science Gateway (Stamatakis 2014) using the RAxML-HPC2 on XSEDE (8.2.12) by discarding the first 25% of generations as “burn-in” (Huelsenbeck & Ronquist 2001). The tree was viewed in Treeview and exported to graphics programs. The final alignment and tree were deposited in TreeBASE under the submission ID 31392 (<http://www.treebase.org>).

Table 1 Details of the taxa used in the phylogenetic analysis of this study.

Taxa	Voucher	Country	GenBank no.		References
			ITS	LSU	
<i>Phallus atrovolvatus</i>	INPA240016	Brazil	MG678531	MG678470	Cabral et al. 2019
<i>P. aureolatus</i>	ICN 176962	Brazil	MF372135	MF372127	Trierweiler-Pereira et al. 2017
<i>P. campanulatus</i>	ICN 176970	Brazil	MF372138	MF372130	Trierweiler-Pereira et al. 2017
<i>P. Chiangmaiensis</i>	BCC 92054	Thailand	MT452882	MT447464	Sommai et al. 2021
<i>P. denigricans</i>	INPA:272383	Brazil	MG678486	MG678455	Cabral et al. 2019
<i>P. denigricans</i>	UFRN-Fungos 2805	Brazil	MG678485	MG678454	Cabral et al. 2019
<i>P. dongsun</i>	GDGM 75402	China	MN264679	MN307397	Li et al. 2020
<i>P. echinovolvatus</i>	TNS-F-34480	Brazil	MF372129	MF372137	Trierweiler-Pereira et al. 2017
<i>P. echinovolvatus</i>	GDGM 79020	China	MN523216		Li et al. 2020
<i>P. fuscoechinovolvatus</i>	GDGM 48589	China	MF039581	MF039585	Song et al. 2018
<i>P. fuscoechinovolvatus</i>	GDGM 48677	China	MF039583	MF039586	Song et al. 2018

Table 1 Continued

Taxa	Voucher	Country	GenBank no.		References
			ITS	LSU	
<i>P. fuscoechinovolvatus</i>	MFLU24-0012	Thailand	PP809093	PP810233	This study
<i>P. fuscoechinovolvatus</i>	MFLU24-0014	Thailand	PP809094	PP810235	This study
<i>P. haitangensis</i>	HKAS:88197	China	–	NR_155668	Li et al. 2016
<i>P. impudicus</i>	GDGM 77656	China	MN264675	MN307393	Li et al. 2020
<i>P. indusiatus</i>	INPA-Fungos 264931	Brazil	MG678500	MG678463	Cabral et al. 2019
<i>P. indusiatus</i>	SP416393	Brazil	MG678507	MG678464	Cabral et al. 2019
<i>P. lutescens</i>	GDGM 72218	China	MN131079	MN131075	Li et al. 2020
<i>P. lutescens</i>	GDGM 76604	China	MN131078	MN131076	Li et al. 2020
<i>P. lutescens</i>	MFLU24-0013		PP809095	PP810234	This study
<i>P. mengsongensis</i>	HKAS:78343	China	NR_158805	–	Li et al. 2014
<i>P. merulinus</i>	CJL-120214-03			KF783250	Trierveiler-Pereira et al. 2017
<i>P. purpurascens</i>	UFRN-Fungos 2808	Brazil	MG678487	MG678456	Cabral et al. 2019
<i>P. purpurascens</i>	SINOP30	Brazil	MG678496	MG678460	Cabral et al. 2019
<i>P. rubrovolvatus</i>	YZS040	China	KF939503	–	Li et al. 2014
<i>P. serratus</i>	HKAS:78340	China	KF052622	–	Li et al. 2014
<i>P. squamulosus</i>	UFRN-Fungos 2806	Brazil	NR_166234	–	Cabral et al. 2019
<i>P. ultraduplicatus</i>	HMAS:253050	China	KJ591584	KJ591586	Adamčík et al. 2015
<i>P. ultraduplicatus</i>	HMAS:253051	China	KJ591585	KJ591587	Adamčík et al. 2015
<i>Mutinus zenkeri</i>	MA-2013 JD781	Belgium	KC128650	KC128654	Degreef et al. 2013

Notes: Sequences used in this study are in bold.

The effect of media, pH, and temperature on mycelium growth

An edible *Phallus fuscoechinovolvatus* (MFLU24-0012) was selected to find out the optimal condition for mycelial growth (Luangharn et al. 2019). Four different culture agar media for mycelium, including corn meal agar (CMA), malt extract agar (MEA), potato dextrose agar (PDA), and yeast extract agar (YEA), were used to determine suitable media for promoting mycelium growth. An active mushroom culture (1 cm in diameter) was removed using a sterile cork borer and transferred to each Petri dish medium (Luangharn et al. 2020). All media were incubated in the dark at 25 °C for 21 days. The optimal media for mycelia growth rate was determined by measuring the colony diameter and recording the mushroom mycelium morphology.

The best medium was selected for evaluation at different temperatures (15 °C, 18 °C, 20 °C, 23 °C, 25 °C and 27 °C) and incubated for 18 days. The colony diameter was measured and compared, and the growth rate was calculated to determine the optimal temperature for mycelial growth.

The best media and temperature conditions were applied to evaluate the pH condition. Six pH ranges (4, 5, 6, 7, 8, 9) were adjusted using 1 N NaOH or 1 N HCl. The pH range was measured using a digital pH meter before the autoclave. All media were incubated at the best temperature for 16 days. Mushroom mycelial growth (mm), growth rates (mm/day), and mycelial density were screened to indicate favourable growth conditions. The experiments were done with five replications.

Determination of mushroom mycelium density

The evaluation of mushroom mycelial density was adjusted according to Luangharn et al. (2014, 2019) and Gonkhom et al. (2022). Manual measurement was evaluated using a ruler across the plate to calculate the vertical and horizontal colony diameter averages. The growth rate was calculated by the ratio of colony diameter to time (days). The mycelial density was determined as + very scanty, 2+ scanty, 3+ moderate, 4+ abundant, and 5+ very abundant.

Data collection and statistical analysis

Data analysis was conducted using the SPSS statistical program (Softonic International SA, Barcelona, Spain) with five replicates. All the data were compared to obtain a mean separation using Tukey's test ($p < 0.05$), followed by post-hoc tests that were expressed in a one-way ANOVA.

Results

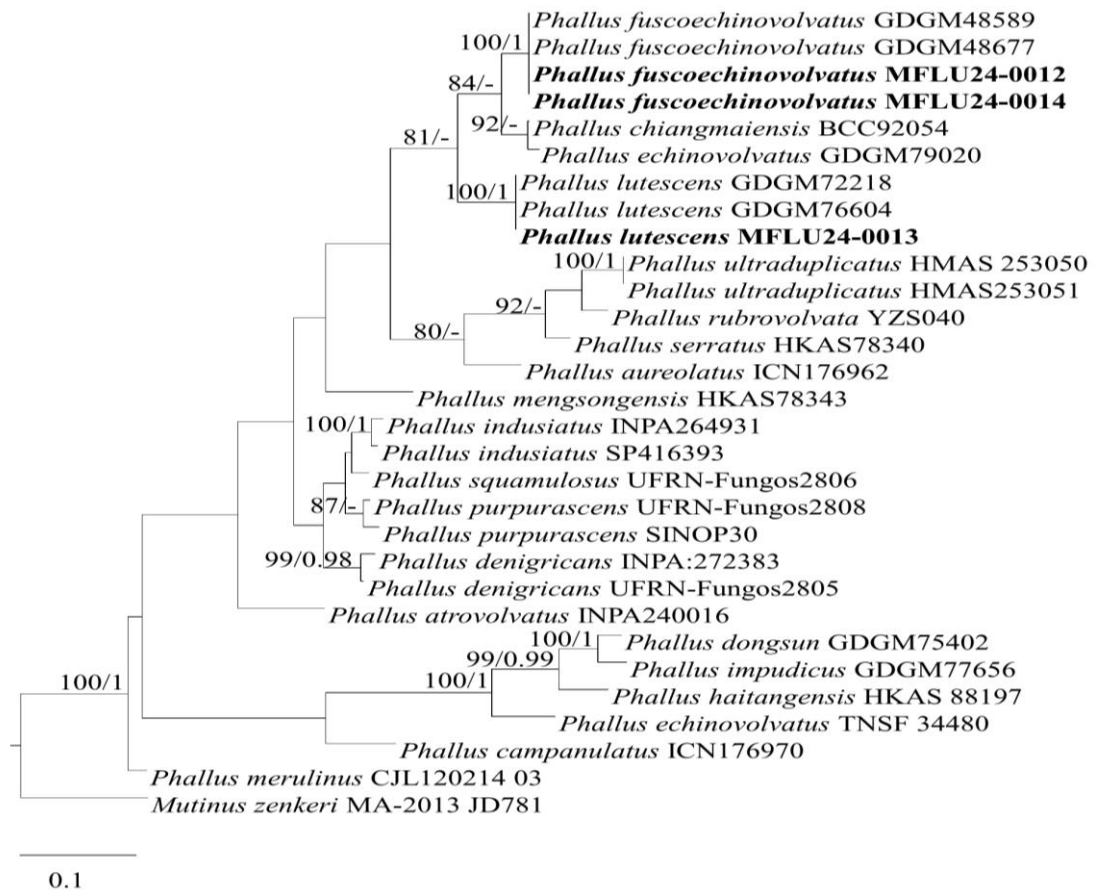


Fig. 1 – Phylogram generated from the maximum likelihood (ML) analysis based on ITS-LSU sequence matrix. The tree is rooted with *Mutinus zenkeri* (MA-2013 JD781). ML bootstrap supports ($\geq 70\%$), and BI posterior probabilities (≥ 0.90) are denoted near the respective nodes. The strains reported in this study are in red bold.

Phylogenetic analyses

The combined ITS and LSU dataset comprised 30 sequences belonging to *Phallus* and its related taxa, including one outgroup taxon, *Mutinus zenkeri* (MA-2013 JD781). The concatenated alignments resulted in similar topologies for both ML and BI analyses. The sequence matrix of two alignment files included ITS (658 bp) and LSU (956 bp) sequence data (a total of 1624 characters). The best-scoring ML tree is shown in Fig. 1, with an optimal log-likelihood value of $-22.890.934$. The proportion of invariable sites was 0.410. Estimated base frequencies were A = 0.275, C = 0.187, G = 0.244, T = 0.294; substitution rates AC = 0.89742, AG = 3.76914, AT = 1.78733, CG = 0.69825, CT = 5.68557, GT = 1.000000; and the gamma distribution shape parameter $\alpha = 0.642$.

Phylogenetic analyses showed that the placements of strains clustered within the *Phallus* clade, revealing new records of *Phallus fuscoechinolvatus* (MFLU2024-0012 and MFLU2024-0014) and *P. lutescens* (MFLU2024-0013) from Thailand. These new records were found to be clustered within their respective clades, with 100% bootstrap support (BS) and 1.00 posterior probability (PP) value for *P. fuscoechinolvatus*, and BS = 100%, PP = 1.00 for *P. lutescens*.

Taxonomy

Phallus fuscoechinolvatus T.H. Li, B. Song & T. Li (2018) (Fig. 2)

Description: – Egg-shaped at first, 3.5–8 cm in height, 2–5 cm in width, obovate to subglobose, zonate with dull green (30D4-5) reticulate cap, violet brown (11F6) to dark brown (14F5) when immature, and the exoperidium rupturing to reveal the mature fruiting body when developing to mature state. Mature basidiomata net-like with bubble-like, spongy, white (1A1) pseudostipe. Gleba dark magenta (13F4), gelatinous, with strong odor. Exoperidium papery, purple-brown (8E4-7), dark brown (8F5-8), usually violet brown (12F4-6), dark magenta (13F4) at the centre, greyish violet (17C3-5), white (1A1) when sectioned, and dark brown (4F5) when exposed. Endoperidium 2–5.2 mm thick, mostly gelatinous, transparent, with yellowish-grey (3B2, 3C2), endoperidium membranous, thin layer, with white (1A1), partially greyish lilac (15C2) at the base of peridium when exposed, and covering the upper surface of gleba. Immature basidiomata 26–38 mm in length and 18–32 mm in width at the base, oblong or slightly bell-shape, strongly rugose, strongly reticulate, yellowish white (4A2) to pale yellow (4A3), usually pits-like with irregular surface and irregular ridges, white (1A1) when the gleba is removed, which is dull green (30D4-5), slimy and sticky. Receptacle 26–40 mm long, 18–28 mm wide, and covered with mucilaginous dull green (30E2-3) gleba. Indusium (skirt-like net structure) 4–12 mm in width, large, coarsely latticed, reticulate surface, white (1A1), without the serrated margin in the hole, occurred between the reticulate cap and volva, developed at distal and composed of round to angular shapes, with some hexagonal or polygonal. Pseudostipe 45–85 mm long, 22–46 mm wide, net-like with a bubble-like shape, hollow. Pseudostipe wall consists of two layers of different chamber sizes, spongy, fragile, friable, and soft, usually cylindrical or fusiform, becoming narrower upwards, especially at the apex, and enlarged downwards, from white (1A1) to yellowish white (1A2). Volva 28–44 mm high, 25–42 mm broad, obovate to slightly globose, some globose, concolorous with the surface of immature basidiomata, dark brown (8F5-8) to dark magenta (13F4), with white (1A1), yellowish white (3A2) to pale yellow (4A3) spines and short white basal rhizomorphs. Odour is a distinctive foetid that is attractive to flies. The foetid odour is from the gleba; other parts of the basidiomata without gleba have a pleasant odour. Taste mild.

Basidiospores (2.3–) 3.4–3.9(–4.1) \times (1.3–) 1.7–2.5(–2.9) μm , Q = (2.23–) 2.49–2.65(–2.78), Qm = 2.58 ± 0.25 , cylindrical to broadly ellipsoid, hyaline, inamyloid, smooth, thin-walled, very light olivaceous in 5% KOH. Receptacle hyphae 2–6 μm in diameter, hyaline, thin-walled, septate, branched, with clamp connections. Indusium hyphae 28–50 μm diam, subglobose to globose, bubble-like and irregular vesicular cells, hyaline with thin-walled. Pseudostipe hyphae hyaline, with thin-walled. Volva hyphae 2–4 μm diam, smooth, thin-walled, septate, branched, with clamp connections. Basidia and cystidia were not seen.

Known distribution: – Known only from Guangdong province in southern China and Thailand (this study).

Habitat: – Solitary on soil in the rubber plantation.

Material examined: – Thailand, Chiang Rai Province, Muang, Thasud village, at 20°0'59"N, 99°52'27"E, alt. 392 m, June 17, 2022, coll. Arttapon Walker (MFLU24-0012, LT 2022-44); Mae Fah Luang University, at 20°0'52"N, 99°52'27"E, alt. 392 m, September 24, 2022, coll. Thatsanee Luangharn (MFLU24-0014, LT2022-165).

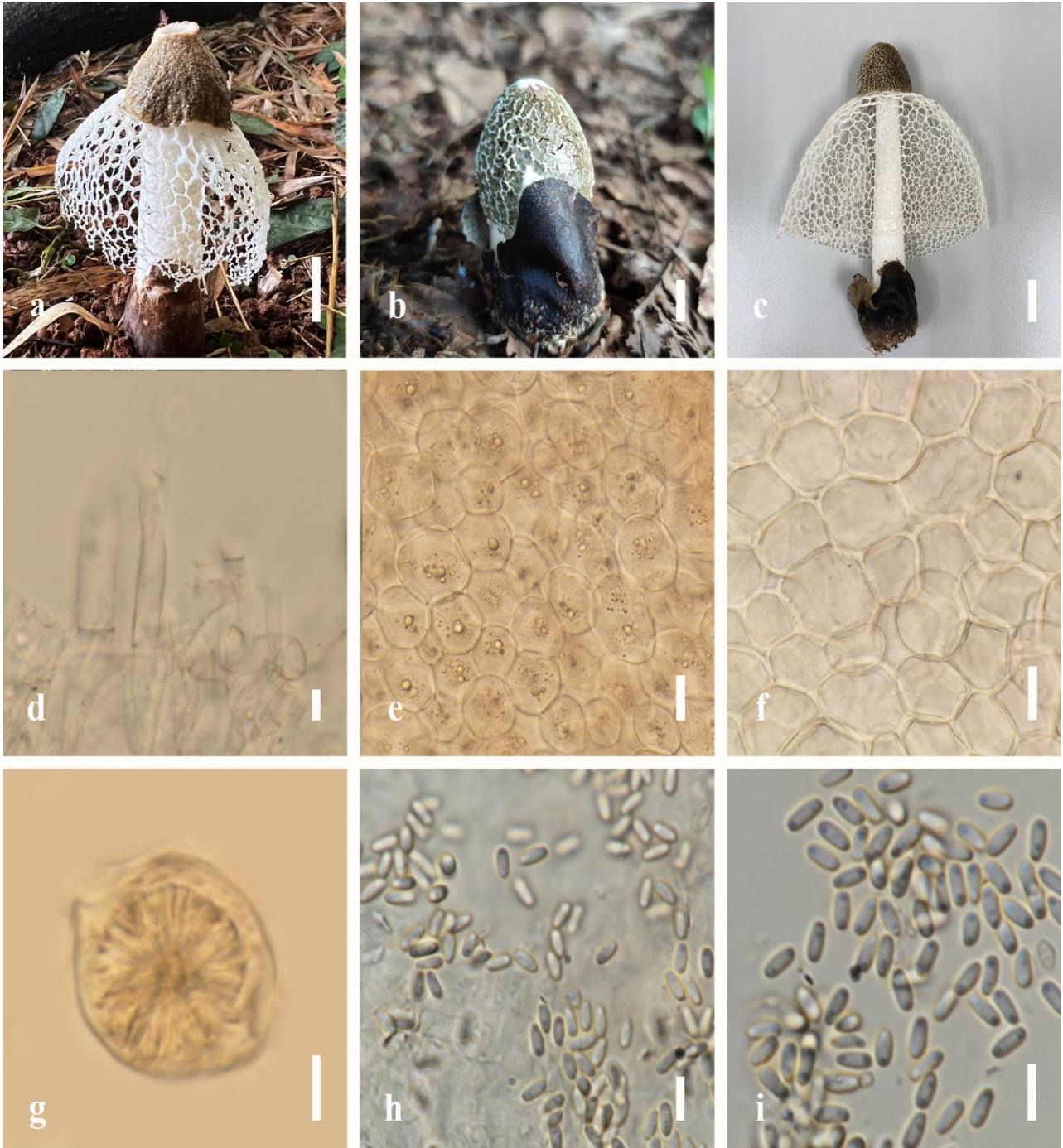


Fig. 2 – Morphological features of *Phallus fuscoechinovolvatus*. a, c Mature basidiomata (MFLU24-0012). b Young basidiomata (MFLU24-0014). d Hyphae from the volva. e Pseudoparenchymatous hyphae from indusium. f Pseudoparenchymatous hyphae from the pseudostipe. g Crystals deposit on globose cells. h, i Basidiospores. Scale bars: a = 3 cm, b = 1 cm, c = 2 cm, d = 10 μ m, e–f = 10 μ m, g–i = 5 μ m.

Phallus lutescens T.H. Li, T. Li & W.Q. Deng

(Fig. 3)

Description: – Egg-shaped or some globose shape at first. Mature basidiomata net-like with bubble-like, spongy, white (1A1) pseudostipe, up to 120 cm long. Exoperidium membranous with endoperidium gelatinous. Gleba olive brown (4E4–8, 4F3–8), mucilaginous. Endoperidium 1.2–3.1 mm thick, mostly gelatinous, transparent, with greenish-grey (1B2, C2) to olive grey (1E2); endoperidium membranous, thin layer, with white (1A1), partially greyish lilac (16C2) at the base of peridium when exposed, and covering the upper surface of gleba. Receptacle subovoid to campanulate, obviously densely reticulated with irregular ridges, usually covered with mucilaginous greyish green (30E5) to dull green (30E2-3) gleba, some with yellowish-white (1A2) to light yellow (2A4) under gleba, with 11–17 mm in length, 13–23 mm in width. Indusium 4–10 mm in width, latticed, white, cream-white, yellowish white (1A2) to pale yellow (3A3) when young and fresh, and orange white (6A2) to pale orange (6A2) when mature, orange (6A7–6B8) when dried, usually developed at distal and composed of round with some angular shapes. Pseudostipe 58–86 mm high, 7–8/13–16.5/18–22 mm thick (apex/middle/base), cylindrical usually tapered upwards and enlarged downwards, white (1A1), soft, spongiform, net-like with bubble-like shape, hollow; pseudostipe 1.4–3.1 mm thick wall two layers of different chambers size, usually cylindrical or fusiform, narrower upwards, especially at the apex and enlarged downwards, white (1A1) to yellowish white (1A2). Volva 19–32 mm length, 17–24 mm width globose or slightly obovate, smooth or lightly rugose, dull white (4A1–2) to yellowish grey (4B2), more often pinkish white (10A2) at the base. Rhizomorph simple to branched, dull white (4A1) to pale red (9A3), 1.2–3.4 mm thick. Odor foetid (mainly from gleba). Taste mild.

Basidiospores (2.8–) 3.3–3.6(–4.4) × (1.3–) 1.5–1.9(–2.1) μm, Q = (2.02–) 2.20–2.48 (–2.72), Qm = 2.39 ± 0.25, cylindrical, some ellipsoid to long ellipsoid, hyaline, inamyloid, smooth, thin-walled, some slightly truncate, and light olivaceous in 5% KOH. Receptacle hyphae 2–5 μm in diameter, hyaline, thin-walled, septate, branched, with clamp connections. Indusium hyphae up to 38–41 μm in diameter, consisting of globose to subglobose, some irregularly globose, hyaline, thin-walled, and pseudoparenchymatic. Pseudostipe hyphae hyaline with thin-walled. Volva hyphae 2.5–8 μm in diam., tubular and branched, thin-walled, smooth, septate, with clamp-connections. Hyphae of rhizomorph filamentous, 2.2–6.6 μm in diam., thin-walled, smooth, septate, rarely branched.

Known distribution: – Known only from Guangdong province in southern China and Thailand (this study).

Habit and habitat: – Solitary on soil with decaying broad-leaved trees.

Material examined: – Thailand, Chiang Rai Province, Muang, Mae Fah Luang University, at 20°0'52"N, 99°52'27"E, alt. 392 m, September 24, 2022, coll. Thatsanee Luangharn (MFLU24-0013, LT 2022-105).

Optimal media for mycelial growth

Phallus fuscoechinovolvatus mycelium was observed on all agar media tested (Table 2). The largest mycelium colony diameter was observed on PDA (43.91 ± 0.10), followed by MEA (39.44 ± 0.11), YEA (34.03 ± 0.25), and CMA (28.04 ± 0.24) media. After 3 days of incubation, the mushroom mycelium had fully colonized the media with white (6A1) mycelium. The morphology of mushroom mycelia typically exhibits a radial pattern extending from the center toward the edge of the petri dish. The colony colour was consistent across different agar media after 21 days of incubation. Mycelial growth on PDA and MEA exhibited similar morphology. On PDA, the mycelium formed a somewhat abundant (4+) massive cotton-like colony with white colour (5A1) and turned orange-white (5A2) after 35 days of incubation. On MEA, the mushroom mycelium also exhibited a somewhat abundant (4+) white cotton-like colony. In contrast, the colonies on YEA and CMA were moderate (3+) white.

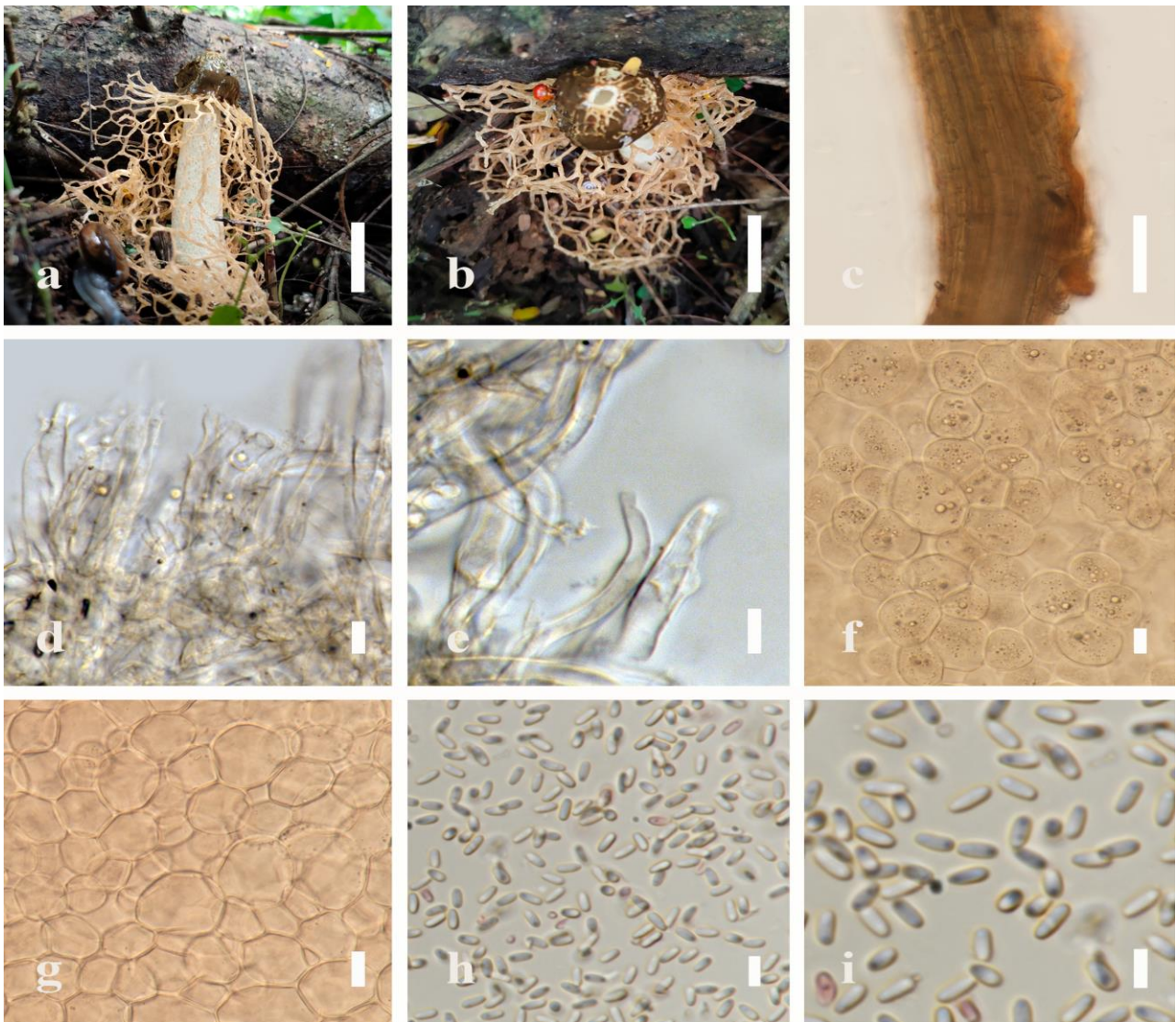


Fig. 3 – Macro-microscopic features of *Phallus lutescens* (MFLU24-0013). a, b Fresh basidiomata in the field. c Hyphae from rhizomorphs. d, e Hyphae from the volva. f Pseudoparenchymatous hyphae from indusium. g Pseudoparenchymatous hyphae from the pseudostipe. h, i Basidiospores. Scale bars: a–b = 3 cm, c = 30 μ m, d–e = 10 μ m, f–g = 20 μ m, h–i = 5 μ m.

Table 2 The effect of varying agar media conditions, pH levels, and temperatures on the mycelial growth (mm) and growth rates (mm/day) of *Phallus fuscoechinovolvatus* (MFLU24-0012).

Conditions	Treatment	Colony diameter	Mycelium growth rate	Mycelial density
Agar media	PDA	43.91 \pm 0.10 ^a	8.80	4+
	MEA	39.44 \pm 0.11 ^{ab}	8.50	4+
	YEA	34.03 \pm 0.25 ^{bc}	7.50	3+
	CMA	28.04 \pm 0.24 ^c	5.40	3+
pH	4	41.78 \pm 0.52 ^c	6.60	3+
	5	47.06 \pm 0.46 ^b	7.80	4+
	6	51.23 \pm 0.24 ^a	8.40	5+
	7	50.33 \pm 0.43 ^a	8.30	5+
	8	46.86 \pm 0.73 ^b	7.60	4+
	9	38.08 \pm 0.82 ^c	5.80	3+

Table 2 Continued

Conditions	Treatment	Colony diameter	Mycelium growth rate	Mycelial density
Temperature	15 °C	32.94 ± 0.26 ^e	5.50	3+
	18 °C	36.03 ± 0.36 ^d	7.30	3+
	20 °C	46.86 ± 0.20 ^c	7.90	4+
	23 °C	53.63 ± 0.30 ^a	8.70	5+
	25 °C	50.98 ± 0.11 ^b	8.50	5+
	27 °C	37.33 ± 0.23 ^d	7.40	4+

Notes: Values with the same letter are not significantly different ($p < 0.05$).

Optimal pH and temperature conditions

PDA was chosen for optimal pH condition tests. All pH values from 4 to 9 supported mycelium growth, with the most favorable pH being 6 and 7, followed by 5, 8, 9, and 4. The optimal pH 6–7 was expressed as very abundant (5+) and exhibited a massive cottony appearance. At pH 5 and 8, it showed an abundant (4+), and at pH 9 and 4, it showed a moderate (3+). The suitable PDA medium at pH 7 was used to test the optimal temperature conditions. The most favorable temperature for mycelial growth was between 23 °C and 25 °C with very abundant (5+), followed by 20 °C, 18 °C, 15 °C, and 27 °C (Fig. 4). At 20 °C and 27 °C, the colony morphology exhibited denser hyphae arranged in concentric rings, extending from the center to the margin in a clear circular shape. At lower temperatures of 15 °C and 18 °C, the colonies were white, with aggregated mycelium forming irregular shapes and branching from the center with a jagged edge.

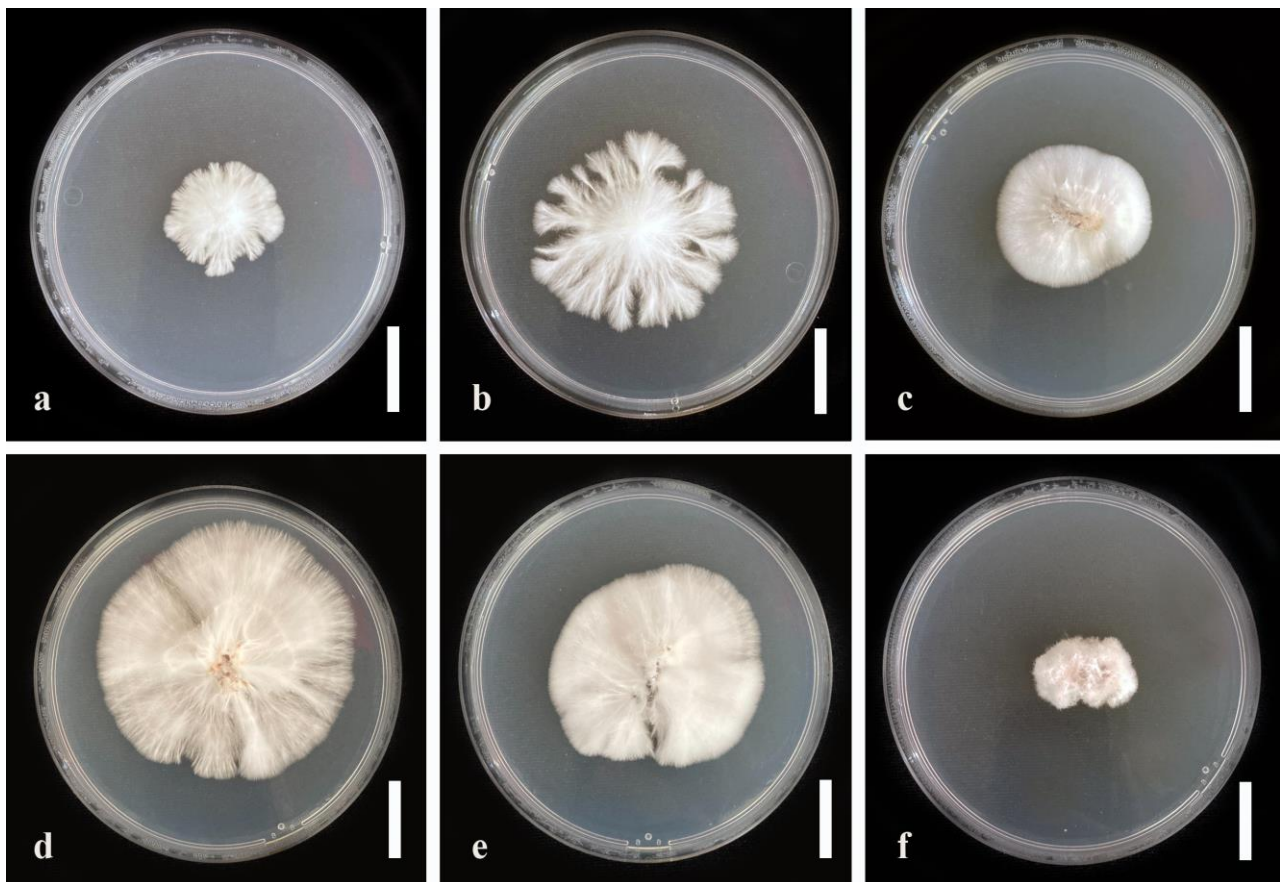


Fig. 4 – Morphological characteristics of *Phallus fuscoechinovolvatus* (MFLU24-0012) on PDA medium after incubation for 10 days at different temperatures. a 15 °C. b 18 °C. c 20 °C. d 23 °C. e 25 °C. f 27 °C. Scale bar = 2 cm.

Discussion

Phallus is distinguished as a fungus by its foul odour, hollow, spongy pseudostipe, and bell-shaped to campanulate receptacle. Species of this genus are often found in temperate to tropical environments. In this study, we present two new geographical records of *Phallus* species, specifically, *Phallus fuscoechinovolvatus* and *P. lutescens*, collected from Chiang Rai, Thailand. The species confirmation was illustrated based on macro- and micro-morphological characteristics and molecular data. Our phylogenetic analysis has unveiled new records. The ITS-LSU dataset revealed that Thai *Phallus fuscoechinovolvatus* and *P. lutescens* form a distinct clade. Each *Phallus* species is grouped with its holotype from Guangdong Province, China. Our two collections of *P. fuscoechinovolvatus* (MFLU24-0012 and MFLU24-0014) clustered in the same clade as the holotype (GDGM 48589), with strong support values (BS = 100%, PP = 1.00). Similarly, the collection of *P. lutescens* (MFLU24-0013) was closely related to its holotype (GDGM 72218), also with high support values (BS = 100%, PP = 1.00). Phylogenetic analysis reveals that these two species form a sister clade, indicating a close evolutionary relationship.

Morphological description details showed our *P. fuscoechinovolvatus* is a distinctive white pseudostipe, obovate to subglobose, zonate with dull reticulate cap, brown to dark brown exoperidium and volva, and dull green gleba. Those characteristics are consistent with Song et al. (2018), who reported a new species from China. However, our collections are smaller in size than the holotype collection. *Phallus fuscoechinovolvatus* was classified as a white or nearly white indusium mushroom. This group also includes species such as *P. atrovolvatus* (Calong et al. 2005), *P. indusiatus*, *P. merulinus*, and *P. serrata* (Trierveiler-Pereira et al. 2017, Cabral et al. 2019). Our two strains of *P. fuscoechinovolvatus* share similarities to *P. chiangmaiensis*, such as a long white indusium. However, *P. fuscoechinovolvatus* can be easily distinguished from *P. chiangmaiensis*, which has a non-echinulate and milk-white volva that never turns blackish, unlike our collections.

Species with different indusium color, such as the yellow indusium of *P. lutescens*, have been reported in over ten species (Lloyd 1909, Kreisel 1996, Kasuya 2008, Li et al. 2016). The described of our *P. lutescens* is similar to the holotype reported from the Chinese collection, with white pseudostipe, olive-brown gleba, and usually yellowish-white when young, orange-white to yellow on maturity and drying. However, the Chinese collection can be observed as the receptacle 16 – 23 × 15 – 20 mm with subovoid to campanulate shape, while our strain can be observed a small size with 11–17 mm in length, 13–23 mm in width with obviously densely reticulated with irregular ridges shapes (Li et al. 2020).

The mycelium of *Phallus fuscoechinovolvatus* was observed on various agar media, with the largest colony diameter on PDA. The morphology of the mycelium colonies on PDA and MEA was similar, forming abundant white cotton-like colonies. Optimal mycelial growth was observed at pH 6–7 on PDA media, with temperatures between 23 °C and 25 °C being the most favorable for growth. The colony morphology varied with temperature, displaying denser hyphae at 20 °C and 27 °C and irregular shapes at lower temperatures of 15 °C and 18 °C. This study is consistent with Cheong et al. (2020), who reported that *Phallus indusiatus* exhibited the largest colony diameter on PDA, with MEA also supporting the growth of abundant, white cotton-like mycelial growth. However, there could be differences in the optimal pH and temperature conditions. For instance, *Phallus indusiatus* might show optimal mycelial growth at a slightly different pH range, such as 5–6, and at a different temperature range, such as 20–22 °C. Additionally, the colony morphology at varying temperatures could differ, perhaps exhibiting more uniform growth across a broader temperature range without the same degree of irregular shapes at lower temperatures.

Despite numerous reports of *Phallus* collections in Thailand, taxonomic confusion exists, as some specimens previously identified as *Phallus* have been classified as *Dictyophora* (Chandrasrikul et al. 2008). Recent advancements include the description of two novel *Phallus* species: *P. merulinus* (Hosaka et al. 2012) and *P. chiangmaiensis* (Sommai et al. 2021). The present study contributes to the ongoing effort by documenting two additional *Phallus* collections from Thailand. Further research, including detailed morphological and molecular analyses, is crucial to bridge the knowledge gap regarding *Phallus* diversity in Thailand. Moreover, a

comprehensive understanding of the ecological roles and significance within the genus is also necessary.

In this study, we aim to evaluate a potential species of *Phallus fuscoechinovolvatus*, recognized as an edible species commercially cultivated in China. In contrast, *Phallus indusiatus* has only recently been identified as a new species from China, with no official reports confirming its edibility (Li et al. 2020). Consequently, this study focuses on determining the optimal conditions for mycelial growth and cultivation of *P. fuscoechinovolvatus*, whereas the cultivation aspects of *P. indusiatus* were not investigated. In addition, these highlights further emphasize the inherent species-specific nature of mycelial growth requirements within the genus *Phallus*. Although common traits include the propensity for growth on agar media and the formation of white, cotton-like colonies, the precise optimal pH and temperature can exhibit variation between species. This highlights the potential for unique ecological adaptations and physiological requirements among *Phallus* species. Further investigation into a broader range of *Phallus* species would strengthen our understanding of this fungal genus' adaptability and niche preferences.

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