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ARTICLE

Natarajania thailandica sp. nov. (Stilbosporaceae, Diaporthales) from Thailand

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ABSTRACT

A fungus similar to the monotypic genus *Natarajania*, isolated from dead wood and collected in Thailand, is reported. Analysis of partial ribosomal LSU and a protein coding gene (RPB2) demonstrated that the new isolate belonged to Stilbosporaceae, Diaporthales and genetically different from *N. indica*. It is unique in producing synnematous conidiophores, smooth-walled conidiogenous cells and a flared collarette but lacks an elongated collar-canal which is distinct in the type species. Therefore, sequence data and morphological traits are used to introduce the new species, *Natarajania thailandica*.

KEYWORDS

Fungi; hyphomycete; one new species; saprobes; taxonomy

1 Introduction

Link [1] introduced Stilbosporaceae to include *Prosthecium* with its asexual morph. Stilbosporaceae has been synonymized under different families [2–4]. Voglmayr et al. [5] established Stilbosporaceae in Diaporthales based on phylogenetic investigation of LSU sequence data and accommodated *Stegonsporium* and *Stilbospora* within the family, and synonymized *Prosthecium* under *Stilbospora*. The type species of *Stilbospora*, *S. macrosperma*, has been linked to its asexual morph *Prosthecium ellipsosporum*, the generic type of *Prosthecium* [6]. *Natarajania* was placed in Stilbosporaceae based on phylogenetic analyses of concatenated LSU, SSU, TEF and RPB2 sequence data by Maharachchikumbura et al. [7]. This is the only hyphomycetous taxon affiliated to the diaporthales which are known to have coelomycetous asexual morphs. *Crinitospora*, *Natarajania*, *Stegonsporium* and *Stilbospora* are presently placed within this family [8]. The reliability of available sequence data and identification of taxon require further investigation [2].

The monotypic dematiaceous hyphomycete genus, *Natarajania*, introduced by Pratibha and Bhat [9] is typified by *N. indica* Pratibha and Bhat. The genus is charactarized by mononemaotus, macronematous, erect, branched conidiophores, monophialidic, verrucose conidiogenous cells with a distinct collar-canal and dark-brown, slimy, smooth conidia [9]. This genus shares similar features of Cryphonectriaceae in Diaporthales which largely comprises coelomycetous asexual morphs [10].

We are carrying out inventories of fungi throughout Thailand where the diversity is proving to be extremely diverse with numerous new species [11,12]. The aim of the present paper is to introduce a second species of *Natarajania* with evidence from phylogenetic analyses of combined LSU and RPB2 sequence data and morphology. A comprehensive morphological description and illustrations are provided.

2 Materials and Methods

2.1 Specimen Collection

Fresh material was collected from Cha-Am, Phetchaburi Province, Thailand in August, 2017. Samples were labeled and brought to the laboratory in Zip lock plastic bags.

2.2 Incubation, Specimen Examination and Isolation

Specimen were incubated in plastic boxes with moistened sterilized tissue papers at room temperature over one week after which they were examined with a Motic SMZ 168 dissecting microscope for fungal fruiting bodies. Scrape mounts of the fungal structures were mounted in water on clean glass slides and stained with Melzer's reagent or Indian ink or Congo red for microscopic studies and photomicrography. Micro-morphological structures of the fungus were examined and photographs were taken by Nikon ECLIPSE 80i compound microscope fitted with a Canon 600D digital camera. Measurements of photomicrographic structures were made with the Tarosoft®Image Frame Work version 0.9.7. program and images used for figures were assembled with Adobe Photoshop CS6 Extended version 13.0.1 software (Adobe Systems, USA). Isolates were made from single spores following the modified method of Chomnunti et al. [13]. Conidial suspensions were incubated at 25–28°C. Germinating conidia were transferred to potato dextrose agar (PDA) media. Pure cultures were obtained after sub-culturing. The cultural characteristics (mycelium color, shape, texture, and growth rate) were recorded [14]. Cultures and herbarium specimens of isolated fungi of this study were deposited in Mae Fah Luang University culture collection (MFLUCC) and Mae Fah Luang University Herbarium (Herb. MFLU) respectively.

2.3 DNA extraction, Polymerase Chain Reactions (PCR) and Sequencing

Total fungal DNA were obtained from fresh fungal mycelium grown on PDA media at 16–25°C for four weeks using Biospin Fungus Genomic DNA Extraction Kit (BioFlux®, China), (Hangzhou, P. R. China) following the instructions of the manufacturer. DNA amplifications were performed by polymerase chain reaction (PCR) using the primer pairs listed in Tab. 1. Amplifications were performed in 25 µl of PCR mixtures containing 12.5 µl of PCR Master Mix, 9.5 µl of ddH_2O, 1 µl of DNA template and 1 µl of each primer set (10 µM). The polymerase chain reactions (PCR) for LSU and RPB2 were performed according to Senanayake et al. [2]. The PCR conditions for LSU was as follows: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 90 s and a final extension at 72°C for 10 min and for RPB2 was: initial denaturation at 94°C for 120 s, followed by 35 amplification cycles of denaturation at 95°C for 45 s, annealing at 57°C for 50 s and extension at 72°C for 90 s. PCR products were visualized and confirmed on 1% agarose electrophoresis gels stained with green stain. Purification and sequencing of PCR products were carried out at Tsingke Company, Beijing, P.R. China. Newly generated DNA sequences were deposited in GenBank database.

2.4 Molecular Phylogenetic Analyses

DNA sequences obtained in this study were analyzed and compared with other sequences retrieved from GenBank based on BLAST searches and recently published data. Sequence data were aligned by MAFFT [18,19] and manually improved with BioEdit v.7.2.5 [20]. Maximum likelihood (ML) and Bayesian Inference (BI) analyses of the combined LSU and RPB2 dataset were used.

Table 1: Partial gene regions and PCR primers used in this study

Gene/Locus	Primer		Reference
	Forward	Reverse	
Large subunit (LSU)	LR0R	LR5	[15,16]
RNA polymerase II subunit 2, (RPB2)	fRPB2-5f	fRPB2-7cR	[17]

Maximum-likelihood analysis was performed using the RAxML-HPC2 on XSEDE (v. 8.2.10) [21] in the CIPRES Science Gateway platform [22]. In this analysis nonparametric bootstrap iterations [23] was run in 1,000 replicates with the GTR model and a discrete gamma distribution [24]. Best-fit models for Bayesian and maximum likelihood analyses were selected using MrModeltest v. 2.2 [25] and the best model was GTR + I + G.

Bayesian analysis was performed using MrBayes v. 3.1.2 [26] to evaluate Posterior probabilities (PP) [27,28] by Markov Chain Monte Carlo sampling (MCMC). Six simultaneous Markov chains were run for 1000000 generations and trees were sampled at every 100th generation in two parallel runs. The first 20% of trees, representing the burn-in phase of the analyses were discarded. The remaining 80% trees were used to calculate PP in the majority rule consensus tree (Fig. 1). Phylograms were visualized with FigTree v1.4.0 program [29] and reorganized in Microsoft power point (2007) (Tab. 2).

3 Results

3.1 Phylogenetic Analysis

The combined LSU and RPB2 dataset belonging to Coryneaceae, Cytosporaceae, Diaporthaceae, Lamproconiaceae, Macrohilaceae, Prosopidicolaceae, Stilbosporaceae and Sydowiellaceae comprised 39 taxa with *Phaeoacremonium aleophilum* (CBS 631.94) and *P. vibratile* (CBS 117115) as the outgroup taxa. RAxML analysis of the combined dataset resulted a best tree with a final ML optimization likelihood value of −17816.176831 (Fig. 1). The matrix comprised 1304 distinct alignment patterns, with 51.11% of undetermined characters. Estimated base frequencies were as follows; gamma distribution shape parameter α = 0.226432; A = 0.240879, C = 0.269773, G = 0.278187, T = 0.211160; substitution rates AC = 1.679941, AG = 2.987693, AT = 1.497541, CG = 1.259527, CT = 7.992461, GT = 1.000000. Phylogenetic trees obtained from BI were similar in topology to the ML tree. Phylogenetic results indicated that the isolate of *N. thailandica* (MFLUCC18-0394) clustered with *N. indica* (GUFCC 5240) with strong support (100% ML, 1.00 PP) within the Stilbosporaceae (Fig. 1).

3.2 Taxonomy

Natarajania J. Pratibha & Bhat, Kavaka 33: 129 (2006) [2005] **amend.**

Saprobic on dead wood or leaves. **Sexual morph:** Undetermined. **Asexual morph:** *Colonies* numerous, effuse, greyish or dark brown to black, hairy, velvety. *Mycelium* partly superficial, partly immersed in the substrate, composed of smooth, hyaline to pale brown, branched, septate, thick-walled hyphae. *Conidiophores* macronematous, mononematous or synnematous, septate, branched above, erect, straight or moderately flexuous, solitary or fasciculate, hyaline or brown to dark brown, smooth and thick-walled; when synnematous compactly intertwined in below half, producing spherical fertile heads at apex. *Conidiogenous cells* monophialidic, terminal, integrated, elongated, cylindrical, entirely smooth-walled or upper-hall distinctly verrucose and smooth below, pale brown, terminating in a collarette, sometimes with a narrow, elongated, cylindrical, smooth, colourless collar-canal. *Conidia* slimy, solitary, subglobose to ellipsoidal, dark brown, smooth walled, aseptate, straight or slightly curved, with a truncate base.

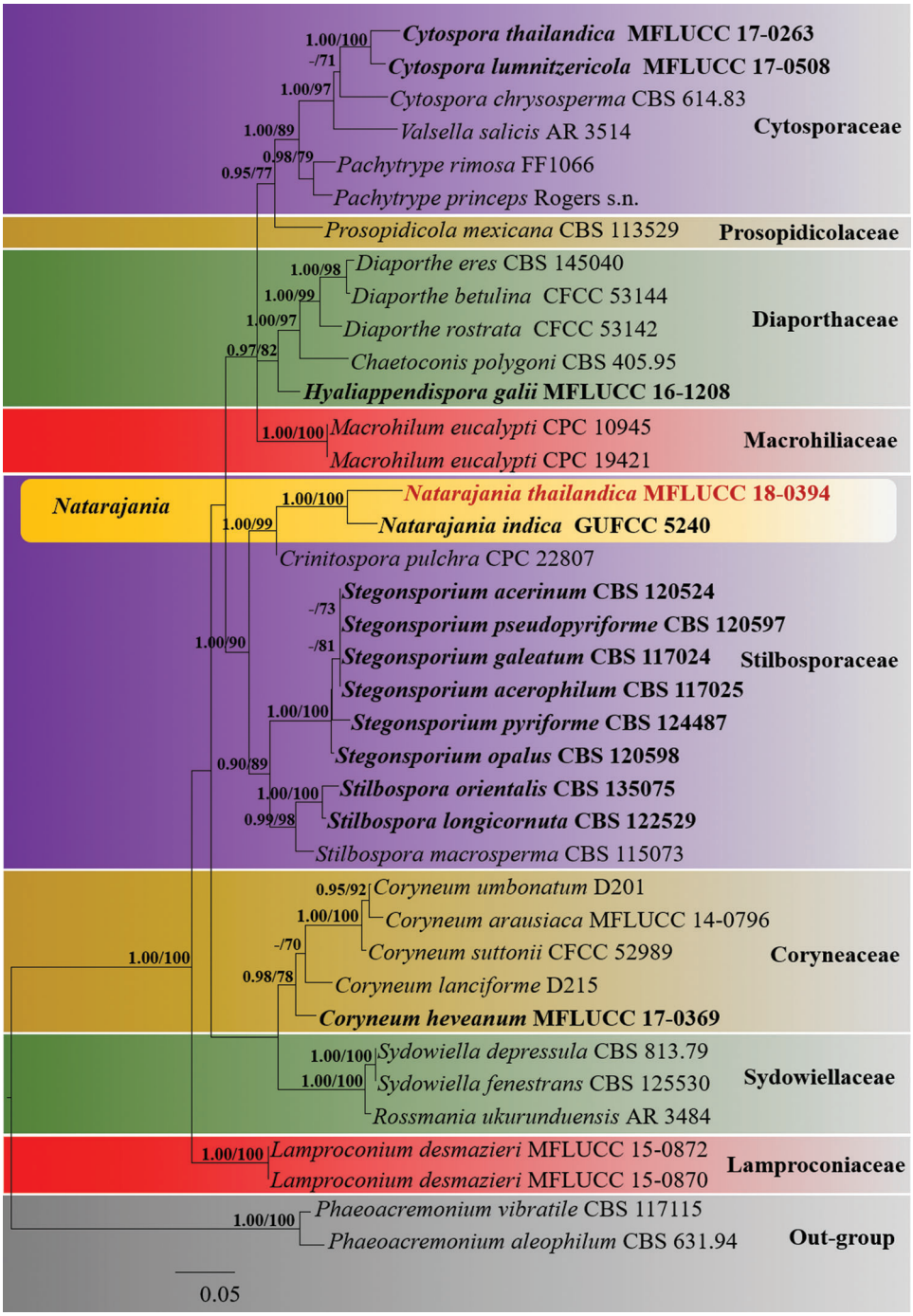

Figure 1: The Maximum likelihood phylogenetic tree based on a combined LSU and RPB2 sequence dataset which comprised 39 strains including *Phaeoacremonium aleophilum* (CBS 631.94) and *P. vibratile* (CBS 117115) as the outgroup taxa. Maximum likelihood bootstrap (ML) values >65% and Bayesian posterior probabilities (PP) >0.90 are indicated at the nodes. The ex-type strains are in bold black and the new isolate is in red bold

Table 2: GenBank accessions of taxa used in the phylogenetic analysis. Those of the novel taxon generated in this study are in blue bold and ex-types strains are in black bold

Species name	Strain No.	GenBank accessions	
		LSU	RPB2
Chaetoconis polygoni	CBS 405.95	EU754141	–
Coryneum arausiaca	MFLUCC 14-0796	MF190067	MF377609
C. heveanum	**MFLUCC 17-0369**	**MH778703**	**-**
C. lanciforme	D215	–	MH674336
C. suttonii	CFCC 52989	MK429926	MK578111
Crinitospora pulchra	CPC 22807	KJ710443	**-**
C. umbonatum	D201	–	MH674333
Cytospora chrysosperma	CBS 614.83	KX965400	KX965554
C. lumnitzericola	**MFLUCC 17-0508**	**NG064534**	**MH253461**
C. thailandica	**MFLUCC 17-0263**	**NG064536**	**MH253464**
Diaporthe betulina	CFCC 53144	MN265874	MN315498
D. eres	CBS 145040	MK442521	MK442663
D. rostrata	CFCC 53142	MN265878	MN315489
Hyaliappendispora galii	**MFLUCC 16-1208**	**MF190095**	**-**
Lamproconium desmazieri	MFLUCC 15-0870	KX430135	–
L. desmazieri	MFLUCC 15-0872	KX430139	–
Macrohilum eucalypti	CPC 10945	DQ195793	–
M. eucalypti	CPC 19421	KR873275	–
Natarajania indica	**GUFCC 5240**	HM171321	–
N. thailandica	**MFLUCC 18-0394**	**MT371074**	**MT364367**
Pachytrype princeps	Rogers s.n.	FJ532382	–
P. rimosa	FF1066	FJ532381	–
Phaeoacremonium aleophilum	CBS 631.94	AB278175	–
P. vibratile	CBS 117115	DQ649065	HQ878611
Phaeodiaporthe appendiculata	CBS 123821	–	–
Prosopidicola mexicana	CBS 113529	KX228354	–
P. mexicana	CBS 113530	–	–
Rossmania ukurunduensis	AR 3484	EU683075	–
Stegonsporium acerinum	**CBS 120524**	**EU039995**	**KF570171**

(Continued)

Table 2 (continued).

S. acerophilum	**CBS 117025**	**EU039993**	**-**
S. galeatum	**CBS 117024**	**EU039989**	**-**
S. opalus	**CBS 120598**	**EU039997**	**-**
S. pseudopyriforme	**CBS 120526**	**-**	**KF570185**
S. pyriforme	**CBS 124487**	**-**	**KF570190**
Stilbospora longicornuta	**CBS 122529**	**-**	**KF570194**
S. macrosperma	CBS 115073	–	KF570195
S. orientalis	**CBS 135075**	**-**	**KF570197**
Sydowiella depressula	CBS 813.79	EU683077	–
S. fenestrans	CBS 125530	EU683078	–
Valsella salicis	AR 3514	EU255210	EU219346

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Index Fungorum Number: IF557508; Fig. 2

Etymology: Species epithet refers to the country, Thailand where it is originated.

Holotype: MFLU 18-0588

Saprobic on unidentified wood. **Sexual morph**: Undetermined. **Asexual morph**: *Colonies* on substrate hairy, numerous, effuse, dark brown to black, velvety. *Conidiophores* 250−950 × 8−16 µm ($\bar{x}$ = 560 × 14 µm, n = 25), macronematous, synnematous, compactly intertwined in below half, septate, branched above, erect, straight or moderately flexuous, producing spherical fertile heads at apex, 345−555 µm diam. ($\bar{x}$ = 485 µm, n = 20), brown to dark brown, smooth- and thick-walled. *Conidiogenous cells* 40−48 × 8−12 µm ($\bar{x}$ = 45 × 10 µm, n = 30), monophialidic, terminal, integrated, elongated, cylindrical, smooth-walled, pale brown, terminating in a collarette. *Conidia* 15−22 × 10−18 µm diam. ($\bar{x}$ = 18.5 × 14 µm, n = 30), solitary, subglobose to ellipsoidal, dark brown, smooth-walled, aseptate.

Culture characteristics: Colonies becoming 3 cm diam. on PDA withing 30 days at 25°C, circular, with smooth margin, white at the beginning, becoming yellowish white/cream after six weeks, flat from the surface, lacking aerial mycelium, reverse yellowish brown.

Material examined: THAILAND, Phetchaburi Province, Cha-Am district, on an unidentified terrestrial wood, 31 Aug. 2017, Dayarathne M.C., MCD 010 (MFLU 18-0588, **holotype**), ex-type living culture MFLUCC18-0394.

Notes: *Natarajania thailandica* is morphologically and phylogenetically closely related to *N. indica* (Figs. 1 and 2). In our phylogenetic analyses, *Natarajania thailandica* formed a sister lineage to *N. indica* with high statistical support (100% ML, 1.00 PP) (Fig. 1). Considering there are 2.06% base pair differences in the LSU region between *N. thailandica* and *N. indica* (18 bp out of 872 bp without gaps) we consider they are different species. However, RPB2 sequence data are unavailable for *N. indica*. Furthermore, *Natarajania thailandica* is clearly distinguished from *N. indica* by the characteristics of conidiophores, conidiogenous cells and conidia. Conidiophores of *N. thailandica* are synnematous and spirally arranged, while *N. indica* has mononematous conidiophores [9]. Additionally, conidiophores of

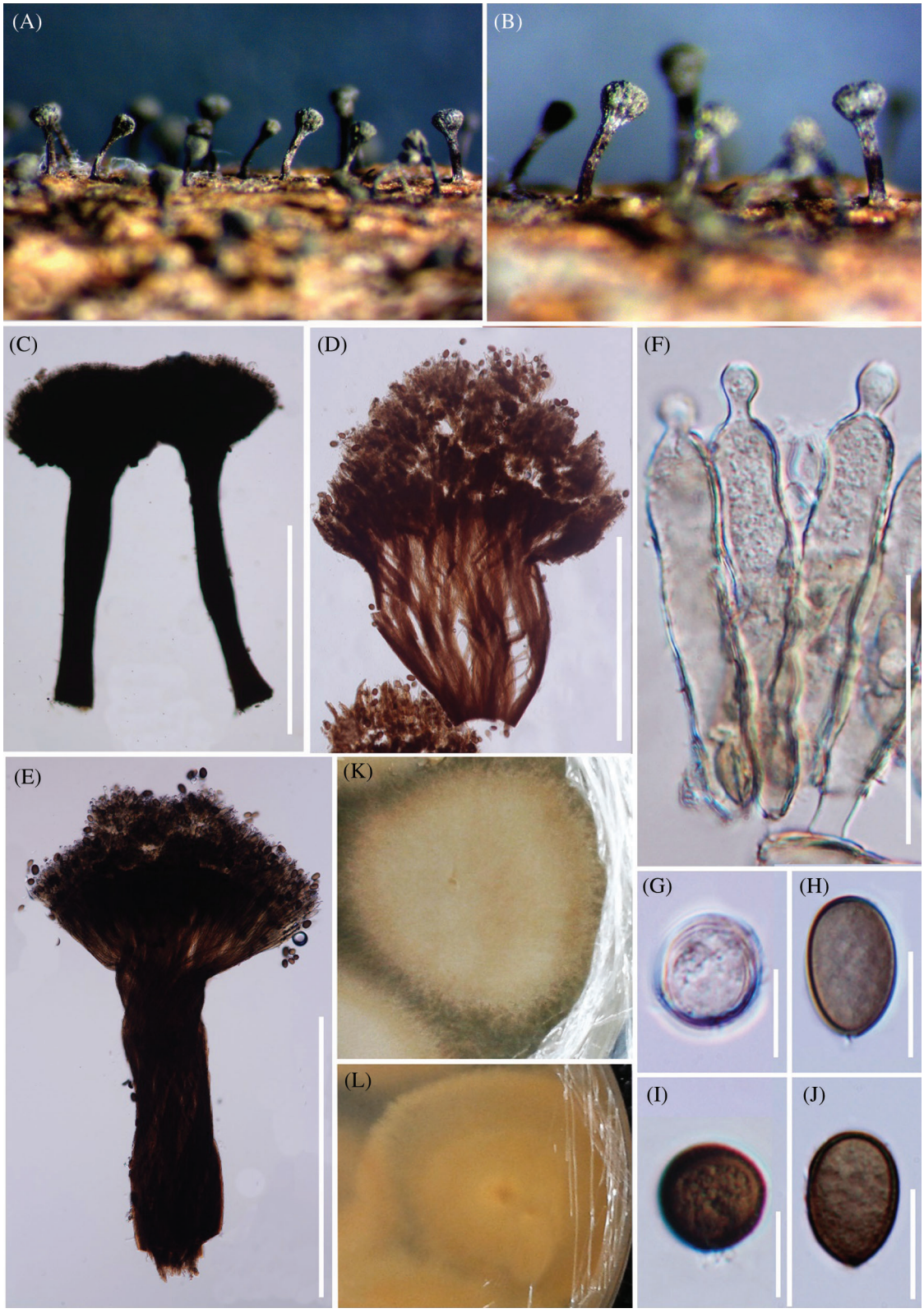

Figure 2: *Natarajania thailandica* (MFLU 18-0588, holotype). A Substrate. B Colonies on substrate. C-E Conidiophores. F Conidiogenous cells. G-J Conidia. K, L Culture on PDA (K upper, L lower). Scale bars: C, E = 500 µm, D = 100 µm, F–J = 20 µm

N. thailandica are longer than those of *N. indica* (250−950 × 8−16 μm *vs.* 50−120 × 2−4.5 μm) [9]. Conidiogenous cells of *N. thailandica* are significantly different from those of *N. indica* in size (40−48 × 8−12 μm *vs.* 30−45 × 2−3 μm) and by the lack of a collar-canal. Further, the conidiogenous cells of *N. indica* are verrucose in the upper part whereas *N. thailandica* has smooth-walled conidiogenous cells. The conidia of *N. thailandica* are comparatively larger than those of *N. indica* (15−22 × 10−18 μm *vs.* 5−7.5 × 3−5 μm) [9]. Considering the morpho-molecular differences, we establish this species as a novel taxon within *Natarajania*.

4 Discussion

Natarajania, typified by *N. indica*, is characterized by mononematous conidiophores, phialidic verrucose conidiogenous cells with a narrow, elongated, cylindrical, smooth, colorless collar-canal and apically flared collarette [9]. Our novel species *N. thailandica* widens the morphological diversity of the genus by having synnematous conidiophores and smooth conidiogenous cells without a collar-canal. *Crinitospora pulchra* B. Sutton & Alcorn showed close phylogenetic affinities to *Natarajania* in our phylogenetic analyses (Fig. 1). However, *Crinitospora pulchra* can be easily distinguished from *Natarajania* by being a coelomycete species with acervular conidiomata and median eu-septate conidia with divergent cellular appendages [30]. Furthermore, *Natarajania* was earlier confined to India [31], but our sampling from Thailand extends the geographical range to the ASEAN region. *Natarajania indica* has been reported from leaf litter of *Antiaris toxicaria* [9], while *N. thailandica* is isolated from dead wood.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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