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Morphology and phylogeny of *Yunnanomyces phoenicis* sp. nov. (Sympoventuriaceae) from Thailand

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Abstract

A new hyphomycetous species, associated with monocotyledonous *Phoenix paludosa* (mangrove date palm) was isolated and is introduced in this study. Multi-gene (LSU, SSU and *RPB2*) phylogenetic analyses showed that the new taxon clustered together with *Yunnanomyces pandanicola* and formed a well-supported clade within the family Sympoventuriaceae (Venturiales). The taxon is characterized by it semi-macronematous conidiophores, reduced to conidiogenous cells, and bears globose to broadly ellipsoidal, muriform conidia. It is morphologically similar to *Yunnanomyces pandanicola*, but differs from the latter in its reduced conidiophores and larger, brown conidia. The phylogenetic result also confirms that this fungus is a distinct species of *Yunnanomyces*. Therefore, *Yunnanomyces phoenicis* sp. nov. is introduced herein, and its morphological features, sporulation in culture are described and illustrated, as well as its phylogenetic placement is provided.

Key words – 1 new taxon – Asexual – Dothideomycetes – Muriform conidia – Taxonomy

Introduction

Plant tissues harbour vast heterotrophic microfungi which exhibit parasitic, saprobic or symbiotic life strategies. The family Sympoventuriaceae Yin. Zhang, C.L. Schoch & K.D. Hyde (Zhang et al. 2011) is typified by *Sympoventuria* Crous & Seifert (Crous et al. 2007), and comprising a group of plant-pathogens or -saprobes, as well as oligotrophic saprobes and some opportunistic species causing infections in vertebrates (Machouart et al. 2014). Phylogenetic analysis indicated that Sympoventuriaceae and Venturiaceae E. Müll. & Arx ex M.E. Barr are sister groups; belonging to the order Venturiales within Dothideomycetes (Zhang et al. 2011, Hyde et al. 2013, Liu et al. 2017). Currently, seven genera: *Clavatispora* Boonmee & K.D. Hyde (Boonmee et al. 2014), *Mycosisymbrium* (Pratibha & Prabhugaonkar 2016), *Ochroconis* (Machouart et al. 2014), *Sympoventuria*, *Veronaeopsis* Arzanlou & Crous (Arzanlou et al. 2007), *Verruconis* (Samerpitak et

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al. 2014), *Yunnanomyces* Tibpromma & K.D. Hyde (Tibpromma et al. 2018), as well as species from *Fusicladium* Bonord (Machouart et al. 2014), *Neocoleroa* Petr. (Johnston & Park 2016) and *Scolecobasidium* E.V. Abbott (Machouart et al. 2014) are referred to the family *Sympoventuriaceae* based on multi-gene phylogeny.

Asexual morphs of *Sympoventuriaceae* are known as fusicladium-like, sympodiella-like, *Veronaeopsis* (Zhang et al. 2011), trichocladium-like (Boonmee et al. 2014) and *Yunnanomyces* (Tibpromma et al. 2018). However, *Yunnanomyces* can be easily distinguished from other asexual morphs by the globose to broadly oval, yellow to brown, muriform conidia. We are carrying out a fungal diversity survey on palms and several new taxa have been described from Thailand (Konta et al. 2016a, b, c, 2017, Wanasinghe et al. 2018, Zhang et al 2018, 2019a, b). In this study, a new asexual fungus *Yunnanomyces phoenicis* is introduced. Its morphology fits well with the genus *Yunnanomyces*, but can be distinguished from the type *Y. pandanicola* by having reduced conidiophores and larger, brown conidia; the phylogenetic results also confirm its distinction. A detailed description and illustration are provided.

Materials & Methods

Specimens collection, examination and single spores isolation

Decaying rachides and leaves of mangrove date palm *Phoenix paludosa* were collected from Ranong and Chanthaburi provinces in Thailand. Specimens were sorted and placed into plastic bags in the field along with the collecting information. Fungal fruiting bodies were observed using Motic SMZ 168 stereo microscope and were made into slides mounted in water by using a syringe needle. Morphological characters photographed by using a Carl Zeiss stereo microscope fitted with an AxioCam ERC 5S camera and a Nikon ECLIPSE 80i compound microscope fitted with a Canon EOS 600D digital camera. Measurements were carried out by Tarosoft Image Frame Work program v. 0.97 (Liu et al. 2010) based on the calibrations fitted with the microimaging system, and which was used to take the photos mentioned above. Photo plate was processed by using Adobe Photoshop CS6 Extended v. 13.0. Single spores were isolated by diluting spores into small amount of sterilized deionized water, and then sprinkled onto potato dextrose agar (PDA) medium using a sterile pipette. The PDA plates with the spore suspension were incubated at 25ºC–28ºC. Germinating spores were observed the next day using Motic SMZ 168 stereo microscope, and transferred to a new PDA medium. Herbarium specimens of the new taxon were deposited at the herbaria of Mae Fah Luang University (MFLU), Chiang Rai, Thailand; Kunming Institute of Botany Academia Sinica (KUN), Kunming, China; the ex-type living culture is deposited in Mae Fah Luang University Culture Collection (MFLUCC). Index Fungorum number (2019) and Facesoffungi number (Jayasiri et al. 2015) are obtained and provided.

DNA extraction, PCR amplification and sequencing

Fungal mycelia were scraped from the margin of a colony on PDA incubated at 25ºC–28ºC for one month. Fungal genomic DNA was extracted by using the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech (Shanghai) Co., Ltd, China). Four genes were used for polymerase chain reaction (PCR) of the new collections: the nuclear rDNA operon spanning the 3' end of the 18S nrRNA gene, the large subunit of the nuclear ribosomal RNA genes (LSU), the small subunit of the nuclear ribosomal RNA (SSU), and the translation elongation factor 1-alpha (*TEF1α*) and the second largest subunit of RNA polymerase II (*RPB2*). The primers used were ITS5/ITS4 for ITS (White et al. 1990), LR0R/LR5 for LSU (Vilgalys & Hester 1990), NS1/NS4 for SSU (White et al. 1990), EF1-983F/EF1-2218R for *TEF1α* (Rehner & Buckley 2005), and fRPB2-5F/fRPB2-7cR for RPB2 (Liu et al. 1999). The amplification reactions were performed in 25μL of PCR mixtures containing 9.5μL ddH_2O, 12.5μL 2× PCR MasterMix (BBI Life Sciences Corporation, Shanghai, China), 1μL DNA temple and 1μL of each primer. The PCR thermal cycle program for LSU, SSU and *TEF1α* amplification were as follows: initial denaturing step of 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 56 °C for 50

seconds, elongation at 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR thermal cycle program for *RPB2* was followed as initially 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min, elongation at 72 °C for 90 seconds, and final extension at 72 °C for 10 min. Purification and sequencing of PCR products were carried out with primers mentioned above at Sangon Biotech (Shanghai) Co., Ltd, China.

Phylogeny analyses

Phylogenetic analysis was performed based on three gene regions: LSU, SSU and *RPB2* (Machouart et al. 2014, Tibpromma et al. 2018). Sequences with the inclusion of reference taxa related to Sympoventuriaceae (Table 1) were downloaded from GenBank. Multiple sequences of each gene region were aligned by online version of MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013), and then manually optimized using BioEdit v.7.0.9 (Hall 1999). A concatenated data set of LSU, SSU and *RPB2* sequences were used for phylogenetic analyses. The alignment of multi-gene dataset used for phylogenetic analyses is deposited in TreeBASE under the submission number 24507.

A maximum likelihood (ML) analysis was performed at the CIPRES web portal (Miller et al. 2010) using RAxML v.7.2.8 as part of the "RAxML-HPC Blackbox (8.2.10)" tool (Stamatakis 2006, Stamatakis et al. 2008). A general time-reversible model (GTR) was applied with a discrete GAMMA distribution and four rate classes. Fifty thorough ML tree searches were carried out in RAxML v.7.2.7 under the same model. One thousand non-parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution. The resulting replicates were plotted onto the best scoring tree obtained previously.

Maximum parsimony (MP) analysis was performed using the heuristic search option with 1000 random taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equally weight, gaps were treated as missing data. Maxtrees setting was 1000, and zero-length branches were collapsed, and all parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Hillis & Bull 1993). Tree length [TL], Consistency index [CI], Retention index [RI], Rescaled consistency index [RC], Homoplasy index [HI] were calculated.

The Bayesian analysis was performed using PAUP v.4.0b10 (Swofford 2002) and MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003). The best model for different genes partition in the concatenated data set was determined by MrModeltest 2.3 (Nylander 2004). Posterior probabilities (Rannala & Yang 1996) were determined by Markov Chain Monte Carlo sampling (MCMC) (Larget & Simon 1999) in MrBayes v.3.1.2. Four simultaneous Markov chains were run for 10 million generations and trees were sampled every 1000th generation, thus 10,000 trees were obtained. The suitable burn-in phases were determined by inspecting likelihoods and parameters in Tracer version 1.6 (Rambaut et al. 2013). Based on the tracer analysis, the first 1,000 trees representing 10% were discarded as the burn-in phase in the analysis. The remaining trees were used to calculate posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01). Phylogenetic tree was visualized by FigTree v.1.4.2 (Rambaut 2014). The new taxon was established based on recommendations outlined by Jeewon & Hyde (2016).

Results

Phylogeny

The individual phylogenetic analyses based on LSU, SSU and *RPB2* genes were carried out and yielded similar topologies. The alignments of each gene were combined to perform multi-gene phylogenetic analyses, resulting in a matrix of 23 taxa and 2976 characters (LSU: 865 bp; SSU: 1022 bp and RPB2: 1089 bp) including gaps. RAxML, MP and Bayesian analyses were conducted and resulted in generally congruent topologies. The best scoring RAxML tree (Fig. 1) with a final

optimization likelihood value of –12059.011217. The matrix had 876 distinct alignment patterns, with 44.63% undetermined characters and gaps. Estimated base frequencies were as follows: A = 0.255690, C = 0.224381, G = 0.291544, T = 0.228385; substitution rates AC = 1.261200, AG = 2.964321, AT = 1.535962, CG = 1.214748, CT = 6.758988, GT = 1.000000; gamma distribution shape parameter α = 0.511302. Maximum parsimony analyses indicated that 2,063 characters were constant, 278 variable characters parsimony uninformative and 635 characters are parsimony-informative. A heuristic search yield two equally most parsimonious trees (TL = 1777, CI = 0.732, RI = 0.699, RC = 0.512, HI = 0.268). The best-fit model of GTR+I+G was selected by Akaike information criterion (AIC) in Mrmodeltest and used for Bayesian settings.

All genera of *Sympoventuriaceae*, including the type species of *Venturiaceae*, were selected to represent taxa in the order *Venturiales*. The resulting tree (Fig. 1) is congruent with topologies from previous studies (Zhang et al. 2011, Machouart et al. 2014, Boonmee et al. 2014, Johnston & Park 2016, Tibpromma et al. 2018), and the genus *Yunnanomyces* close to several fusicladium-like taxa nested in *Sympoventuriaceae*. In this study, the new isolates of *Yunnanomyces phoenicis* clustered with *Y. pandanicola* and formed a well-supported clade.

Table 1 Taxa used in this study and their GenBank accession numbers. The new generated sequences are indicated in bold.

Taxa	Strains	GenBank Accession numbers			References
		LSU	SSU	*RPB2*	
Clavatispora thailandiaca	MFLUCC 100107	KF770458	_	_	Boonmee et al. 2014
Fusicladium africanum	CPC 12828	EU035423	_	_	Machouart et al. 2014
Fusicladium cordae	CBS 675.82	FN398149	_	_	Machouart et al. 2014
Fusicladium intermedium	CBS 110746	EU035432	_	_	Machouart et al. 2014
Fusicladium pini	CBS 463.82	EU035436	_	_	Machouart et al. 2014
Fusicladium ramoconidii	CBS 462.82	EU035439	_	_	Machouart et al. 2014
Fusicladium sicilianum	CBS 105.85	FN398150	_	_	Machouart et al. 2014
Mycosisymbrium cirrhosum	MTCC12435	KR259884	KR259885	KR349124	Pratibha & Prabhugaonkar 2016
Neocoleroa metrosideri	ICMP 21139	KU131677	_	_	Johnston & Park 2016
Ochroconis constricta	CBS 211.53	KF282653	KF282671	KF282686	Machouart et al. 2014
Phaeotrichum benjaminii	CBS 541.72	AY004340	AY016348	DQ677946	Liu et al. 2017
Scolecobasidium excentricum	CBS 469.95	KF282669	KF282683	_	Machouart et al. 2014
Sympoventuria capensis	CBS120136	DQ885906	_	_	Crous et al. 2007
Sympoventuria capensis	CPC12840	DQ885904	_	_	Crous et al. 2007
Trichodelitschia bisporula	CBS 262.69	GU348996	GU349000	GU371802	Liu et al. 2017
Venturia inaequalis	CBS 594.70	GU301879	GU296205	_	Zhang et al. 2011

Table 1 Continued.

Taxa	Strains	GenBank Accession numbers			References
		LSU	SSU	*RPB2*	
Venturia inaequalis	CBS 815.69	GU301878	GU296204	_	Zhang et al. 2011
Veronaeopsis simplex	CBS 588.66	EU041877	_	_	Machouart et al. 2014
Verruconis gallopava	CBS 118.91	KF282655	KF282673	KF282688	Machouart et al. 2014
Verruconis gallopava	CBS 437.64	KF282656	KF282674	KF282689	Samerpitak et al. 2014
Yunnanomyces pandanicola	MFLUCC 17-2260	MH376743	MH388333	MH412736	Tibpromma et al. 2018
Yunnanomyces phoenicis	**MFLUCC 19-0253**	**MK976737**	**MK976739**	**MK986483**	**This study**
Yunnanomyces phoenicis	**MFLUCC 19-0254**	**MK976738**	**MK976740**	**MK986484**	**This study**

Abbreviations: CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Collection of Pedro Crous housed at CBS; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand. MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; MTCC: Microbial type culture collection, Institute of Microbial Technology, Chandigarh, India.

Note: Additional sequences for the new taxa in this study are provided as follows: MFLUCC 19-0253 (*TEF1α* = MK986485); MFLUCC 19-0254 (*TEF1α* = MK986486).

Taxonomy

Yunnanomyces phoenicis S.N. Zhang & J.K. Liu, sp. nov. Fig. 2

Index Fungorum number: IF556745; Facesoffungi number: FoF06149

Etymology – referring to the host on which the fungus was collected.

Saprobic on rachides and leaves of palms. Sexual morph: Undetermined. Asexual morph: Hyphomycetous. *Colonies* scattered, granular, black, glistening, punctiform distributed on the substrate. *Mycelium* mostly immersed in the substrate, composed of branched, septate, subhyaline to pale brown, smooth hyphae. *Conidiophores* semi-macronematous, reduced to conidiogenous cells, subhyaline to pale brown, smooth, thin-walled. *Conidiogenous cells* 0.7–1.2 μm, integrated, determinate, holoblastic, monoblastic, terminal, cylindrical, subhyaline to pale brown. *Conidia* (14–)18–34 × (8.5–)12−22 ($\bar{x}$ = 24.6 × 17.7 μm, n = 30), acrogenous, solitary, globose to broadly ellipsoidal, muriform, brown and hyaline immature, thick-walled. The number of cells per conidium varies from 10–30, apical row with 1−3 cells.

Culture characteristics – Colonies growing well on PDA, and attaining a diameter of 22 mm after 21 days at 25ºC, circular, flat, velvety, with brown filamentous margin, obverse grey-green to brown, reverse dark brown. Mycelium hyaline to brown, septate, branched. Conidial sporulation was apparent in culture. Conidia in culture are slightly larger than on palm substrate, 17–35 × 12−24 ($\bar{x}$ = 27.8 × 19.4 μm, n = 65).

Material examined – Thailand, Ranong, Ngao Mangrove Forest Research Center, on fallen rachides and leaves of *Phoenix paludosa* Roxb. (Arecaceae), 7 December 2016, S.N. Zhang, SNT72 (MFLU 19-0811, holotype; HKAS 105456, isotype), ex-type living culture: MFLUCC 19-0253. *Ibid.,* Chanthaburi, Amphoe Khlung, Tambon Wan Yao, attached on leaf litter of *Phoenix paludosa,* 12°26′43″ N, 102°15′47″ E, 0m Elevation, 25 April 2017, S.N. Zhang, SNT158 (MFLU 19-0814 = HKAS 105478, paratype), living culture MFLUCC 19-0254.

Notes – Muriform ascospores or conidia are common in Ascomycota especially in Dothideomycetes, but rare in the family *Sympoventuriaceae.* The genus *Yunnanomyces* was

introduced by Tibpromma et al. (2018) as a monotypic genus with *Y. pandanicola* as the type species. Herein we introduce a second *Yunnanomyces* species. *Yunnanomyces phoenicis* resembles *Y. pandanicola* in the globose to broadly ellipsoidal, muriform conidia. But it differs from *Y. pandanicola* in its semi-macronematous conidiophores, reduced to conidiogenous cells and bearing relatively larger brown conidia. The phylogenetic results showed that the isolates of *Yunnanomyces phoenicis* clustered together with *Y. pandanicola* and formed a monophyletic clade which represents a distinct species from *Y. pandanicola* (Fig. 1). In addition, to further justify the establishment, the single gene region comparison (Jeewon & Hyde 2016) was carried out between these two species, there are 35 nucleotide substitutions (4.32%) at specific positions in total 810 base pair of LSU, 23 nucleotide substitutions (2.33%) at specific positions and six gaps in total 986 base pair of SSU, and 25.34% nucleotide substitutions in *RPB2* gene region.

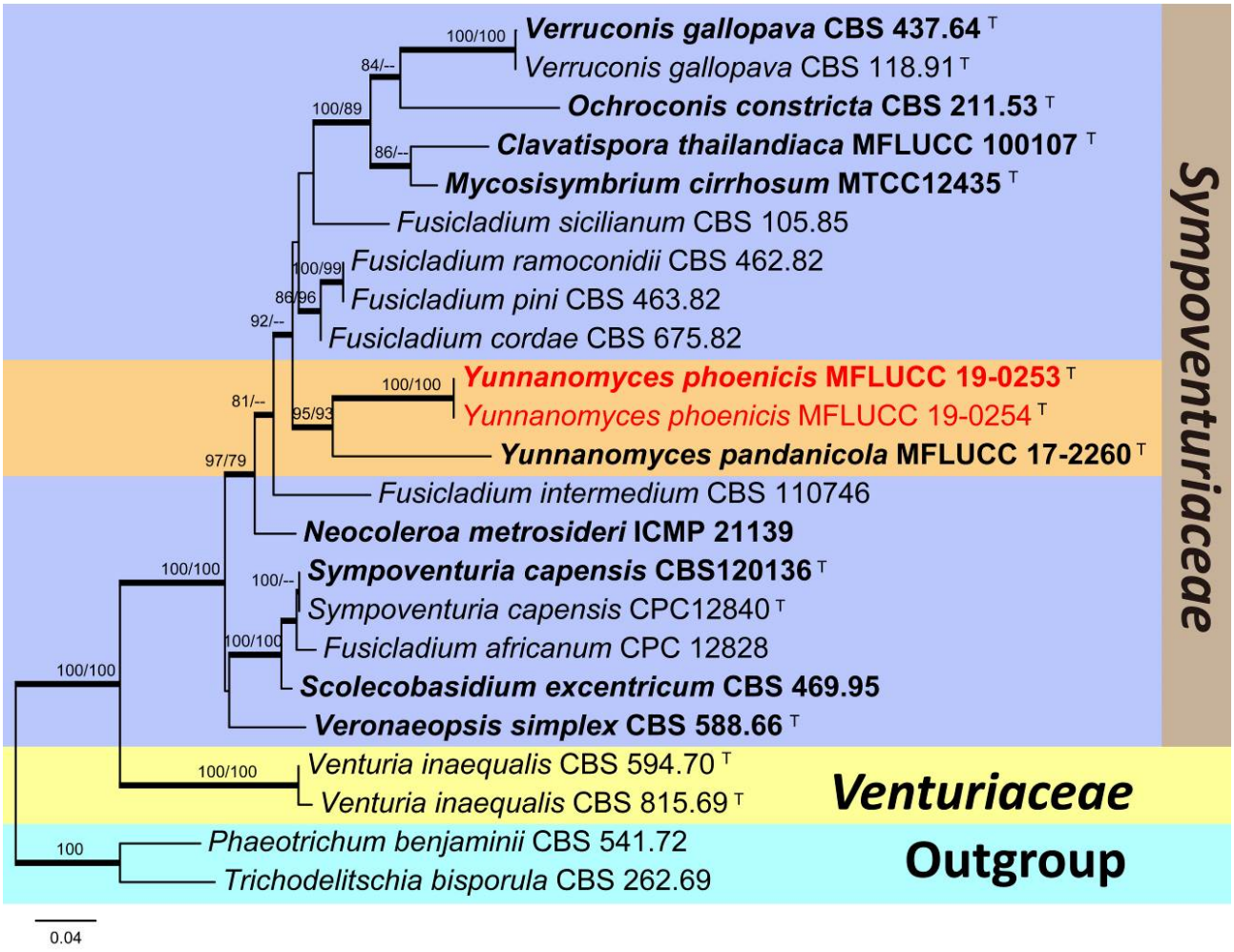

Fig. 1 – RAxML tree of representatives in *Venturiales* based on analysis of LSU, SSU and *RPB2* gene region sequences data. Bootstrap values for ML and MP equal to or greater than 75 are placed (ML/MP) above the branches respectively. Branches with Bayesian posterior probabilities (PP) from MCMC analysis equal or greater than 0.95 are in bold. The type species are indicated with superscript ^{T} and ex-type strains are in bold. New generated sequences are indicated in red. The tree is rooted with *Phaeotrichum benjaminii* (CBS 541.72) and *Trichodelitschia bisporula* (CBS 262.69).

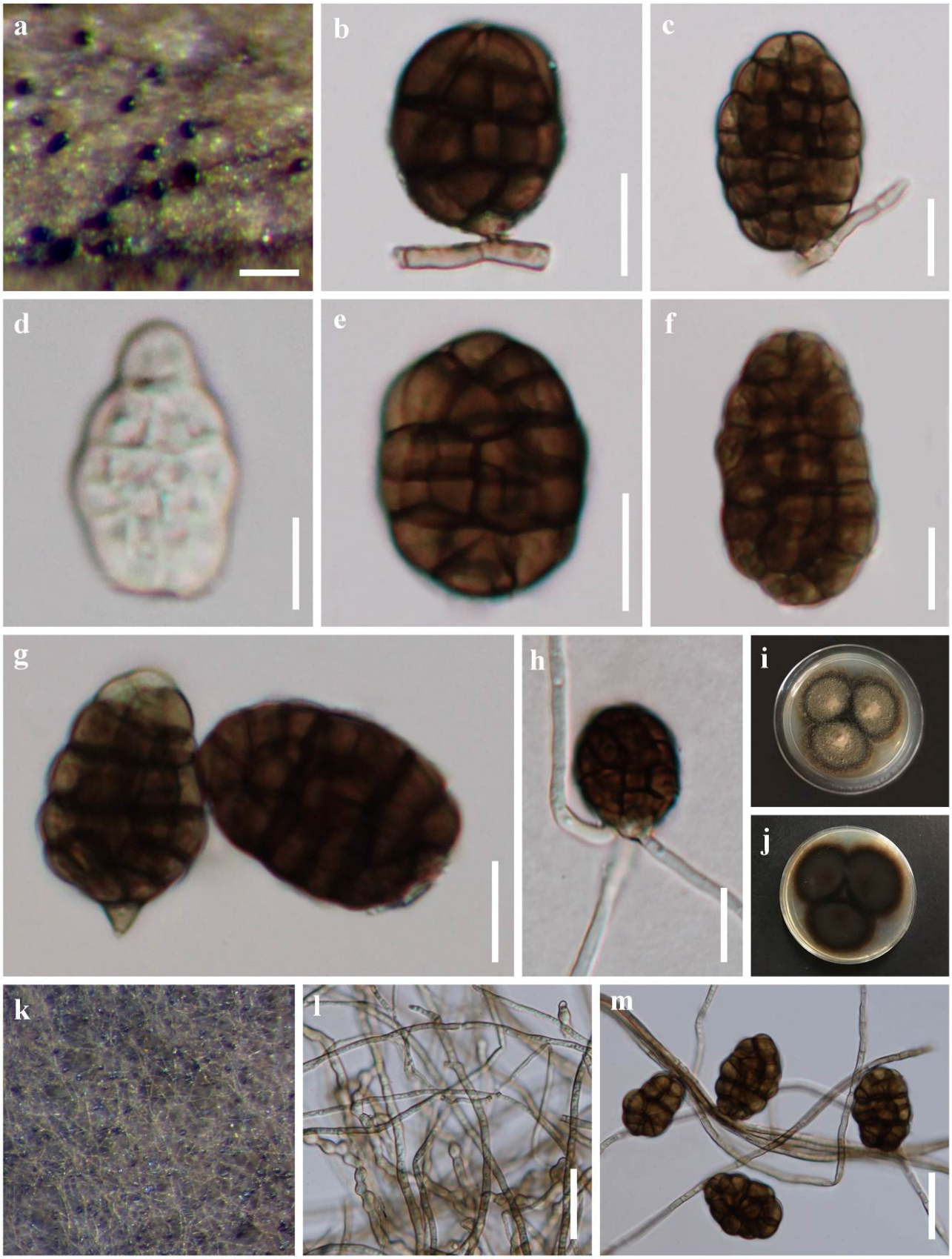

Fig. 2 – *Yunnanomyces phoenicis* (MFLU 19-0811, holotype). a Colonies on host substrate. b,c Conidiogenous cells developing conidia. d Hyaline, immature conidium. e–g Mature conidia. h Germinating conidium. i–j Culture on PDA. k–m Mycelium and sporulation in culture. Scale Bars: a = 50 µm, b,c, e–h = 10 µm, d = 5 µm, l–m = 20 µm.

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