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Phylogeny and morphology of *Helicotubeufia* gen. nov., with three new species in *Tubeufiaceae* from aquatic habitats

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Abstract

We are participating in an investigation of freshwater fungi along a north-south latitudinal gradient in the Asian region. Five new collections of asexual and sexual morphs of *Tubeufiaceae* from decaying wood in freshwater streams were obtained from China and Thailand. Morphologically, they line well with *Tubeufiaceae* in having superficial, solitary, scattered, subglobose to ellipsoidal-ovate, dark brown to black ascomata; cylindrical asci with hyaline, fusiform, multi-septate, slightly curved and guttulate ascospores and hyphomycetous helicosporous asexual morphs. Phylogenetic analyses based on combined LSU, ITS and *TEF1α* sequences data indicate that they formed a monotypic clade among the genera of *Tubeufiaceae*, but as a phylogenetically distinct lineage. Therefore, we introduce a new genus *Helicotubeufia* gen. nov, with three species (*H. guangxiensis*, *H. hydei* and *H. jonesii*) to accommodate these new taxa. Detailed descriptions and illustrations are provided, as well as the comparisons with similar taxa.

Key words – 4 new taxa – asexual morph – Dothideomycetes – freshwater – Taxonomy

Introduction

Freshwater fungi are a taxonomically highly diverse group that plays an important role in nutrient cycling and ecosystem functioning (Wong et al. 1998, Vijaykrishna et al. 2006, Shearer et al. 2009, Hyde et al 2016). Most freshwater fungi are distributed in the classes Dothideomycetes and Sordariomycetes of the Ascomycota (Vijaykrishna et al. 2006, Shearer et al. 2009, Hyde et al. 2013, Maharachchikumbura et al. 2015). The Tubeufiales (order of Dothideomycetes) is a large order comprising more than 20 genera of freshwater ascomycetes (Brahmanage et al. 2017), which include both asexual and sexual morphs (Wijayawardene et al. 2017, 2018). The order Tubeufiales was introduced by Boonmee et al. (2014) with a modern treatment based on the examinations of the type specimens and phylogenetic analyses. Three families are currently included in this order (Liu et al. 2017, Wijayawardene et al. 2018). Members of Tubeufiaceae are mostly saprobic and widely distributed, and often found on woody substrates in terrestrial and freshwater habitats (Hyde & Goh

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1998, Ho et al. 2001, Cai et al. 2002, Zhao et al. 2007, Boonmee et al. 2011, 2014, Hyde et al. 2013, Lu et al. 2017a, c) and sometimes in peat swamps (Pinnoi et al. 2006, Pinruan et al. 2007).

We are carrying out a study of lignicolous freshwater fungi along a north-south latitudinal gradient in Asia (Hyde et al. 2016). In this study, fresh collections of tubeufiaceous taxa were obtained from submerged wood in freshwater stream in China and Thailand respectively. Five taxa formed a monotypic clade among the genera of *Tubeufiaceae* and showed closely phylogenetic relationship with *Aquaphila* and *Chlamydotubeufia*. A new genus *Helicotubeufia* with three species is introduced and detailed descriptions and illustrations are provided for these new taxa. The phylogenetic analysis of combined LSU, ITS and *TEF1α* sequences data is performed to justify the establishment of the new genus, as well as the phylogenetic relationships among the genera of *Tubeufiaceae.*

Materials & Methods

Isolation and morphology

Decaying wood specimens were collected from Guangxi, China and Trat, Thailand. The samples were processed followed by Boonmee et al. (2014). Morphological observations were made using a Motic SMZ 168 Series stereomicroscope and photographed by a Nikon E80i microscope-camera system. Measurements were made with the Tarosoft (R) Image Frame Work (Liu et al. 2010).

Isolations were made from single spores as described in Liu et al. (2010). Type material is deposited at the herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS), Kunming, Guizhou Academy of Agricultural Sciences (GZAAS), Guiyang, China, and Mae Fah Luang University (MFLU), Chiang Rai, Thailand. The strains isolated in our study are deposited at Mae Fah Luang University Culture Collection (MFLUCC), Chiang Rai, Thailand and Guizhou Culture Collection (GZCC), Guiyang, China. Facesoffungi numbers and Index Fungorum numbers are provided as outlined in Jayasiri et al. (2015) and Index Fungorum (2018).

DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA for 14 days at 28 ℃ in the dark. Genomic DNA was extracted from the fresh mycelium using Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China).

DNA amplification was performed by Polymerase Chain Reaction (PCR). Two partial gene portions and one protein coding gene were used in this study: the large subunits of the nuclear ribosomal RNA genes (LSU), the internal transcribed spacers (ITS) and the translation elongation factor 1-alpha gene (*TEF1α*). The primers used were LROR and LR5 (Vilgalys & Hester 1990) for LSU, ITS5 and ITS4 (White et al. 1990) for ITS, EF1-983F and EF1-2218R (Rehner & Buckley 2005) for *TEF1α*. The PCR thermal cycle program for LSU, ITS and *TEF1α* amplification were as follows: initially denaturing step of 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 50 seconds, elongation at 72 °C for 1 min, and final extension at 72 °C for 10 min.

PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham product code: 27–9602–01). Sequence analysis was carried out by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, P.R. China).

Phylogenetic analysis

Sequences generated from different primers were analyzed with other sequences obtained from GenBank. The related sequences were determined by using a BLAST search to reveal the closest matches with taxa in *Tubeufiaceae* and recent relevant publications (Boonmee et al. 2011, 2014, Brahmanage et al. 2017, Lu et al. 2017a, b, c, 2018, Luo et al. 2017, Phookamsak et al. 2018). Sequences were aligned using Bioedit 7.2.5 (Hall 1999) and ClustalX v. 1.83 (Thompson et

al. 1997). The alignments were checked visually and improved manually where necessary. Phylogenetic analyses were performed by using PAUP v. 4.0b10 (Swofford 2002) for maximum-parsimony (MP) and MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001) for Bayesian analyses.

A maximum likelihood analysis was performed at the CIPRES webportal (Miller et al. 2010) using RAxML v.7.2.8 as part of the "RAxML-HPC2 on TG" tool (Stamatakis 2006). A general time reversible model (GTR) was applied with a discrete gamma distribution and four rate classes. Fifty thorough maximum likelihood (ML) tree searches were done 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 on to the best scoring tree obtained previously.

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally 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). The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Four simultaneous Markov chains were run for 10,000,000 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 trees were drawn using Treeview (Page 1996) and MEGA5 (Tamura et al. 2011). Sequences derived in this study are deposited in GenBank (Table 1).

Results

Phylogenetic analysis

Five isolates of tubeufiaceous taxa obtained from the submerged decaying wood in the freshwater stream were identified in the family *Tubeufiaceae*. LSU, ITS and *TEF1α* sequence data and morphological characters were used to determine their placement and to describe novel taxa with a comparison with similar taxa.

The combined LSU, ITS and *TEF1α* data set comprised 49 taxa with *Botryosphaeria dothidea* (CBS 115476) as the outgroup taxon. The dataset comprises 2,267 positions (LSU: 1–820; ITS: 821–1437; *TEF1α*: 1438–2267) after alignment, including gaps. The maximum parsimonious dataset consists of 2,267 characters, of which 1,431 characters were constant, and 165 variable characters are parsimony-uninformative. Maximum parsimony analysis of the remaining 671 parsimony-informative characters resulted in 1000 trees with TL = 3697, CI = 0.382, RI = 0.582, RC = 0.223, HI = 0.618. RAxML, Maximum-parsimony (MP) and Bayesian analysis of the combined dataset resulted in phylogenetic reconstructions with largely similar topologies, and the Bayesian tree is shown in Fig. 1.

Representatives of the sequenced genera (with molecular data) of *Tubeufiaceae* (Tsui & Berbee 2006, Tsui et al. 2006, 2007, Promputtha & Miller 2010, Boonmee et al. 2011, 2014, Rajeshkumar & Sharma 2013, Brahmanage et al. 2017, Doilom et al. 2017, Lu et al. 2017a, b, 2018, Luo et al. 2017, Phookamsak et al. 2018) are included in our phylogenetic analysis (Fig. 1). Twenty genera are represented by at least one species in *Tubeufiaceae* included the asexual and sexual morphs. The five newly generated isolates formed a well-supported monotypic clade and can be identified as a new genus (namely as *Helicotubeufia*) in *Tubeufiaceae*, and three species were recognized in *Helicotubeufia*. *Helicotubeufia* is phylogenetically close to *Aquaphila* and

Table 1 Isolates used in this study. Newly deposited sequences are shown in bold

Taxon	Culture Accession No.[1]	GenBank Accession No.[2]			References
		ITS	LSU	*TEF1α*	
Acanthohelicospora aurea	GZCC 16-0059 [T]	KY321322	KY321325	KY792599	Lu et al. (2017a)
Acanthohelicospora pinicola	MFLUCC 10–0116 [T]	KF301526	KF301534	KF301555	Boonmee et al. (2014)
Acanthostigma chiangmaiensis	MFLUCC 10–0125 [T]	JN865209	JN865197	KF301560	Boonmee et al. (2014)
Acanthostigma perpusillum	UAMH 7237	AY916492	AY856892	[a]	Tsui and Berbee (2006)
Aquaphila albicans	BCC 3543	DQ341096	DQ341101		Tsui et al. (2007)
Aquaphila asiana	BCC 3463	DQ341097	DQ341100		Tsui et al. (2007)
Boerlagiomyces macrospora	MFLUCC 12–0388	KU144927	KU764712	KU872750	Doilom et al. (2017)
Botryosphaeria dothidea	CBS 115476 [T]	KF766151	DQ678051	DQ767637	Schoch et al. (2006)
Chlamydotubeufia aquatica	MFLUCC 16–1131	KY873625	KY873620	KY873284	Brahamanage et al. (2017)
C. chlamydosporum	CBS 160.69	AY916466	AY856875		Tsui et al. (2006)
C. helicospora	MFLUCC 16–0213	KX454169	KX454170	KY117035	Hyde et al. (2016)
C. huaikangplaensis	MFLUCC 10–0926 [T]	JN865210	JN865198		Boonmee et al. (2011)
C. khunkornensis	MFLUCC 10–0117	JN865201	JN865189		Boonmee et al. (2011)
C. khunkornensis	MFLUCC 10–0118	JN865202	JN865190	KF301564	Boonmee et al. (2011)
C. krabiensis	MFLUCC 16–1134	KY678767	KY678759	KY792598	Hyde et al. (2017)
Dictyospora thailandica	MFLUCC 16-0001 [T]	KY873627	KY873622	KY873286	Brahamanage et al. (2017)
Dictyospora thailandica	MFLUCC 16-0215	KY873628	KY873623	KY873287	Brahamanage et al. (2017)
Helicangiospora lignicola	MFLUCC 11–0378 [T]	KF301523	KF301531	KF301552	Boonmee et al. (2014)
Helicoma guttulatum	MFLUCC 16–0022 [T]	KX454171	KX454172	MF535254	Hyde et al. (2016)
Helicoma khunkornense	MFLUCC 10–0119 [T]	JN865203	JN865191	KF301559	Boonmee et al. (2011)
Helicoma muelleri	CBS 964.69	AY916453	AY856877		Tsui et al. (2006)
Helicomyces roseus	CBS 283.51	AY916464	AY856881		Tsui and Berbee (2006)
Helicomyces talbotii	MUCL 33010	AY916465	AY856874		Tsui and Berbee (2006)
Helicosporium flavum	MFLUCC 16–1230	KY873626	KY873621	KY873285	Brahmanage et al. (2017)
Helicosporium luteosporum	MFLUCC 16–0226 [T]	KY321324	KY321327	KY792601	Lu et al. (2017a)
Helicosporium vegetum	CBS 941.72	AY916488	AY856883		Tsui et al. (2006)
Helicotubeufia guangxiensis	MFLUCC 17–0040 [T]	**MH290018**	**MH290023**	**MH290028**	this study
Helicotubeufia guangxiensis	MFLUCC 17–0041	**MH290019**	**MH290024**	**MH290029**	this study
Helicotubeufia hydei	MFLUCC 17–1980 [T]	**MH290021**	**MH290026**	**MH290031**	this study
Helicotubeufia hydei	MFLUCC 17–1986	**MH290022**	**MH290027**	**MH290032**	this study
Helicotubeufia jonesii	MFLUCC 17–0043 [T]	**MH290020**	**MH290025**	**MH290030**	this study
Kamalomyces bambusicola	MFLU 11–0228		MF506880		Phookamsak et al. (2018)
Kamalomyces thailandicus	MFLUCC 11–0158	MF506883	MF506881	MF506885	Phookamsak et al. (2018)
Kamalomyces thailandicus	MFLUCC 13–0233 [T]	MF506884	MF506882	MF506886	Phookamsak et al. (2018)
Manoharachariella tectonae	MFLUCC 12–0170 [T]	KF301529	KF301537	KU872762	Doilom et al. (2017)
Muripulchra aquatica	KUMCC 15–0276	KY320534	KY320551	KY320564	Luo et al. (2017)
Muripulchra aquatica	MFLUCC 15–0249 [T]	KY320532	KY320549		Luo et al. (2017)
Neoacanthostigma aquaticum	MFLUCC 17–0049 [T]	KY790444	KY790432	KY792608	Lu et al. (2017b)
Neoacanthostigma fusiforme	MFLUCC 11–0510	KF301529	KF301537		Boonmee et al. (2014)
Neohelicomyces aquaticus	MFLUCC 16–0993 [T]	KY320528	KY320545	KY320561	Luo et al. (2017)
Neohelicomyces grandisporus	KUMCC 15–0470 [T]	KX454165	KX454175		Luo et al. (2017)
Neohelicomyces submersus	MFLUCC 16–1106 [T]	KY320530	KY320547		Luo et al. (2017)
Neohelicosporium aquaticum	MFLUCC 17–1519 [T]	MF467916	MF467929	MF535242	Lu et al. (2018)
Neohelicosporium parvisporum	MFLUCC 17–1523 [T]	MF467926	MF467939	MF535252	Lu et al. (2018)
Neotubeufia krabiensis	MFLUCC 16–1125 [T]	MG012031	MG012024	MG012010	Chaiwan et al. (2017)
Tamhinispora indica	NFCCI 2924 [T]	KC469282	KC469283		Rajeshkumar & Sharma (2013)
Thaxteriellopsis lignicola	MFLUCC 10–0124	JN865208	JN865196	KF301561	Boonmee et al. (2011)
Tubeufia filiformis	MFLUCC 16–1135 [T]	KY092416	KY092411	KY117032	Lu et al. (2017b)
Tubeufia javanica	MFLUCC 12–0545 [T]	KJ880034	KJ880036	KJ880037	Boonmee et al. (2014)

Notes: Additional sequences (*RPB2*) for the new taxa in this study are provided as follows (same order as listed in table 1): MH290033, MH290034, MH290036, MH290037 and MH290035

[a] No data in GenBank.

[b] Abbreviations of isolates and culture collections: BCC, BIOTEC Culture Collection, Thailand; CBS, Centraalbureau voor Schimmel cultures, Utrecht, The Netherlands; GZCC, Guizhou Culture Collection, Guizhou Academy of Agricultural Sciences, Guiyang, China; KUMCC, Culture Collection of Kunming Institute of Botany, Kunming, China; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; *NBRC*, the NITE Biological Resource Center; NFCCI, the National Fungal Culture Collection of India. UAMH, UAMH Centre for Global Microfungal Biodiversity, University of Toronto, Canada. T ex-type/ex-epitype isolates.

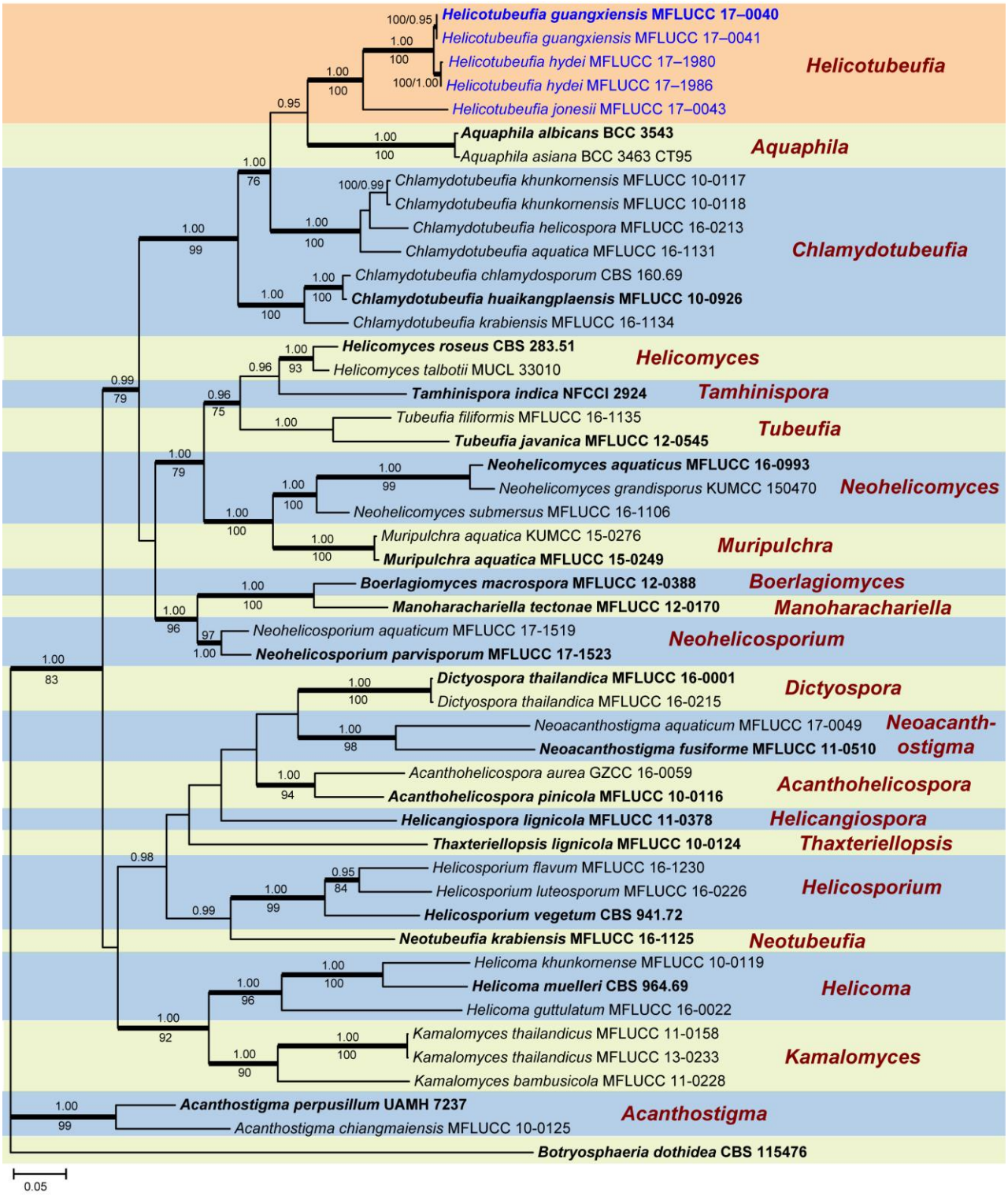

Figure 1 – Consensus phylogram (50 %) majority rule resulting from a Bayesian analysis of a combined LSU, ITS and *TEF1α* sequence alignment of *Tubeufiaceae*. Bayesian posterior probabilities (PP) above 0.95 and ML bootstrap proportion (BP) greater than 75 % are presented at the nodes as BP/PP. Branches with more than 75% bootstrap (ML/ MP) and 0.95 (PP) are in **thickened**. The original isolate numbers are noted after the species names, the type species of each genus are in **bold**, and the new taxa in blue. The scale bar shows 0.05 changes and the tree is rooted to *Botryosphaeria dothidea* (CBS 115476)

Chlamydotubeufia, and show closer relationship to *Aquaphila*. Many of the genera in *Tubeufiaceae* showed the stable phylogenetic positions and relationships, *Boerlagiomyces* and

Manoharachariella showed to be a sister group; the genera *Helicomyces*, *Muripulchra*, *Neohelicomyces* and *Tubeufia* formed a monotypic clade, *Helicoma* and *Kamalomyces* formed a sister group, and these clades could be recognized as good genera. However, some of the genera, such as *Acanthohelicospora*, *Acanthostigma*, *Neoacanthostigma*, *Neotubeufia* and *Tamhinispora*, their phylogenetic position are unstable. In addition, seven taxa of *Chlamydotubeufia* formed two distinct monotypic clades, which *C. chlamydosporum*, *C. huaikangplaensis* (the type) and *C. krabiensis* clustered together, and *C. aquatica*, *C. helicospora* and *C. khunkornensis* formed a separated clade.

Taxonomy

Helicotubeufia Y.Z. Lu & J.K. Liu., gen. nov.

Index Fungorum number: IF554759; Facesoffungi number: FoF04385

Etymology – "helico" referring to the spiral or helical shape of the conidia and "tubeufia" referring the type genus (Tubeufia) of the Tubeufiaceae.

Saprobic on submerged decaying wood in a freshwater stream. Sexual morph: *Ascomata* superficial, seated on a subiculum, solitary, scattered, subglobose to ellipsoidal-ovate, dark brown to black, with a central ostiolate. *Peridium* composed of cells of *textura angularis*, with inner cells pale brown and outer cells brown. *Hamathecium* comprising numerous, filiform, septate, branched, hyaline pseudoparaphyses. *Asci* 8-spored, bitunicate, cylindrical, short or long-pedicellate, apically rounded. *Ascospores* fusiform, tapering towards rounded ends, slightly curved, guttulate, 5–8-septate, not constricted at septa, hyaline, smooth-walled. Asexual morph: hyphomycetous, helicosporous. *Conidiophores* hyaline, macronematous, partially erect, partially immersed, cylindrical, septate, smooth-walled. *Conidiogenous cells* holoblastic, polyblastic, sympodial, integrated, terminal or intercalary, cylindrical, with a truncate apex, hyaline, smooth-walled. *Conidia* acropleurogenous, helicoid, coiled 2½–3½ times when tightly coiled, becoming loosely coiled in the water, basal cell rounded at tip, multi-septate, not constricted at septa, hyaline, smooth-walled.

Type species – *Helicotubeufia guangxiensis* Y.Z. Lu & J.K. Liu

Notes – *Helicotubeufia* appears to be a typical tubeufiaceous taxon and shares similar morphological characters with species of *Tubeufiaceae*. *Helicotubeufia* consists of three species, namely *H. guangxiensis*, *H. hydei* and *H. jonesii*, and formed a well-separated clade from all other genera of *Tubeufiaceae*. Morphologically, *Helicotubeufia* is characterized by its superficial, solitary, scattered, subglobose to ellipsoidal-ovate, dark brown to black ascomata; cylindrical asci with hyaline, fusiform, multi-septate, slightly curved and guttulate ascospore and hyphomycetous helicosporous asexual morphs. The new genus is introduced based on multi-gene phylogenetic analysis (Fig. 1).

Helicotubeufia guangxiensis Y.Z. Lu & J.K. Liu., sp. nov. Figs 2–3

Index Fungorum number: IF554760; Facesoffungi number: FoF04386

Etymology – Named referring to the location where the fungus was collected, Guangxi, China.

Holotype – HKAS 97423

Saprobic on submerged decaying wood in a freshwater stream. Sexual morph: *Ascomata* 220–275 μm high × 250–290 μm diam., superficial, seated on a subiculum, solitary, scattered, subglobose, dark brown to black, with a central ostiolate. *Peridium* 50–60 μm wide, composed of cells of *textura angularis*, with inner cells pale brown and outer cells brown. *Hamathecium* comprising numerous, filiform, septate, branched, hyaline pseudoparaphyses. *Asci* 120–165 × 13–15.5 μm ($\bar{x}$ = 141 × 14 μm, n = 20), 8-spored, bitunicate, cylindrical, pedicellate, apically rounded. *Ascospores* 55–70 × 5–6 μm ($\bar{x}$ = 60 × 5.5 μm, n = 50), fusiform, tapering towards rounded ends, slightly curved, guttulate, 7–8-septate, not constricted at septa, hyaline, smooth-walled.

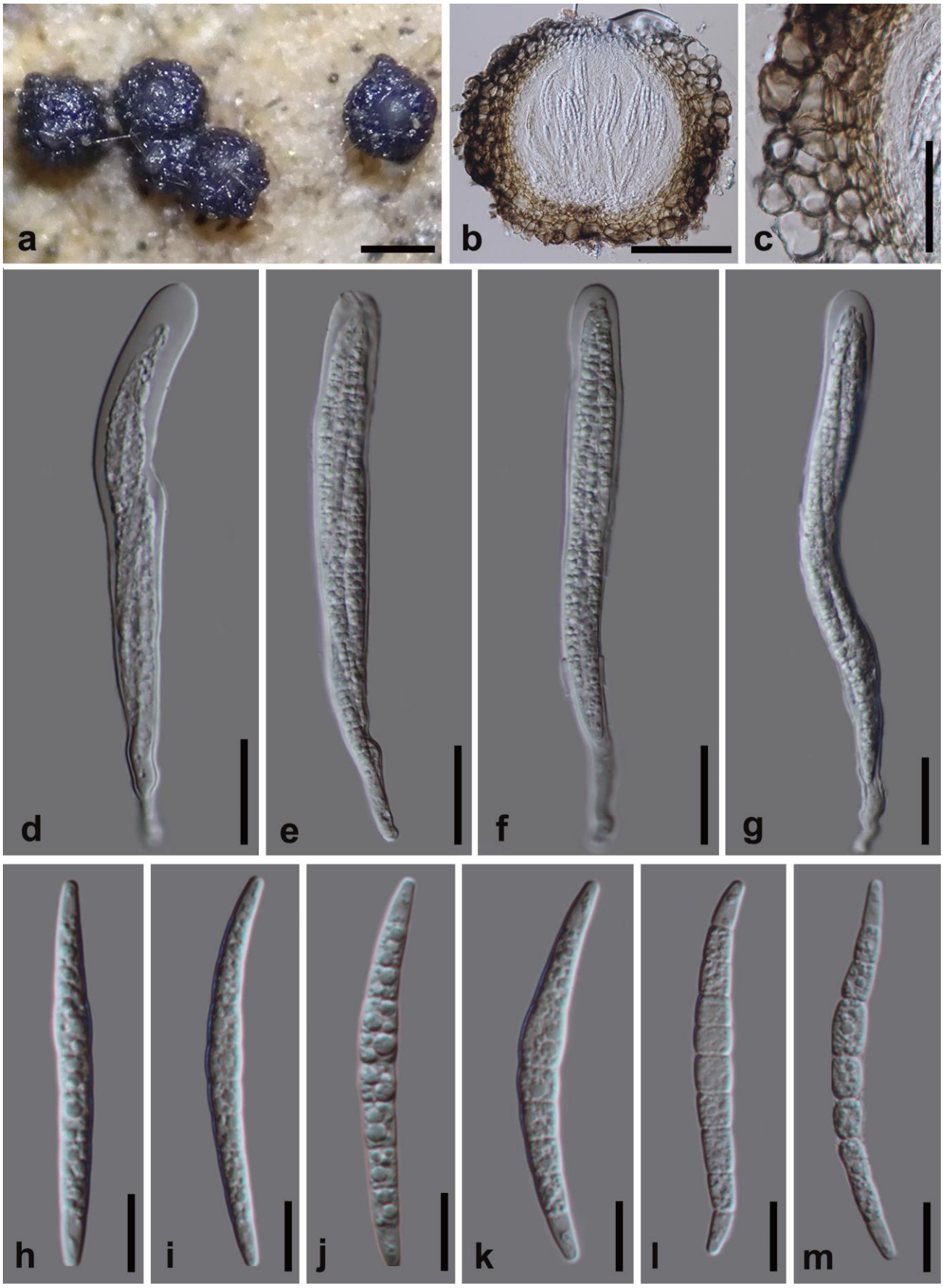

Figure 2 – *Helicotubeufia guangxiensis* (HKAS 97423, holotype). a Superficial ascomata on substrate. b Ascoma. c Peridium. d–g Asci. h–m Ascospores. Scale bars: a = 200 *μm*, b = 100 *μm*, c = 50 *μm*, d–g = 20 *μm*, h–m = 10 *μm*

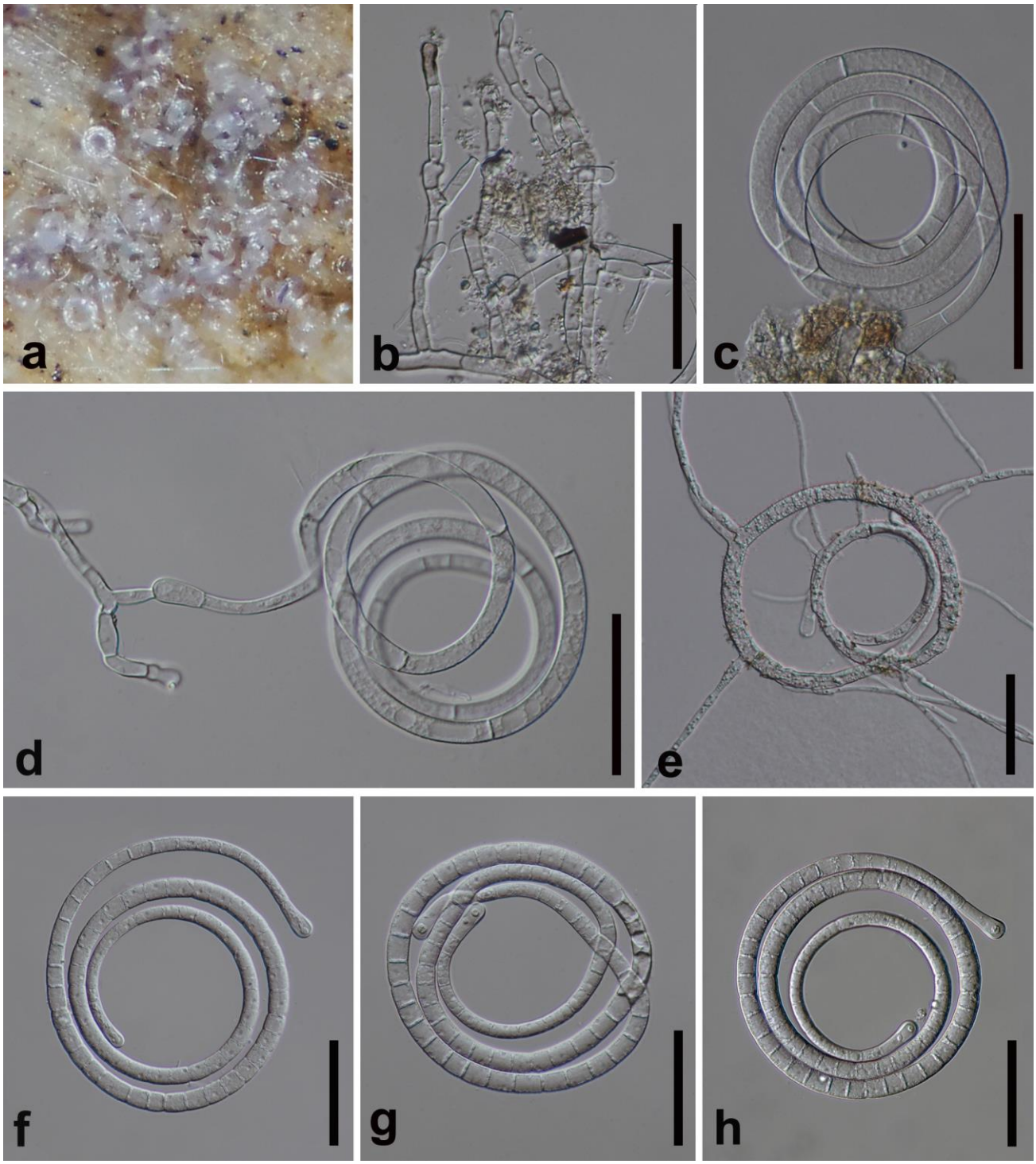

Figure 3 – *Helicotubeufia guangxiensis* (HKAS 97424, paratype). a Colony on decaying wood. b Conidiophores. c–d Conidiophores with attached conidia. e Germinating conidium f–h Conidia. Scale bars: b–h = 50 *μm*.

Asexual morph: hyphomycetous, helicosporous. *Conidiophores* macronematous, partially erect, partially immersed, cylindrical, septate, 70–140 μm long, 4–6 μm wide, hyaline, smooth-walled. *Conidiogenous cells* holoblastic, monoblastic, sympodial, integrated, terminal or intercalary, cylindrical, with a truncate apex, 10–18 μm long, 4–6 μm wide, hyaline, smooth-walled. *Conidia* acropleurogenous, helicoid, basal cell rounded at tip, 85–120 μm diam. and conidial filament 7–10 μm wide ($\bar{x}$ = 105 × 9 μm, n = 20), 590–845 μm long, coiled 2½–3½ times when tightly coiled, becoming loosely coiled in the water, multi-septate, up to 83-septate, not constricted at septa, hyaline, smooth-walled.

Culture characteristics – Conidia germinating on water agar (WA) within 24 h and germ tubes produced from conidia. Colonies growing on PDA, circular, with center umbonate surface, edge entire, reaching 11 mm in 3 weeks at 28 °C, pale brown to brown in PDA medium. mycelium superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Material examined – CHINA, Guangxi Province, Fang Cheng Gang City, on submerged decaying wood in a freshwater stream, 15 May 2016, Yong-Zhong Lu, JHC04–1 (HKAS 97423, holotype; GZAAS 16–0045, isotype), ex-type living culture, MFLUCC 17–0040 = GZCC 16–0033; *Ibid*., JHC04–2 (HKAS 97424 = GZAAS 16–0106, paratype), living culture, MFLUCC 17–0041 = GZCC 16–0094.

Notes – *Helicotubeufia guangxiensis* is designated as the type species of *Helicotubeufia* with both asexual and sexual morphs found from the substrates in nature. Morphologically, the sexual morph of *H. guangxiensis* resembles to *Neotubeufia krabiensis* in having subglobose ascomata with a central ostiolate, thick peridium, cylindrical bitunicate asci and fusiform ascospores, but can be distinguished by its smaller ascomata (220–275 × 250–290 μm vs. 340–390 × 365–400 μm) (Chaiwan et al. 2017). The asexual morph of *H. guangxiensis* differs from other helicosporous genera by its mycelium and conidiophores with a large sized conidia are hyaline, while most of other helicosporous hyphomycetes are mycelium and conidiophores pale brown to brown, or hyaline conidiophores with a small sized conidia (Linder 1929, Moore 1957, Goos 1985, 1986, 1989, Zhao et al. 2007, Boonmee et al. 2014, Lu et al. 2017c, Luo et al. 2017). In addition, all of previously described helicosporous species which conidial filament is wider than 7 μm and have pale brown to brown conidia, but the conidia of our new isolates are hyaline (Goos 1989, Lu et al. 2017c).

Helicotubeufia hydei Y.Z. Lu & J.K. Liu., sp. nov. Fig. 4

Index Fungorum number: IF554761; Facesoffungi number: FoF04387

Etymology: Named in honour of Kevin D. Hyde for his contributions to Asian mycology.

Holotype: MFLU 17–1122

Saprobic on decaying wood in a dry freshwater stream. Sexual morph: *Ascomata* 285–350 μm high × 270–375 μm diam., superficial, seated on a subiculum, solitary, scattered, subglobose, dark brown to black, with a central ostiolate. *Peridium* 80–105 μm wide, composed of cells of *textura angularis*, with inner cells pale brown and outer cells brown. *Hamathecium* 1–2 μm wide, comprising numerous, filiform, septate, branched, hyaline pseudoparaphyses. *Asci* 90–110 × 13–16 μm ($\bar{x}$ = 100 × 14.5 μm, n = 10), 8-spored, bitunicate, cylindrical, short-pedicellate, apically rounded. *Ascospores* 45–65 × 4.5–6.5 μm ($\bar{x}$ = 55 × 5.5 μm, n = 50), fusiform, tapering towards rounded ends, slightly curved, guttulate, 5–8-septate, not constricted at septa, hyaline, smooth-walled. Asexual morph: undetermined.

Culture characteristics – *Ascospores* germinating on water agar (WA) within 36 h and germ tubes produced from ascospores. Colonies growing on PDA, circular, with flat surface, edge entire, reaching 10 mm in 2 weeks at 28 °C, pale brown to brown in PDA medium. *Mycelium* superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Material examined – THAILAND, Trat, Amphoe Ko Chang, Yuttha Navi Ko Chang Memorial, on decaying wood in a dry stream, 27 April 2017, Yong-Zhong Lu, TD05 (MFLU 17–1122, holotype; HKAS100792, isotype); ex-type living culture, MFLUCC 17–1980; *Ibid*., TD12 (HKAS100798, paratype); living culture, MFLUCC 17–1986.

Notes – Morphologically, *Helicotubeufia hydei* resembles to *H. guangxiensis*, but can differ by its larger ascomata (285–350 × 270–375 μm vs. 220–275 × 250–290 μm) and thicker peridium (80–105 μm vs. 50–60 μm), as well as the shorter asci (90–110 μm vs. 120–165 μm). Phylogenetically, they are distinct species (Fig. 1).

Helicotubeufia jonesii Y.Z. Lu & J.K. Liu., sp. nov. Fig. 5

Index Fungorum number: IF554762; Facesoffungi number: FoF04388

Etymology – Named in honour of Prof. E.B. Gareth Jones for his contributions to Asian mycology.

Holotype – HKAS 97426

Saprobic on submerged decaying wood in a freshwater stream. Sexual morph: *Ascomata* 170–215 μm high × 175–215 μm diam., superficial, seated on a subiculum, solitary, scattered, subglobose, ellipsoidal-ovate, dark brown to black, with a central ostiolate. *Peridium* 35–45 μm wide, composed of cells of *textura angularis*, with inner cells brown and outer cells dark brown. *Hamathecium* comprising numerous, filiform, septate, branched, hyaline pseudoparaphyses. *Asci* 95–120 × 15–17.5 μm ($\bar{x}$ = 105 × 16 μm, n = 20), 8-spored, bitunicate, cylindrical, short-pedicellate, apically rounded. *Ascospores* 35–43 (–47) × 4.5–7 μm ($\bar{x}$ = 40 × 5.5 μm, n = 50), fusiform, tapering towards rounded ends, enlarged at the 3rd cell, slightly curved, guttulate, 5–6-septate, not constricted at septa, hyaline, smooth-walled. Asexual morph: undetermined.

Culture characteristics – Ascospores germinating on water agar (WA) within 12 h and germ tubes produced from ascospores. Colonies growing on PDA, circular, with flat surface, edge entire, reaching 14 mm in 3 weeks at 28 °C, brown to dark brown in PDA medium. *Mycelium* superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Material examined – CHINA, Guangxi Province, Fang Cheng Gang City, on submerged decaying wood in a freshwater stream, 15 May 2016, Yong-Zhong Lu, JHC08–2 (HKAS 97426, holotype; GZAAS 16–0052, isotype), ex-type living culture, MFLUCC 17–0043, GZCC 16–0040.

Notes – *Helicotubeufia jonesii* morphologically resembles to *H. guangxiensis* and *H. hydei* in ascomata and asci, but can be easily distinguished by its ascospores which are obviously enlarged at the 3rd cell, while the other two species lack this characteristic. In addition, the ascomata of *H. jonesii* are smaller and its peridium is thinner than those of *H. guangxiensis* and *H. hydei*. The phylogenetic analysis of combined LSU, ITS and *TEF1α* sequences data showed that they are phylogenetically distinct species, and *H. guangxiensis* and *H. hydei* have a close phylogenetic relationship.

Discussion

The asexual ascomycetes comprise a large number of genera that have never been linked to a sexual morph (Shenoy et al. 2007). Helicosporous hyphomycete genera are commonly found in *Tubeufiaceae*, and several genera have been recognized. Many share a similar morphology and could be morphologically assigned as a group, but they are phylogenetically distinct (Boonmee et al. 2011, 2014, Lu et al. 2017a, c, 2018, Luo et al. 2017). This is not only at the species level, but even genus (Lu et al. 2017a, 2018, Luo et al. 2017). Lu et al. (2018) suggested that many helicosporous species may be wrongly named, even at the genus level; and this can be confirmed by future studies which bring more molecular data into the study. With more and more molecular data of the Tubeufiales become available, the understanding of the taxonomy and phylogeny of the order have been well investigated (Kodsueb et al. 2006, Boonmee et al. 2011, 2014, Liu et al. 2015, Brahmanage et al. 2017, Doilom et al. 2017, Lu et al. 2017a, b, Luo et al. 2017). Boonmee et al. (2014) provided a modern classification of the order Tubeufiales based on the examinations of the types and phylogenetic analysis of multi-gene molecular data, including the asexual and sexual morphs. Subsequently, several new taxa were introduced mainly based on the phylogenetic evidences (Hyde et al. 2016, Brahmanage et al. 2017, Chaiwan et al. 2017, Lu et al. 2017b, c, 2018, Luo et al. 2017) which the morphological characters are similar.

One interesting finding in this study is that the phylogenetic position of *Chlamydotubeufia* is questionable. In previous studies (Brahmanage et al 2017, Chaiwan et al 2017, Lu et al 2017a, b, 2018, Luo et al 2017, Phookamsak et al. 2018), *Chlamydotubeufia* and *Aquaphila* have been shown to be a stable sister group, and close to *Boerlagiomyces* and *Manoharachariella*. However, in this study, where five taxa of *Helicotubeufia* were added to the phylogenetic analysis of *Tubeufiaceae*, the seven isolates representing six species of *Chlamydotubeufia* formed two clades, where *C. chlamydosporum*, *C. huaikangplaensis* (the type) and *C. krabiensis* clustered together, and *C. aquatica*, *C. helicospora* and *C. khunkornensis* formed a separated clade.

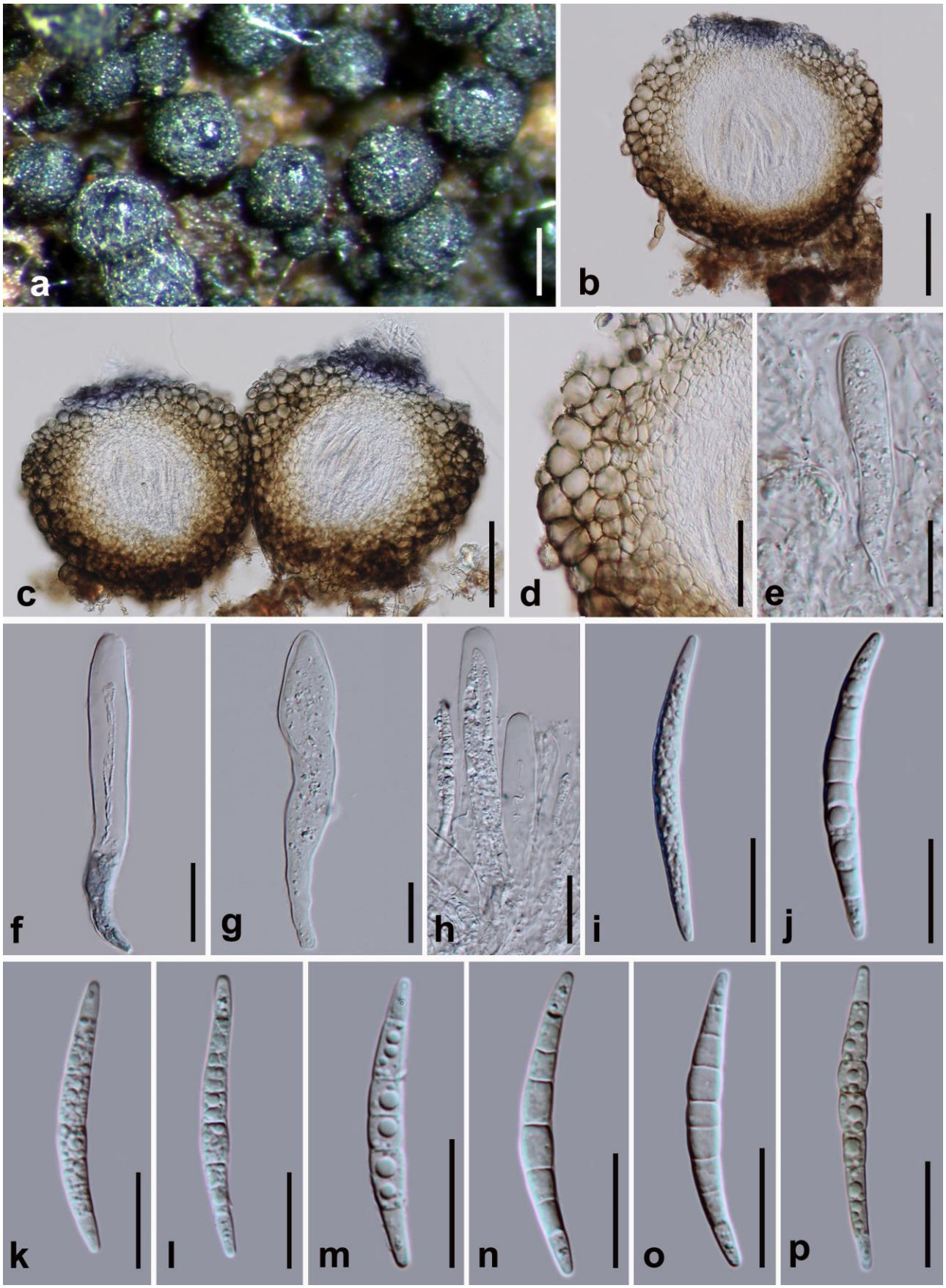

Figure 4 – *Helicotubeufia hydei* (MFLU 17–1122, holotype). a Superficial ascomata on substrate. b–c Ascoma. d Peridium. e–h Asci. i–p Ascospores. Scale bars: a = 200 *µm*, b–c = 100 *µm*, d = 50 *µm*, e–p = 20 *µm*.

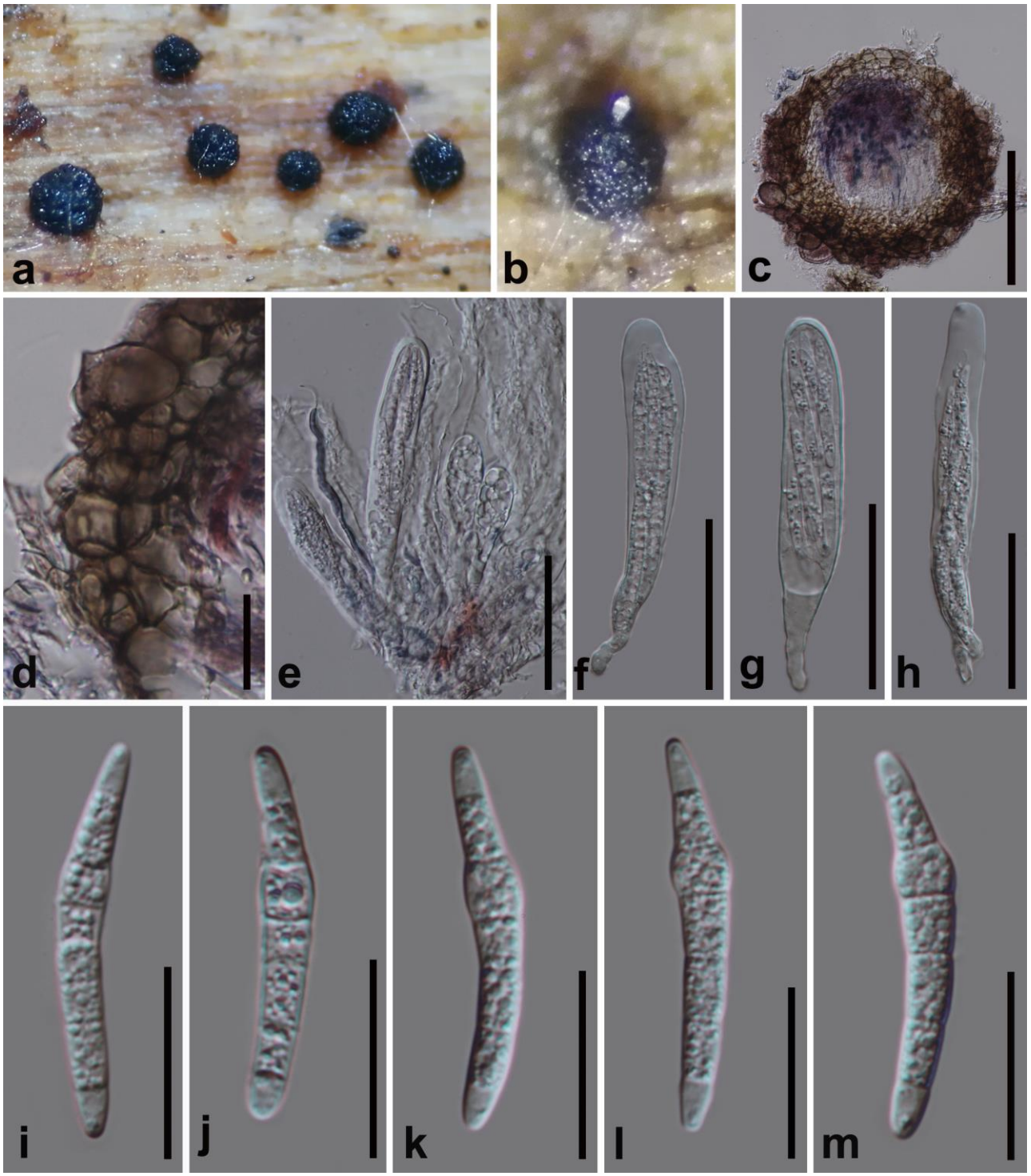

Figure 5 – *Helicotubeufia jonesii* (HKAS 97426, holotype). a–b Superficial ascomata on substrate. c Ascoma. d Peridium. e–h Asci. i–q Ascospores. r–s Colonies on PDA from above and below. Scale bars: c = 100 *μm*, d = 20 *μm*, e–h = 50 *μm*, i–m = 20 *μm*.

Based on both morphology and phylogeny, we can treat *C. aquatica*, *C. helicospora* and *C. khunkornensis* as *Chlamydotubeufia sensu lato* and *C. chlamydosporum*, *C. huaikangplaensis* and *C. krabiensis* as *Chlamydotubeufia sensu stricto*. This also indicates that *C. aquatica*, *C. helicospora* and *C. khunkornensis* could be recognized as a new genus and the monotypic clade including *C. chlamydosporum*, *C. krabiensis* and the type species *C. huaikangplaensis* could represent the genus *Chlamydotubeufia*. However, we are not willing to follow this conclusion until more taxa and sequences data are available, as well as more sampling and taxa population included

in the analysis. This funding also confirms that the tubeufiaceous taxa support a high diversity and the phylogenetic studies give a better understanding towards the classification of both asexual and sexual morphs of Tubeufiales.

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