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Novel *Neoacanthostigma* species from aquatic habitats

*Yong-Zhong LU[a, b, c], Saranyaphat BOONMEE[b], Jian-Kui LIU[d,e], Kevin D. HYDE[b], D. Jayarama BHAT[f, g], Prapassorn Damrongkool EUNGWANICHAYAPANT[h] & Ji-Chuan KANG[a]**

[a]*The Engineering and Research Center for Southwest Bio-Pharmaceutical Resources of National Education Ministry of China, Guizhou University, Guiyang, Guizhou Province 550025, P.R. China*

[b]*Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*

[c]*School of Pharmaceutical Engineering, Guizhou Institute of Technology, Guiyang, Guizhou Province 550003, P.R. China*

[d]*Guizhou Institute of Biotechnology, Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou Province 550006, P.R. China*

[e]*Guizhou Key Laboratory of Agricultural Biotechnology, Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou Province 550006, P.R. China*

[f]*Formerly, Department of Botany, Goa University, Goa, India*

[g]*No. 128/1-J, Azad Housing Society, Curca, Goa Velha-403108, India*

[h]*School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*

Abstract – Helicosporous hyphomycetes are a morphologically fascinating group of Tubeufiales. Ten dematiaceous helicosporous asexual morphs and two sexual morphs collected from aquatic habitats are characterized in this study using morphological features and phylogenetic analyses. Four new species of *Neoacanthostigma*, viz. *N. aquaticum*, *N. brunneisporum*, *N. guangxiense* and *N. latisporum*, are described and illustrated. The phylogenetic analyses of combined ITS, LSU and TEF1α sequence data place all taxa in the genus *Neoacanthostigma* (Tubeufiaceae, Tubeufiales) and provide evidence to support the establishment of the new taxa. The differentiating morphological characters of *Neoacanthostigma* with other helicosporous species are compared and discussed. A key to all *Neoacanthostigma* species is also provided.

Freshwater fungi / Phylogeny / Taxonomy / Woody substrates

* Corresponding authors: jckang@gzu.edu.cn

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INTRODUCTION

Helicosporous asexual morphs are a fascinating group common on submerged woody debris submerged in water and in what in damp areas (Cai *et al.* 2003, bamboo, Ho *et al.* 2002, seasonality). Most species are members of Tubeufiales (Linder 1929; Moore 1957; Goos 1985, 1986, 1989; Zhao *et al.* 2007; Boonmee *et al.* 2011, 2014; Hyde *et al.* 2016; Doilom 2017; Lu *et al.* 2017a, b; Luo *et al.* 2017). *Helicomyces* Link (1809), *Helicosporium* Nees (1817) and *Helicoma* Corda (1837) are the three earliest described helicosporous hyphomycete genera. These genera were delineated by Goos (1985, 1986, 1989) based on different aspects of conidia and conidiophores. This scheme is followed to diagnose our new helicosporous collections in this study. The helicosporous asexual morphs are linked to sexual genera of Tubeufiales, for example, the asexual morph of *Helicosporium vegetum* (BPI 447464) has been linked to its sexual morph (BPI 1107327) by Boonmee *et al.* (2014) according to phylogenetic analyses. Lu *et al.* (2017a) introduced the sexual morph of *Tubeufia filiformis* linked to its helicosporous asexual morph based on phylogenetic evidence.

The genus *Neoacanthostigma* Boonmee, Bhat & K.D. Hyde was introduced by Boonmee *et al.* (2014) to accommodate three species, viz. *N. filiforme* (Promp. & A.N. Mill.) S. Boonmee & K.D. Hyde, *N. fusiforme* Boonmee, Bhat & K.D. Hyde and *N. septoconstrictum* (Promp. & A.N. Mill.) S. Boonmee & K.D. Hyde. These three species, collected from terrestrial habitats (Promputtha & Miller 2010; Boonmee *et al.* 2014), were morphologically similar to *Acanthostigma* species in that ascomata are covered with setae, asci bitunicate, ascospores cylindrical to narrowly fusiform and the asexual morphs are helicosporous hyphomycetes.

We are studying fungi on submerged wood in streams, rivers and lakes along a north-south longitudinal gradient (Hyde *et al.* 2016) and have collected numerous helicosporous hyphomycetes. In this study, we report on ten dematiaceous helicosporous asexual morphs and two ascomatal sexual morphs collected from submerged decaying woody substrates in freshwater streams in southern China and Thailand. New taxa are characterized based on morphological features and phylogenetic analyses. We introduce four novel species of *Neoacanthostigma* with descriptions, illustrations, and phylogenetic analysis. A key to all *Neoacanthostigma* species is also provided.

MATERIALS AND METHODS

Sample collection and specimen examination

Decaying wood samples were randomly collected from sites in freshwater streams in south of China and Thailand. The procedures followed here have been described previously (Boonmee *et al.* 2014). Micromorphological structures were photographed using a Nikon ECLIPSE 80i compound microscope fitted with a Canon EOS 600D digital camera and measurements were made using Tarosoft (R) Image Frame Work program. Figures were processed with an Adobe Photoshop CS6 Extended version 10.0 software (Adobe Systems, USA).

Single spore isolations were obtained using the method described by Chomnunti *et al.* (2014). Germinating spores were aseptically transferred to fresh Potato-Dextrose Agar (PDA) or Malt Extract Agar (MEA) plates and incubated at 25-30°C. Cultures were grown for 1-2 months and morphological characters, such as colour, colony shape, and texture were recorded. Type materials are deposited at the Herbarium of Mae Fah Luang University (Herb. MFLU), Chiang Rai, Thailand, Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (Herb. HKAS), Kunming, China and the Guizhou Academy of Agriculture Sciences (Herb. GZAAS), Guiyang, China. Ex-type living cultures are deposited at Mae Fah Luang University Culture Collection (MFLUCC) and Guizhou Culture Collection (GZCC). Facesoffungi and MycoBank numbers are provided (Jayasiri *et al.* 2015).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fungal mycelium grown on MEA at 28°C for 60 days. Three genes were amplified with universal primers, namely the internal transcribed spacer region of ribosomal DNA (ITS: ITS5/ITS4) (White *et al.* 1990), large subunit nuclear ribosomal DNA (LSU: LROR/LR5) (Vilgalys & Hester 1990), and the translation elongation factor 1-alpha gene (TEF1α: EF1-983F/EF1-2218R) (Rehner & Buckley 2005). The PCR products were purified and sequenced with the same primers. The amplification reactions were carried out using the method described by Lu *et al.* (2017a). The quality of PCR products were checked on 1% agarose gel electrophoresis strained with ethidium bromide. The PCR products were sent for sequencing at Sangon Biotech, Shanghai, China.

Phylogenetic analysis

BLAST searches of new sequences were performed to verify the identities of species in GenBank database (Kodsueb *et al.* 2004, 2006; Tsui & Berbee 2006; Tsui *et al.* 2006; Promputtha & Miller 2010; Boonmee *et al.* 2011, 2014). The combined alignments of ITS, LSU and TEF1α sequence data from the closest relatives in Tubeufiaceae were used to generate phylogenetic trees (Boonmee *et al.* 2011, 2014; Hyde *et al.* 2016; Lu *et al.* 2017a, b). *Botryosphaeria dothidea* in the closely related family Botryosphaeriaceae was selected as the outgroup taxon. The sequence accession numbers in the analysis are provided in Table 1. The sequence data were aligned using MAFFT v.7.110 online program (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) and manually adjusted via BioEdit 7.2.3 (Hall 1999). Phylogenetic analyses were performed by using PAUP v.4.0b10 (Swofford 2002) for maximum parsimony (MP) and MrBayes v.3.2.2 (Ronquist *et al.* 2012) for Bayesian analyses.

Phylogeny website tools "ALTER" (Glez-Peña *et al.* 2010) were used to transfer the alignment fasta file for RAxML analysis. Maximum likelihood (ML) analysis was performed at the CIPRES Science Gateway v.3.3 (http://www.phylo.org/portal2/; Miller *et al.* 2010) using RAxML v.8.2.8 as part of the "RAxML-HPC BlackBox" tool (Stamatakis 2006; Stamatakis *et al.* 2008). All free model parameters were estimated by RAxML with ML estimates of 25 per site rate categories. The final ML search was conducted using the GTRGAMMA + I model. The best scoring tree was selected with a final likelihood value of -16526.170950. RAxML bootstrap support values greater than 75% are given above at each nodes (Fig. 1).

Table 1. Taxa used in this study and their GenBank accession numbers for ITS, LSU and TEF1α DNA sequence data

Taxa	*Culture accession No.*[b]	*GenBank Accession No.*			*References*
		ITS	*LSU*	*TEF1α*	
Acanthohelicospora aurea	GZCC 16-0059	KY321322	KY321325	KY792599	Lu *et al.* (2017b)
Acanthohelicospora pinicola	MFLUCC 10-0116	KF301526	KF301534	KF301555	Boonmee *et al.* (2014)
Acanthostigma chiangmaiensis	MFLUCC 10-0125	JN865209	JN865197	KF301560	Boonmee *et al.* (2014)
Acanthostigma perpusillum	UAMH 7237	AY916492	AY856892	–[a]	Tsui & Berbee (2006)
Aquaphila albicans	BCC 3543	DQ341096	DQ341101	–	Tsui *et al.* (2007)
Aquaphila albicans	MFLUCC 16-0010	KX454165	KX454166	KY117034	Hyde *et al.* (2016); Lu *et al.* (2017a)
Boerlagiomyces macrospora	MFLUCC 12-0388	KU144927	KU764712	KU872750	Doilom *et al.* (2017)
Botryosphaeria dothidea	CBS 115476	KF766151	DQ678051	DQ767637	Schoch *et al.* (2006); Slippers *et al.* (2013)
Chlamydotubeufia helicospora	MFLUCC 16-0213	KX454169	KX454170	KY117035	Hyde *et al.* (2016); Lu *et al.* (2017a)
Chlamydotubeufia khunkornensis	MFLUCC 10-0117	JN865201	JN865189	–	Boonmee *et al.* (2011)
Helicangiospora lignicola	MFLUCC 11-0378	KF301523	KF301531	KF301552	Boonmee *et al.* (2014)
Helicoma chiangraiense	MFLUCC10-0115	JN865200	JN865188	–	Boonmee *et al.* (2011)
Helicoma khunkornensis	MFLUCC 10-0119	JN865203	JN865191	KF301559	Boonmee *et al.* (2011)
Helicoma muelleri	CBS 964.69	AY916453	AY856877	–	Tsui *et al.* (2006)
Helicomyces indicum	CBS 374.93	AY916477	AY856885	–	Tsui & Berbee (2006)
Helicomyces paludosa	CBS 120503	DQ341095	DQ341103	–	Tsui *et al.* (2007)
Helicomyces roseus	CBS 283.51	AY916464	AY856881	–	Tsui & Berbee (2006)
Helicomyces talbotii	MUCL 33010	AY916465	AY856874	–	Tsui & Barbee (2006)
Helicosporium vegetum	CBS 941.72	AY916488	AY856883	–	Tsui *et al.* (2006)
Helicosporium vegetum	NBRC 9014	AY916489	AY856903	–	Tsui & Berbee (2006)
Helicosporium luteosporum	MFLUCC 16-0226	KY321324	KY321327	KY792601	Lu *et al.* (2017b)
Manoharachariella tectonae	MFLUCC12-0170	KF301529	KF301537	KU872762	Boonmee *et al.* (2014)
Muripulchra aquatica	KUMCC 15-0276	KY320534	KY320551	KY320564	Luo *et al.* (2017)
Muripulchra aquatica	MFLUCC 15-0249	KY320532	KY320549	–	Luo *et al.* (2017)
Neoacanthostigma aquaticum	**GZCC 16-0034**	**KY790438**	**KY790426**	**KY792602**	This study
Neoacanthostigma aquaticum	**GZCC 16-0039**	**KY790439**	**KY790427**	**KY792603**	This study

Neoacanthostigma aquaticum	**GZCC 16-0044**	**KY790440**	**KY790428**	**KY792604**	This study
Neoacanthostigma aquaticum	**GZCC 16-0055**	**KY790441**	**KY790429**	**KY792605**	This study
Neoacanthostigma aquaticum	**GZCC 16-0056**	**KY790442**	**KY790430**	**KY792606**	This study
Neoacanthostigma aquaticum	**MFLUCC 17-0039**	**KY790443**	**KY790431**	**KY792607**	This study
Neoacanthostigma aquaticum	**MFLUCC 17-0049**	**KY790444**	**KY790432**	**KY792608**	This study
Neoacanthostigma filiforme	ANM 101	–	GQ850495	–	Promputtha & Miller (2010)
Neoacanthostigma filiforme	ANM 514	GQ856146	GQ850494	–	Promputtha & Miller (2010)
Neoacanthostigma fusiforme	MFLUCC 11-0510	KF301529	KF301537	–	Boonmee *et al.* (2014)
Neoacanthostigma brunneisporum	MFLUCC 15-1248	KX454176	–	**KY792614**	Hyde *et al.* 2016; This study
Neoacanthostigma brunneisporum	**MFLUCC 16-0012**	**KY790445**	**KY790433**	**KY792609**	This study
Neoacanthostigma brunneisporum	**MFLUCC 16-0015**	**KY790446**	**KY790434**	**KY792610**	This study
Neoacanthostigma brunneisporum	**MFLUCC 16-1127**	**KY790447**	**KY790435**	**KY792611**	This study
Neoacanthostigma guangxiense	**MFLUCC 17-0042**	**KY790448**	**KY790436**	**KY792612**	This study
Neoacanthostigma latisporum	**MFLUCC 16-0019**	**KY790449**	**KY790437**	**KY792613**	This study
Neoacanthostigma septoconstrictum	ANM 536.1	GQ856143	GQ850491	–	Promputtha & Miller (2010)
Neohelicomyces aquaticus	KUMCC 15-0463	KY320529	KY320546	KY320562	Luo *et al.* (2017)
Neohelicomyces aquaticus	MFLUCC 16-0993	KY320528	KY320545	KY320561	Luo *et al.* (2017)
Neohelicomyces submersus	MFLUCC 16-1106	KY320530	KY320547	–	Luo *et al.* (2017)
Tamhinispora indica	NFCCI 2924	KC469282	KC469283	–	Rajeshkumar & Sharma (2013)
Thaxteriellopsis lignicola	MFLUCC 10-0122	JN865206	JN865194	KF301563	Boonmee *et al.* (2011)
Thaxteriellopsis lignicola	MFLUCC 10-0124	JN865208	JN865196	KF301561	Boonmee *et al.* (2011)
Tubeufia filiformis	MFLUCC 16-1135	KY092416	KY092411	KY117032	Lu *et al.* (2017a)
Tubeufia javanica	MFLUCC 12-0545	KJ880034	KJ880036	KJ880037	Boonmee *et al.* (2014)
Tubeufia latispora	MFLUCC 16-0027	KY092417	KY092412	KY117033	Lu *et al.* (2017a)
Tubeufia laxispora	MFLUCC 16-0232	KY092413	KY092408	KY117029	Lu *et al.* (2017a)

Notes: new sequences are in bold.

[a] No data in GenBank.

[b] *ANM*, A.N. Miller; *BCC*, BIOTEC Culture Collection, Thailand; *CBS*, Centra albureau 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.

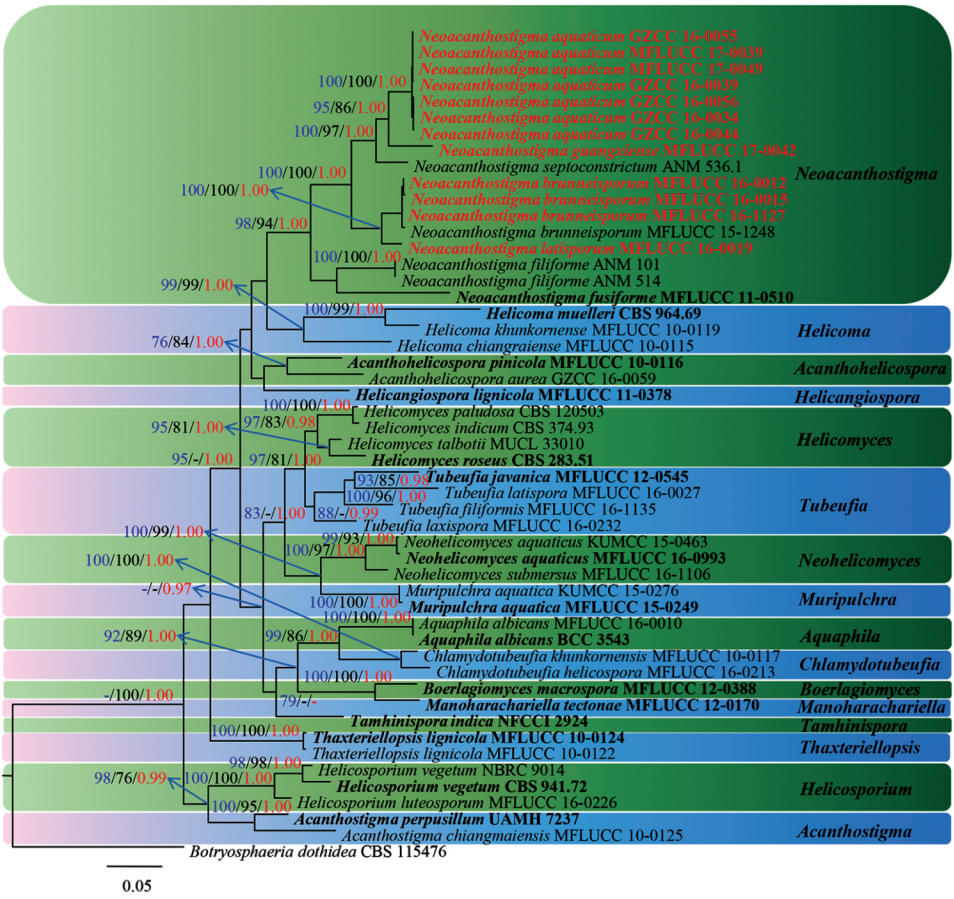


Fig. 1. Phylogenetic tree based on analysis of a combined ITS, LSU and TEF1α dataset. Bootstrap support values for RAxML and maximum parsimony (MP) greater than 75% and Bayesian posterior probabilities greater than 0.95 (PP) are indicated above or below the nodes as MLBS/MPBS/PP. The tree is rooted with *Botryosphaeria dothidea* CBS 115476 (Botryosphaeriaceae). The type species of each genus are in bold and new isolates are in bold and red.

MP 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 ten replicates of random stepwise addition of taxa (Hillis & Bull 1993). Bayesian analysis was carried out using MrBayes v.3.2.2 (Ronquist *et al.* 2012). The best-fit model of sequences evolution was estimated via MrModeltest 2.2 (Nylander 2004). Markov Chain Monte Carlo sampling (MCMC) in MrBayes v.3.2.2 (Ronquist *et al.* 2012) was used to determine the Posterior probabilities (PP) (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002). Phylogenetic trees were sampled every 100th

generation (resulting in 10,000 total trees) in 1,000,000 generations from the running of six simultaneous Markov chains. The first 2,000 trees, which contained the burn-in phase of the analyses were discarded. The remaining 8,000 trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree (Liu *et al.* 2011). Bayesian posterior probabilities with those equal or greater than 0.95 are given at each node (Fig. 1). Phylogenetic trees were visualized using FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/, Rambaut 2012). The sequences are deposited in GenBank (Table 1). The alignment was deposited in TreeBASE (http://www.treebase.org, submission number 20796).

RESULTS

Phylogenetic analysis of combined ITS, LSU, TEF1α sequence data

The combined sequence alignment comprised 51 taxa, with *Botryosphaeria dothidea* as the outgroup taxon. The dataset comprises 2324 characters (ITS, LSU and TEF1α sequence data) after alignment, of which 1568 characters were constant, 179 variable characters were parsimony-uninformative and 593 characters were parsimony informative. RAxML, MP and Bayesian analysis of the combined dataset resulted in phylogenetic reconstructions with largely similar topologies, and the RAxML tree is shown in Fig. 1. Bootstrap support values for RAxML and MP greater than 75% and Bayesian posterior probabilities greater than 0.95 are given at each node (Fig. 1).

Representatives of the sequenced genera (with molecular data) of Tubeufiaceae (Tsui *et al.* 2006, 2007; Tsui & Berbee 2006; Promputtha & Miller 2010; Boonmee *et al.* 2011, 2014; Rajeshkumar & Sharma 2013; Hyde *et al.* 2016; Doilom *et al.* 2017; Lu *et al.* 2017a, 2017b; Luo *et al.* 2017) are included in our phylogenetic analysis (Fig. 1). Sixteen genera are represented by at least one species in Tubeufiaceae. Our twelve isolates were included in the analysis of combined ITS, LSU and TEF1α sequence data. Seventeen isolates representing seven species of *Neoacanthostigma* formed a well-supported clade in Tubeufiaceae. And our twelve isolates were identified as *Neoacanthostigma aquaticum*, *N. brunneisporum*, *N. guangxiense* and *N. latisporum* spp. nov. based on morphology and phylogenetic analysis. The species of *N. aquaticum* was represented by seven isolates which were collected from southern China and it is phylogenetically close to the new species of *N. guangxiense.*

In this study, we found that the strain of *Neoacanthostigma brunneisporum* MFLUCC 15-1248 (Hyde *et al.* 2016, named *N. septoconstrictum*) is wrongly identified as it grouped together with *N. brunneisporum* strains (MFLUCC 16-0012, MFLUCC 16-0015, MFLUCC 16-1127) with 100% RAxML bootstrap support, 100% MP bootstrap support and 1.00 Bayesian posterior probabilities (Fig. 1), and formed a distinct clade from *N. septoconstrictum* (ANM 536.1). This result is also confirmed by comparing their sequence data, therefore we renamed the strain MFLUCC 15-1248 as *N. brunneisporum*. The incorrect identification was caused due to two reasons: 1) phylogenetic analyses lacked any protein gene, while the TEF1α gene is added in this study, so that more genetic variation has been included (Jeewon & Hyde 2016); 2) the phylogenetic result becomes more clear after obtaining more isolates, which is important when resolving cryptic species.

Taxonomy

Neoacanthostigma aquaticum Y.Z. Lu, Boonmee & K.D. Hyde, ***sp. nov.*** **Figs 2, 3**

Index Fungorum number: IF 553170; *Facesoffungi number*: FoF 03239
Holotype: HKAS 97431
Etymology: 'aquaticum' referring to aquatic habitats of this fungus.

Saprobic on decaying wood in a freshwater stream. ***Sexual morph***: *Ascomata* 235-290 µm high × 180-225 µm diam., superficial, seated on a subiculum, solitary, scattered, subglobose, ellipsoidal-ovate, dark brown to black, with a central ostiolate. Setae 35-65 µm long, 3.5-6 µm wide in the middle, one-celled, thick-walled, brown to black, straight to slightly curved, covered with the whole ascomata. *Peridium* 25-34 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* 115-175 × (11-) 12.5-16 (-17.5) µm ($\bar{x}$ = 140 × 14 µm, n = 20), 8-spored, bitunicate, cylindrical, short-pedicellate, apically rounded. *Ascospores* (45-) 50.5-60 × 4.5-6.5 µm ($\bar{x}$ = 54 × 5.5 µm, n = 50), overlapping 2-3-seriate, fusiform, tapering towards rounded ends, slightly curved, guttulate, 7-septate, not constricted at septa, hyaline, smooth-walled. ***Asexual morph***: hyphomycetous, helicosporous. *Conidiophores* (13-) 18-45.5 (-54) × 3.5-6 µm, pale brown, macronematous, erect, short, cylindrical, septate, smooth-walled. *Conidiogenous cells* 6-24 µm long, 3-5 µm wide, holoblastic, mono- to polyblastic, integrated, terminal, cylindrical, truncate at apex after conidial secession, hyaline to pale brown, smooth-walled. *Conidia* 100-150 µm diam. and conidial filament 8-12 (-13.5) µm wide ($\bar{x}$ = 125 × 9.5 µm, n = 50), 500-1070 µm long, coiled 2½-3 times when tightly coiled, becoming loosely coiled in the water, with elongated basal cell, rounded at the tip, multi-septate, up to 93-septate, slightly constricted at septa, pale brown, smooth-walled.

Culture characteristics: *Conidia* germinating on water agar (WA) within 12 h and germ tubes produced from ascospores. Colonies growing on PDA, irregular, umbonate, surface rough and wrinkle, edge undulate, reaching 10 mm in 2 weeks at 28°C, brown in PDA medium, produced brown pigment in 3 weeks. *Mycelium* superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Materials examined: CHINA, Guangxi Province, Fang Cheng Gang, on decaying wood in a freshwater stream, 15 May 2016, Yong-Zhong Lu, JHC23-3 (HKAS 97431, **holotype**; GZAAS 16-0069, **isotype**); ex-type living culture, MFLUCC 17-0049 = GZCC 16-0057; *Ibid*., 15 May 2016, Yong-Zhong Lu, JHC02-1 (HKAS 97421, **paratype**; GZAAS 16-0105), living culture, MFLUCC 17-0039 = GZCC 16-0093; *Ibid*., JHC05-1 (GZAAS 16-0046, **paratype**), living culture, GZCC 16-0034; *Ibid*., JHC08-1 (GZAAS 16-0051, **paratype**), living culture, GZCC 16-0039; *Ibid*., JHC12-3 (GZAAS 16-0056, **paratype**), living culture, GZCC 16-0044; *Ibid*., JHC23-1 (GZAAS 16-0067, **paratype**), living culture, GZCC 16-0055; *Ibid*., JHC23-2 (GZAAS 16-0068, **paratype**), living culture, GZCC 16-0056.

Notes: *Neoacanthostigma aquaticum* was collected from a freshwater stream in Guangxi Province, southern China. Morphologically, the sexual morph of *N. aquaticum* is similar to *N. septoconstrictum* (ANM 536.1) in that ascomata are covered with one-celled setae, asci bitunicate and cylindrical, ascospores fusiform (Promputtha & Miller 2010). However, *N. aquaticum* differs from *N. septoconstrictum* in having narrower asci (115-175 × 12.5-16 µm vs 110-140 × 17-22 µm). The asexual morph of *N. aquaticum* resembles *N. guangxiense* in having macronematous, erect, short conidiophores, terminal conidiogenous cells and helicoid conidia. However, *N. aquaticum* differs from *N. guangxiense* in having different number of

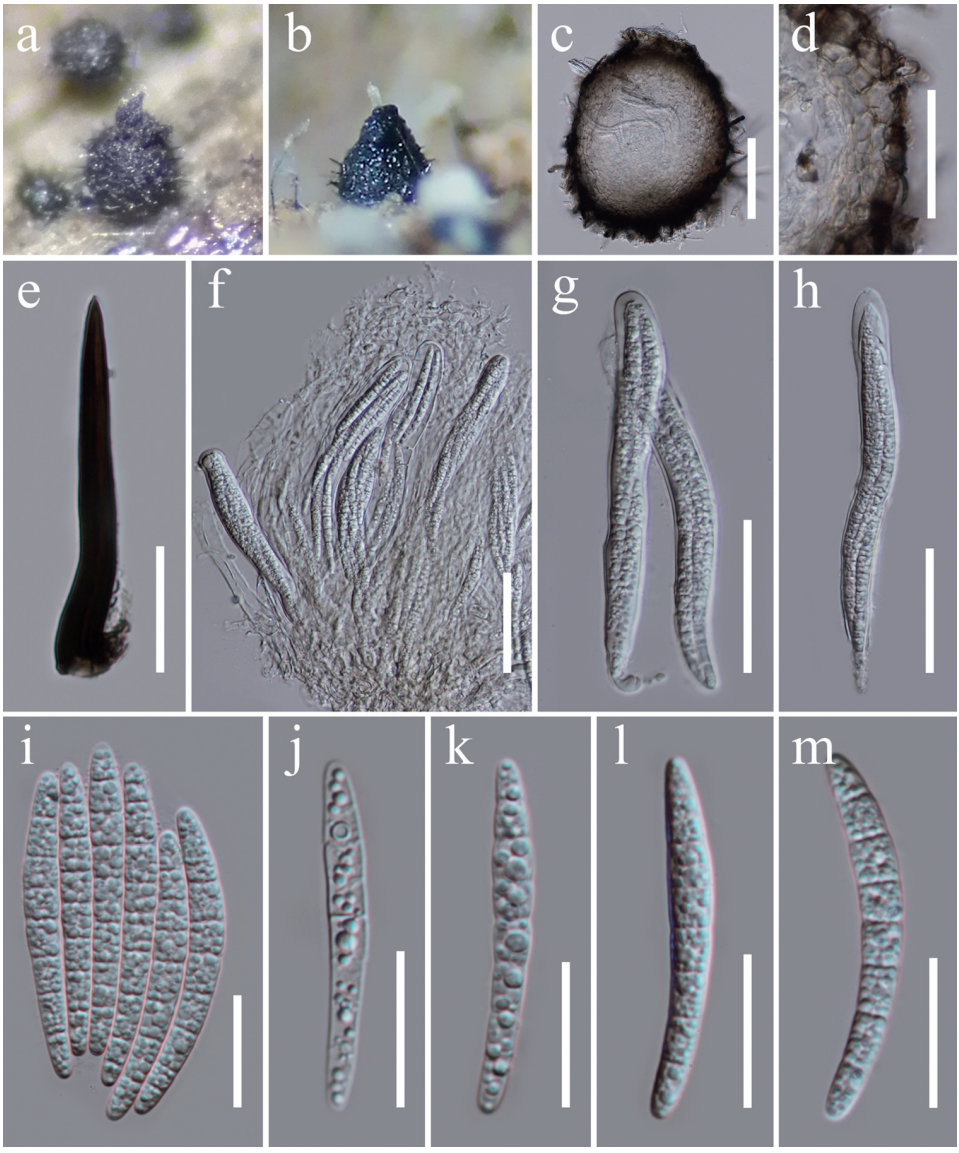
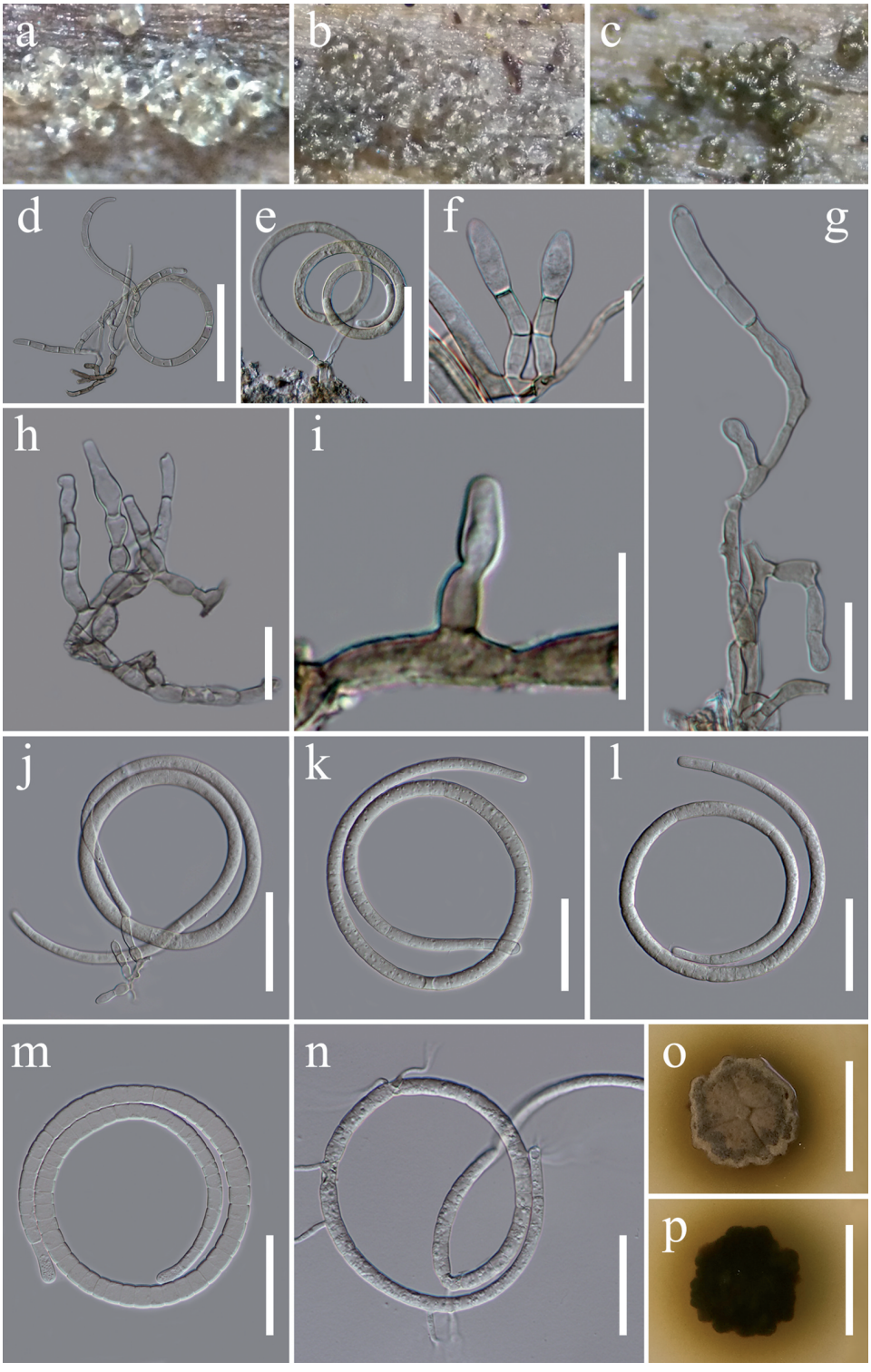


Fig. 2. ***Neoacanthostigma aquaticum*** (HKAS 97431, **holotype**). **a-b.** Superficial ascomata on substrate. **c.** Ascoma. **d.** Peridium. **e.** Seta. **f.** Asci with pseudoparaphyses. **g-h.** Asci. **i-m.** Ascospores. Scale bars: **c** = 100 μm, **d**, **f-h** = 50 μm, **e, i-m** = 20 μm.

septa (up to 93-septate vs up to 62-septate). Phylogenetically, *N. aquaticum* forms a sister clade to *N. guangxiense* with high support (Fig. 1), but the phylogenetic tree clearly showed that they are phylogenetically distinct species. Further, we also compared *N. aquaticum* with *N. guangxiense* by using single gene data, and there are 44 bp (base pair), 3 bp and 37 bp differences in ITS, LSU and TEF1α respectively, which also confirmed that they are phylogenetically distinct species. Therefore, we introduced it as a novel species of *Neoacanthostigma*.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Neoacanthostigma brunneisporum Y.Z. Lu, Boonmee & K.D. Hyde, ***sp. nov.***
Figs 4, 5

Index Fungorum number: IF 553171; *Facesoffungi number*: FoF 03240
Holotype: MFLU 17-0335

Etymology: 'brunneisporum' referring to brown helicosporous conidia on woody substrate.

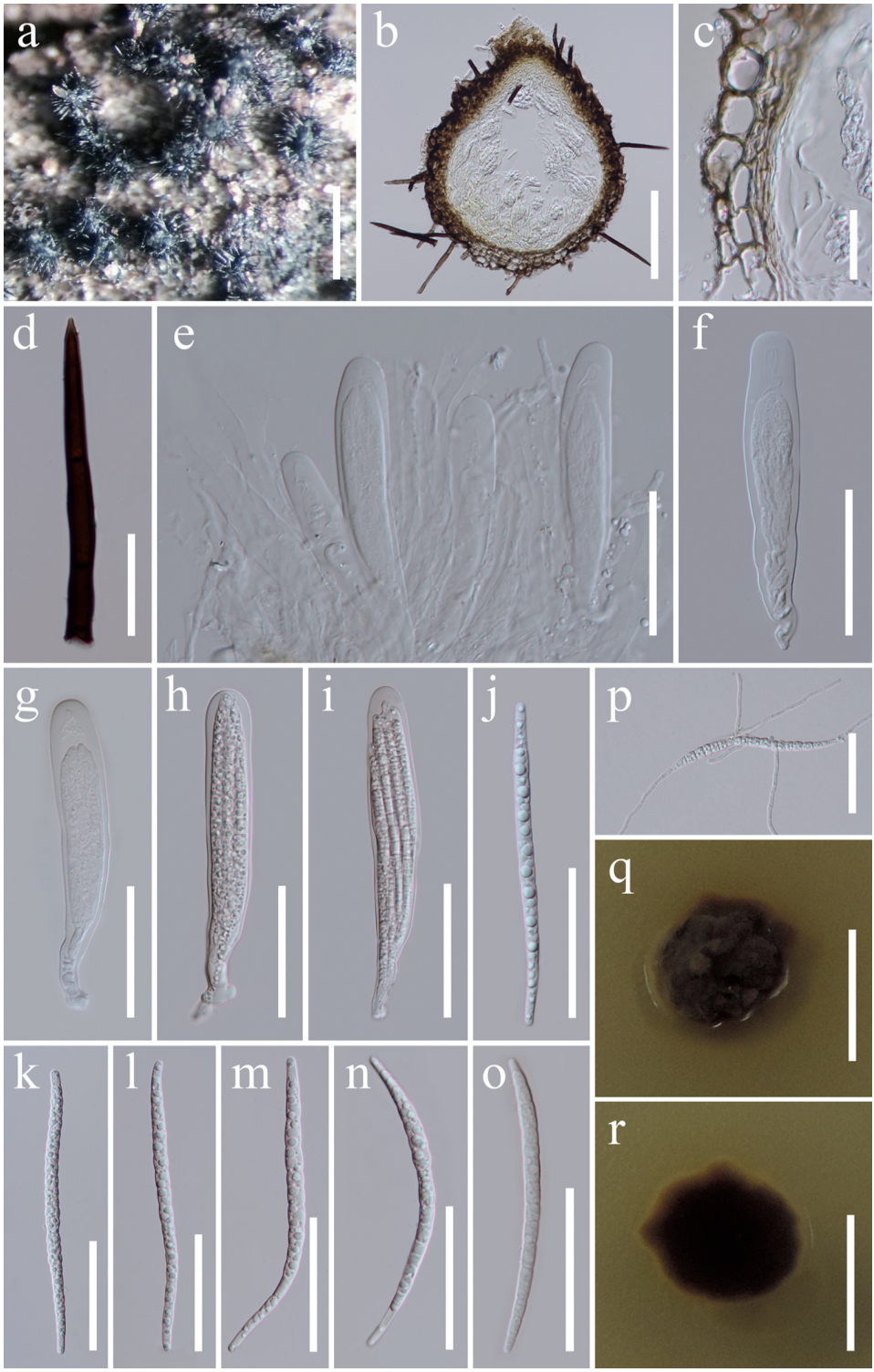
Saprobic on decaying wood in a freshwater stream. ***Sexual morph***: *Ascomata* 190-275 µm high × 175-230 µm diam., superficial, seated on a subiculum, solitary, scattered, subglobose, ellipsoidal-ovate, dark brown to black, with a central ostiolate, covered with brown to dark brown, 45-85 µm long, septate, tapering to an acute apex setae. *Peridium* 20-28 µm wide, composed cells of *textura angularis*, brown, with innermost part comprising thin layers of hyaline to pale brown cells of *textura subprismatica*. *Hamathecium* comprising numerous, filiform, septate, branched, hyaline pseudoparaphyses. *Asci* 110-140 × 17-23 µm ($\bar{x}$ = 125 × 20 µm, n = 20), 8-spored, bitunicate, cylindrical, short-pedicellate, apically rounded. *Ascospores* 95-115 (-128) × 4.5-6.5 µm ($\bar{x}$ = 108 × 5.5 µm, n = 50), fasciculate, broadly filiform, cylindrical to long subfusiform, elongate, rounded at ends, slightly curved, guttulate, 12-13-septate, not constricted at septa, hyaline to pale brown, smooth-walled. ***Asexual morph***: hyphomycetous, helicosporous. *Conidiophores* 10-24 × 4-6 µm, pale brown, macronematous, erect, short, cylindrical, septate, smooth-walled. *Conidiogenous cells* 10-19 µm long, 4-5.5 µm wide, monoblastic, holoblastic, integrated, terminal, cylindrical, truncate at apex, hyaline to pale brown, smooth-walled. *Conidia* 89-155 µm diam. and conidial filament 10.5-14 (-15) µm wide ($\bar{x}$ = 130 × 12 µm, n = 50), 630-950 µm long, coiled 2-3 times when tightly coiled, becoming loosely coiled in the water, basal cell elongated, rounded at tip, multi-septate, up to 60-septate, slightly constricted at septa, pale brown, smooth-walled.

Culture characteristics: *Conidia* germinating on water agar (WA) within 12 h and germ tubes produced from ascospores. Colonies growing on MEA, irregular, umbonate, surface rough and wrinkle, edge undulate, reaching 19 mm in 4 weeks at 28°C, brown in MEA medium. *Mycelium* superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Materials examined: THAILAND, Uttaradit, Laplae, Mae Phun, Ban Ton Klua, on decaying wood in a freshwater stream, 24 October 2015, Saranyaphat Boonmee, UTD15-4 (MFLU 17-0335, **holotype**; GZAAS 17-0002, **isotype**); ex-type living culture, MFLUCC 16-0015 = GZCC 17-0002; *Ibid*., 24 October 2015, Saranyaphat Boonmee, UTD18 (MFLU 17-0337, **paratype**); living culture, MFLUCC 16-0012; THAILAND, Krabi, Plai Praya, Khao To, Ban Bang Thao Mae, on decaying wood in a freshwater stream, 17 December 2015, Saranyaphat Boonmee, BTM12 (MFLU 17-0334, **paratype**) ; living culture, MFLUCC 16-1127.

Notes: Four isolations, collected from submerged decaying wood in freshwater stream of Thailand, are identified as a new *Neoacanthostigma* species, *N. brunneisporum*. Phylogenetically, three new collections cluster together with *Neoacanthostigma septoconstrictum* (MFLUCC 15-1248) and formed a distinct clade from *N. septoconstrictum* (ANM 536.1) (Fig. 1). However, Hyde *et al.* (2016) identified the strain MFLUCC 15-1248 as *N. septoconstrictum* because it clustered

◀ Fig. 3. ***Neoacanthostigma aquaticum*** (HKAS 97421, GZAAS 16-0046, GZAAS 16-0051, GZAAS 16-0056, GZAAS 16-0067 and GZAAS 16-0068, **paratypes**). **a-c.** Colony on decaying wood. **d-g, j.** Conidiophores with attached conidia. **h-i.** Conidiophores with conidiogenous cells. **k-m.** Conidia. **n.** Germinating conidium. **o-p.** Colony on PDA from above and below. Scale bars: **d** = 100 µm, **e, j-n** = 50 µm, **f-i** = 20 µm, **o-p** = 20 mm.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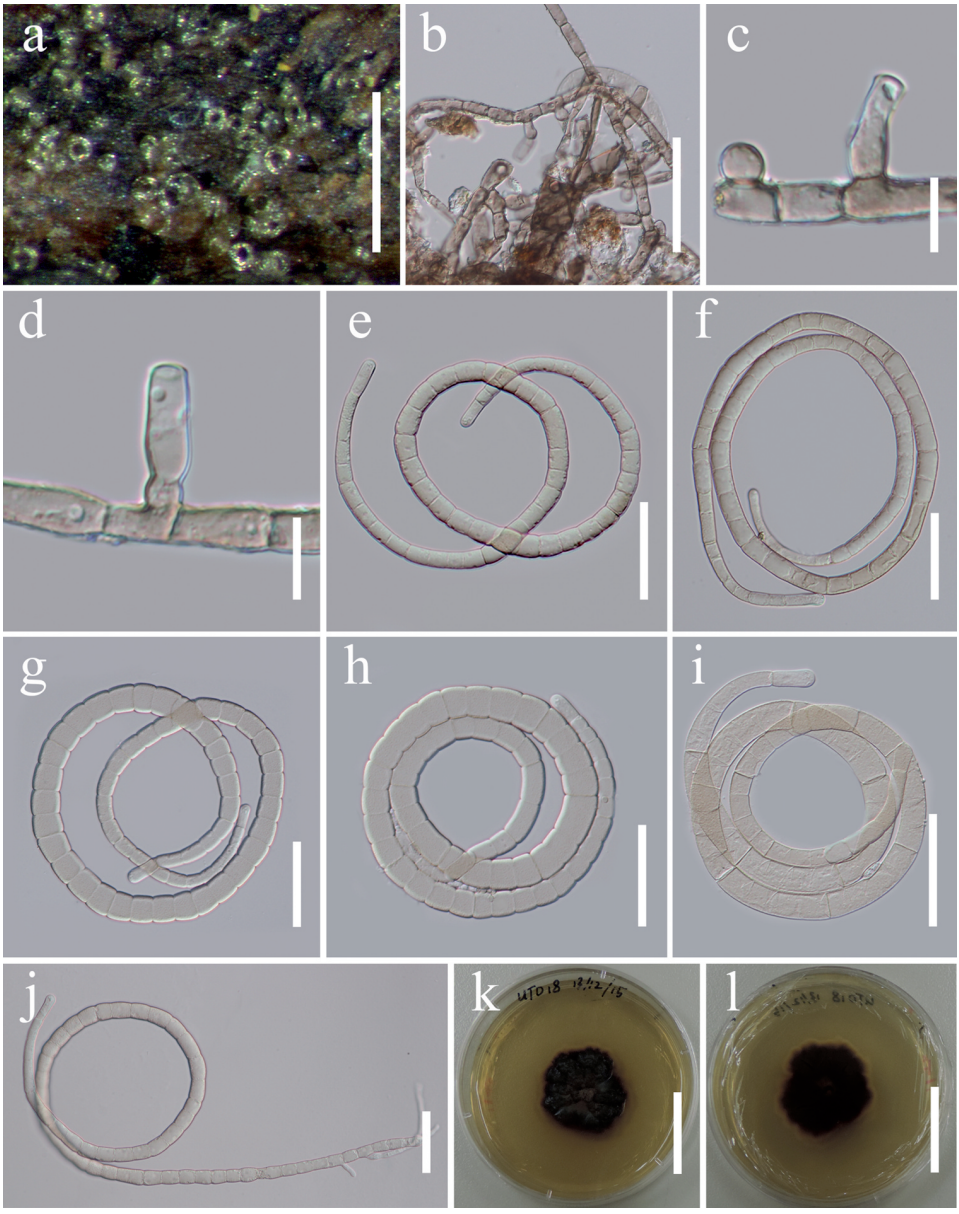


Fig. 5. ***Neoacanthostigma brunneisporum*** (MFLU 17-0334 and MFLU 17-0337, **paratypes**)**. a.** Colony on decaying wood. **b-d.** Conidiogenous cell with hyphae. **e-i.** Conidia. **j.** Germinating conidium. **k-l.** Colony on MEA from above and below. Scale bars: **a** = 500 µm, **b**, **e-j** = 50 µm, **c-d** = 10 µm, **k-l** = 20 mm.

◀ Fig. 4. ***Neoacanthostigma brunneisporum*** (MFLU 17-0335, **holotype**). **a.** Superficial ascomata on substrate. Note ascomata surrounded by dark brown setae. **b.** Ascoma. **c.** Peridium. **d.** seta. **e.** Asci with hamathecium. **f-i.** Asci. **j-o.** Ascospores. **p.** Germinating ascospore. **q-r.** Colony on MEA from above and below. Scale bars: **a** = 500 µm, **b** = 100 µm, **c-d** = 20 µm, **e-p** = 50 µm, **q-r** = 10 mm.

together with *N. septoconstrictum* (ANM 536.1) and this might be because of lack of sequences (gene regions). With more isolates included in the analysis, we compared our new taxa with *N. septoconstrictum* MFLUCC 15-1248 by using single gene data. There are only one bp difference in ITS and TEF1α respectively, which showed that they could be the same species. Moreover, we also compared our new taxa and *N. septoconstrictum* MFLUCC 15-1248 with *N. septoconstrictum* ANM 536.1 by using single gene data. There are 49 bp and 20 bp differences in ITS and LSU respectively, which confirmed that they are phylogenetically distinct species from *N. septoconstrictum* ANM 536.1. Therefore, we identify MFLUCC 15-1248 isolate as *N. brunneisporum*. Morphologically, *N. brunneisporum* differs from *N. septoconstrictum* in having different setae (septate vs one-celled) and longer ascospores (95-115 μm vs 40-50 μm).

Neoacanthostigma guangxiense Y.Z. Lu, Boonmee & K.D. Hyde, ***sp. nov.*** **Fig. 6**

Index Fungorum number: IF 553172; *Facesoffungi number*: FoF 03241
Holotype: HKAS 97425
Etymology: 'guangxi' referring to collecting site.

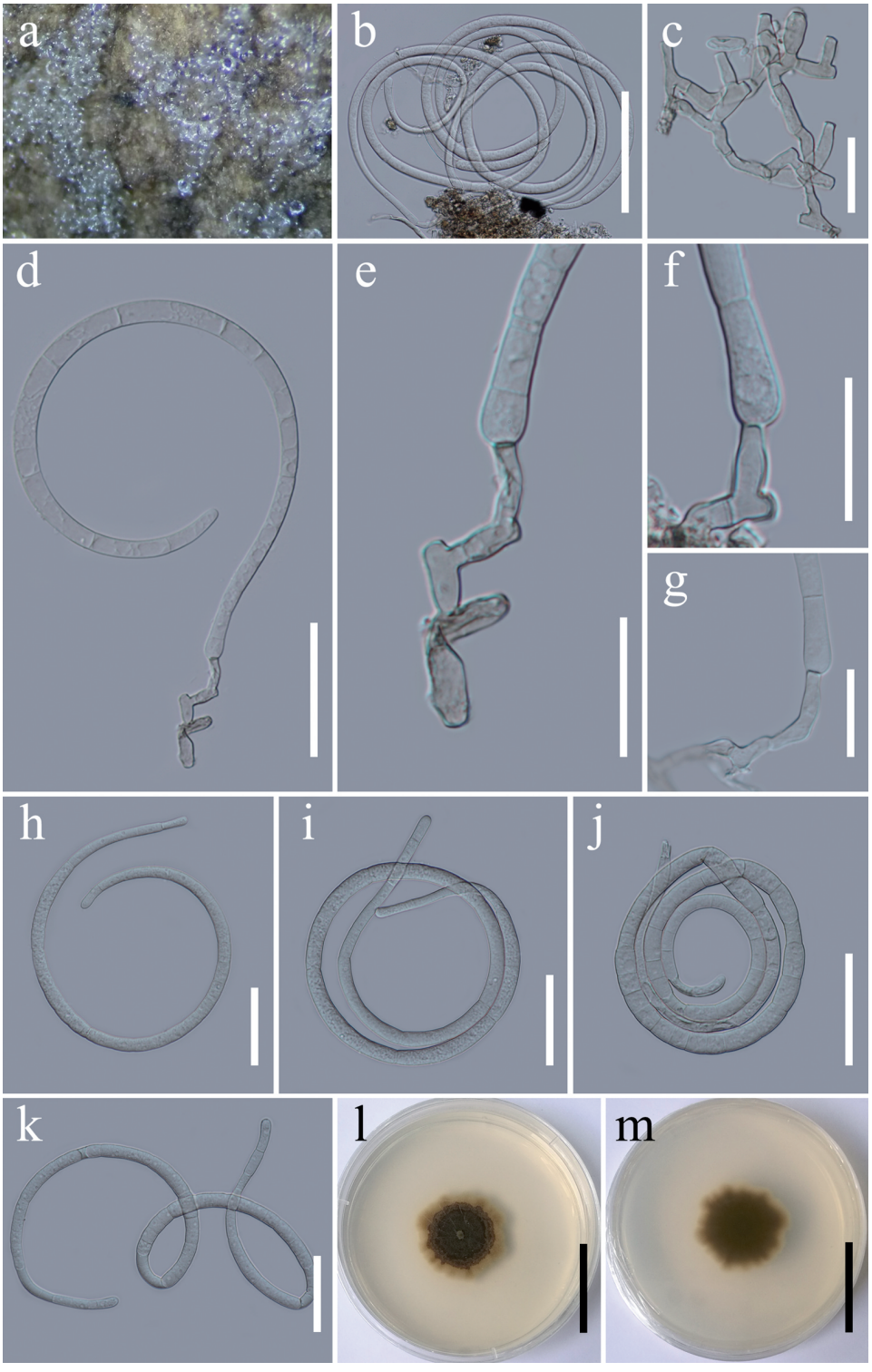
Saprobic on decaying wood in a freshwater stream. *Colonies* effuse, Alice blue. *Mycelium* mostly immersed, partly superficial. *Hyphae* branched, septate, subhyaline to pale brown. ***Sexual morph***: undetermined. ***Asexual morph***: hyphomycetous, helicosporous. *Conidiophores* 19-37 × 3.5-5 μm, pale brown, macronematous, erect, short, cylindrical, septate, smooth-walled. *Conidiogenous cells* 8.5-21.5 μm long, 3.5-5 μm wide, holoblastic, polyblastic, sympodial, integrated, terminal, cylindrical, with a truncate apex, hyaline to pale brown, smooth-walled. *Conidia* 90-130 (-144) μm diam. and conidial filament 8-10 μm wide ($\bar{x}$ = 115 × 9 μm, n = 50), 405-725 (-800) μm long, coiled 2½-3½ times when tightly coiled, becoming loosely coiled in the water, basal cell elongated, rounded at tip, multi-septate, up to 62-septate, not constricted at septa, grey to pale blue, smooth-walled.

Culture characteristics: *Conidia* germinating on water agar (WA) within 12 h and germ tubes produced from conidia. Colonies growing on PDA, irregular, with flat surface, veined and without wrinkle, edge undulate, reaching 11 mm in 2 weeks at 28°C, brown in PDA medium. *Mycelium* superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Materials examined: CHINA, Guangxi Province, Fang Cheng Gang, on decaying wood in a freshwater stream, 15 May 2016, Yong-Zhong Lu, JHC07-1 (HKAS 97425, **holotype**; GZAAS 16-0048, **isotype**); ex-type living culture, MFLUCC 17-0042 = GZCC 16-0036.

Notes: *Neoacanthostigma guangxiense* formed an asexual morph on the natural substrate and is morphologically similar to *N. aquaticum*. It resembles *N. aquaticum* in having macronematous, erect, short conidiophores and terminal to sympodial conidiogenous cells and helicoid conidia, but it differs from *N. aquaticum* in different number of septate (up to 62-septate vs up to 93-septate). Besides, its colony on PDA medium are surface flat and without wrinkled, but the colony of *N. aquaticum* on PDA medium are umbonate, surface roughed and wrinkled. Phylogenetic analyses also confirm our new taxon as a new *Neoacanthostigma* species caused 1) from the topology it clearly distinct species to others with high

Fig. 6. ***Neoacanthostigma guangxiense*** (HKAS 97425, **holotype**). **a.** Colony on decaying wood. **b, d.** Conidiophores with attached conidia. **c.** Conidiophores. **e-g.** Holo- to sympodial conidiogenous cells with part of conidia. **h-k.** Conidia. **l-m.** Colony on PDA from above and below. Scale bars: **b** = 100 μm, **c, e-g** = 20 μm, **d, h-k** = 50 μm, **l-m** = 20 mm. ▶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support; 2) compared its DNA sequence data with the phylogeny and morphology closest species, *N. aquaticum*, there are 44 bp, 3 bp and 37 bp differences in ITS, LSU and TEF1α respectively, which also confirm it is new species.

Neoacanthostigma latisporum Y.Z. Lu, Boonmee & K.D. Hyde, ***sp. nov.*** **Fig. 7**

Index Fungorum number: IF 553173; *Facesoffungi number*: FoF 03242
Holotype: MFLU 17-0336
Etymology: 'latisporum' referring to wide helicosporous conidia.

Saprobic on decaying wood in a freshwater stream. *Colonies* effuse, brown. *Mycelium* mostly immersed, partly superficial. *Hyphae* branched, septate, brown. ***Sexual morph***: undetermined. ***Asexual morph***: hyphomycetous, helicosporous. *Conidiophores* brown, macronematous, erect, short, cylindrical, septate, smooth-walled. *Conidiogenous cells* holoblastic, polyblastic, sympodial, integrated, terminal, cylindrical, with a truncate apex, brown, smooth-walled. *Conidia* 95-120 μm diam. and conidial filament 12-14.5 μm wide ($\bar{x}$ = 105 × 13 μm, n = 10), 540-760 μm long, coiled 1½-3½ times when tightly coiled, becoming loosely coiled in the water, basal cell elongated, rounded at the tip, multi-septate, up to 60-septate, slightly constricted at septa, pale brown, smooth-walled.

Culture characteristics: *Conidia* germinating on water agar (WA) within 12 h and germ tubes produced from conidia. Colonies growing on MEA, circular, umbonate, with rough surface, veined and wrinkle, edge entire, reaching 12 mm in 3 weeks at 28°C, dark brown in MEA medium. *Mycelium* superficial and partially immersed, branched, septate, hyaline to pale brown, smooth.

Materials examined: THAILAND, Uttaradit, Laplae, Mae Phun, Ban Ton Klua, on decaying wood in flowing freshwater stream, 24 October 2015, Saranyaphat Boonmee, UTD15-5 (MFLU 17-0336, **holotype**; GZAAS 17-0003, **isotype**); ex-type living culture, MFLUCC 16-0019 = GZCC 17-0003.

Notes: *Neoacanthostigma latisporum* formed a brown helicosporous conidia colony on natural woody substrate, which is morphologically similar to *N. brunneisporum* (introduced in this paper). We compared its morphology features with other *Neoacanthostigma* species (Table 2), the result showed it share similar

Table 2. Synopsis of Neoacanthostigma asexual species

Species	*Colonies on natural substrates*	*Conidiophores*	*Conidiogenous cells*	*Conidia (μm)*	*Conidia septa*	*Number of coils in conidia (times)*	*References*
Neoacanthostigma aquaticum	Pale brown to brown	18-45.5 × 3.5-6	6-24 × 3-5	100-150 μm diam × 8-12 μm wide, 500-1070 μm long	Up to 93-septate	2½-3	This study
N. brunneisporum	Brown	10-24 × 4-6	10-19 × 4-5.5	89-155 × 10.5-14, 630-950	Up to 60-septate	2-3	Hyde *et al.* 2016; This study
N. fusiforme	–	Up to 5 μm long	–	23-30 × 2	–	1-2½	Boonmee *et al.* 2014
N. guangxiense	Alice blue	19-37 × 3.5-5	8.5-21.5 × 3.5-5	90-130 × 8-10, 405-725	Up to 62-septate	2½-3½	This study
N. latisporum	Brown	–	–	95-120 × 12-14.5, 540-760	Up to 60-septate	1½-3½	This study

morphology characters with *N. brunneisporum*. However, the phylogeny (Fig. 1) clearly indicates that they are different species with strong support (100 MLBS / 100 MPBS / 1.00 PP). Furthermore, we also compared the new species with *N. brunneisporum* by using single DNA gene sequences data, and there are 14 bp, 5 bp and 32 bp differences in ITS, LSU and TEF1α respectively (data not contains gap), which also confirmed that they are phylogenetically distinct species even though they share similar morphology features.

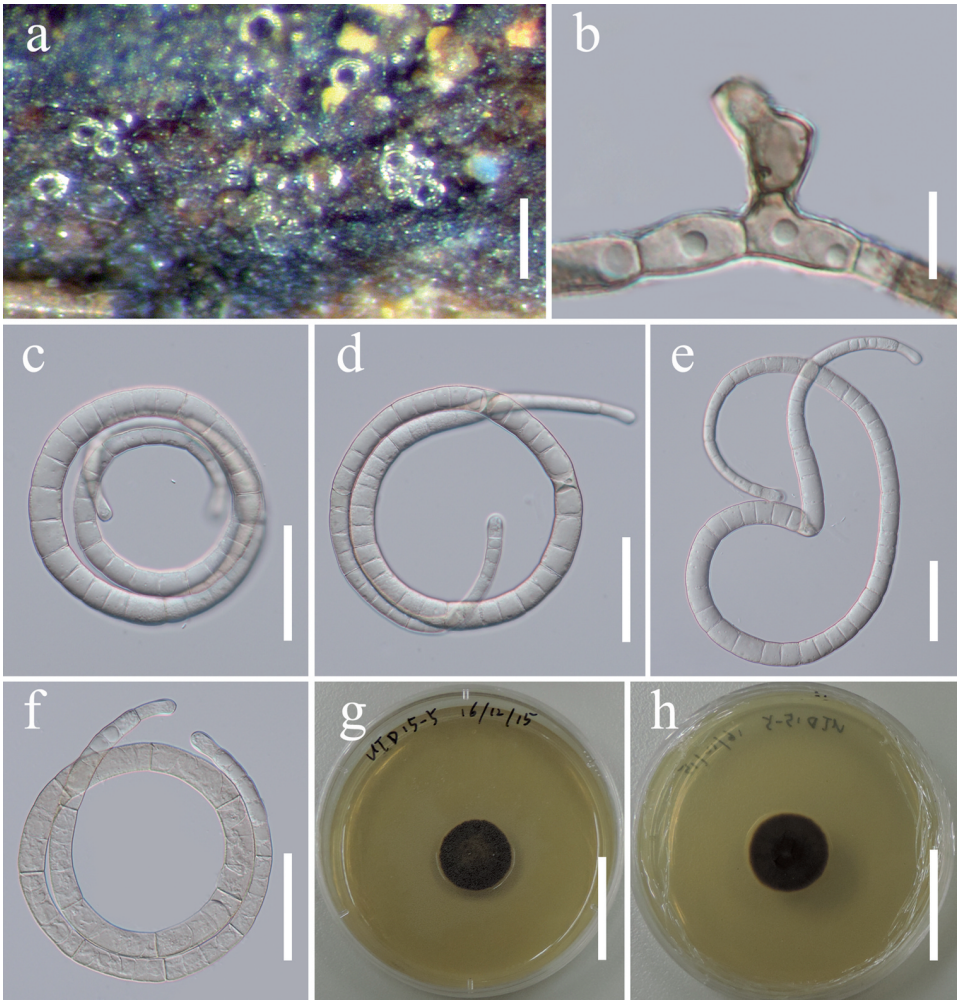

Fig. 7. ***Neoacanthostigma latisporum*** (MFLU 17-0336, **holotype**). **a.** Colony on decaying wood. **b.** Conidiophores and conidiogenous cell with hyphae. **c-f.** Conidia. **g-h.** Colony on MEA from above and below. Scale bars: **a** = 200 µm, **b** = 10 µm, **c-f** =50 µm, **g-h** = 20 mm.

DISCUSSION

In this study, we followed the recommendations of Jeewon & Hyde (2016) in determining species novelty. *Neoacanthostigma aquaticum*, *N. brunneisporum*, *N. guangxiense* and *N. latisporum*, are introduced using morphological and phylogenetic evidence. A key to *Neoacanthostigma* species is provided as well.

Helicosporous hyphomycetes were found to be common on submerged wood in streams in previous studies (Hyde & Goh 1997; Tsui *et al.* 2000; Ho *et al.* 2001; Cai *et al.* 2002, 2003; Sakayaroj *et al.* 2005; Pinnoi *et al.* 2006; Zhao *et al.* 2007). However previous identifications were based on morphology and we suspect that many may be wrongly named, even at the genus level. The lightly dematiaceous helicosporous in the asexual morph could easily have been overlooked. In this study, we showed that *Neoacanthostigma septoconstrictum* (strain MFLUCC 15-1248) clusters with the new species, *N. brunneisporum*. Thus, as more taxa are sequenced, it is likely that more cryptic species will be resolved. Whether streams are isolated habitats where species have evolved independently from common ancestors in other streams, has yet to be determined (Vijaykrishna *et al.* 2006). If this has happened, we could expect to find numerous new taxa as we study more streams.

We collected ten lightly dematiaceous helicosporous hyphomycetes specimens from aquatic habitats. Morphologically, they differ from *Helicoma* and *Helicosporium* in having shorter conidiophores, larger conidiogenous cells and distinct conidia. Morphologically they resemble *Helicomyces* and are especially similar to *Helicomyces macrofilamentosus* (Matsushima 1983; Zhao *et al.* 2007). Our newly *Neoacanthostigma* species however, have wider, polyblastic, conidiogenous cells, wider conidia and distinctly wider conidial filaments. We also compared our new collections with other helicosporous genus, viz. *Acanthohelicospora*, *Chlamydotubeufia*, *Helicangiospora*, *Neohelicomyces* and *Tubeufia*. They differ from these genera in having distinct conidiophores, conidiogenous cells and conidia.

Our DNA sequence data strongly support *N. aquaticum*, *N. brunneisporum*, *N. guangxiense* and *N. latisporum* as new *Neoacanthostigma* species. The phylogenetic tree clearly indicates that they are phylogenetically distinct species. However, we noted that morphologically *N. aquaticum* is similar with *N. guangxiense*, and *N. brunneisporum* is similar with *N. latisporum* in their asexual morphs (Table 2). Phylogenetically their placements also cluster in sister group, therefore, we compared pairwise dissimilarities of DNA sequence data and noted that there are differences in the ITS, LSU and TEF1α sequences which possibly explain why they are different. For, 1) in *N. aquaticum* and *N. guangxiense*, there are 28 bp, 3 bp and 37 bp differences in ITS, LSU and TEF1α respectively (data not contains gap); and 2) in *N. brunneisporum* and *N. latisporum*, there are 14 bp, 5 bp and 32 bp differences in ITS, LSU and TEF1α respectively. The sequences data indicate that they could be different species.

The genus *Neoacanthostigma* now comprises seven species and all species are confirmed by molecular analysis. Four species are reported from freshwater habitats, which include those newly described, viz. *N. aquaticum*, *N. brunneisporum*, *N. guangxiense* and *N. latisporum*. Three sexual morph species, *N. aquaticum*, *N. brunneisporum* and *N. fusiforme* have been linked to their asexual morphs.

Key to species of *Neoacanthostigma*

1. Ascomata present 2
1. Ascomata lacking 6

2. Ascospores with > 7 septa 3
2. Ascospores 50.5-60 × 4.5-6.5 µm, fusiform, 7-septate ..... ***N. aquaticum***
3. Ascospores shorter than 50 µm 4
3. Ascospores longer than 50 µm 5
4. Ascomata 126-138 × 98-100 µm, asci 71-84 × 10-11 µm..... ***N. fusiforme***
4. Ascomata 200-250 × 150-200 µm, asci 110-140 × 17-22 µm..... ***N. septoconstrictum***
5. Asci 110-140 × 17-23 µm, setae septate ..... ***N. brunneisporum***
5. Asci 100-115 × 11.5-13 µm, setae one celled ..... ***N. filiforme***
6. Conidial filament wider than 12 µm, conidia up to 60-septate ..... ***N. latisporum***
6. Conidial filament narrower than 10 µm, conidia up to 62-septate ..... ***N. guangxiense***

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