

Asexual-sexual morph connection in the type species of *Berkleasmium*

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Abstract: *Berkleasmium* is a polyphyletic genus comprising 37 dematiaceous hyphomycetous species. In this study, independent collections of the type species, *B. concinnum*, were made from Eastern North America. Nuclear internal transcribed spacer rDNA (ITS) and partial nuc 28S large subunit rDNA (LSU) sequences obtained from collections and subsequent cultures showed that *Berkleasmium concinnum* is the asexual morph of *Neoacanthostigma septoconstrictum* (*Tubeufiaceae*, *Tubeufiales*). Phylogenies inferred from Bayesian inference and maximum likelihood analyses of ITS-LSU sequence data confirmed this asexual-sexual morph connection and a re-examination of fungarium reference specimens also revealed the co-occurrence of *N. septoconstrictum* ascomata and *B. concinnum* sporodochia. *Neoacanthostigma septoconstrictum* is therefore synonymized under *B. concinnum* on the basis of priority. A specimen identified as *N. septoconstrictum* from Thailand is described as *N. thailandicum* sp. nov., based on morphological and genetic distinctiveness.

Key words:

Ascomycota
molecular systematics
morphology
new species
taxonomy
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Tubeufiales

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INTRODUCTION

Naturalist Thomas Gibson Lea botanized extensively in Ohio until his death in 1844, after which his fungal specimens were sent to his correspondent Miles J. Berkeley (Lea 1849). Berkeley (1845) described *Sporidesmium concinnum* (“A very pretty object under the microscope”) from Lea’s specimen growing on dead wood from a rotten tree trunk (host unknown) in Ohio. Zobel (in Corda 1854) later described the new genus *Berkleasmium* to accommodate *S. concinnum*, illegitimately devising the name *Berkleasmium cordeanum*. More than one century later, Moore (1958) re-established the generic name *Berkleasmium* to accommodate sporodochial species previously placed in *Sporidesmium*, later accepting ten species (Moore 1959). *Berkleasmium* currently comprises 37 species characterized by sporodochial conidiomata bearing macronematous conidiophores and monoblastic conidiogenous cells that give rise to brown or black, dry, rhexolytically-seceding, dictyoconidia (Ellis 1971, Bussaban *et al.* 2001, Seifert *et al.* 2011, <http://www.speciesfungorum.org/>). *Berkleasmium* species are associated with a variety of decaying above-ground tissues of monocot and dicot plants from terrestrial and aquatic habitats. No known sexual morph has yet been connected to *Berkleasmium*.

Available sequences in GenBank are restricted to species described from Thailand or Micronesia, including *Berkleasmium crunisia*, *B. micronesicum*, *B. nigroapicale*, and *B. typhae*. Previous phylogenetic analyses based on SSU and LSU sequences of these species indicate the polyphyly of *Berkleasmium*, with *B. micronesicum* and *B. nigroapicale* placed *incertae sedis* sister to *Sporormiaceae*, and *B. crunisia*

and *B. typhae* placed *incertae sedis* sister to *Mycopezon smithii* (Pinnoi *et al.* 2007, Wang *et al.* 2007, Hyde *et al.* 2016). This polyphyly is not surprising given the treatment of *Berkleasmium* as a genus characterised by the sporodochia bearing dark brown dictyoconidia. For example, *Monodictys monilicellularis* was transferred to *Berkleasmium* because of sporodochia formation, despite the presence of distinctive sterile moniliform appendages, while *B. papillatum* was placed within the genus despite proliferating conidiophores; the taxonomic informativeness of these characters remains to be tested by molecular phylogenetic analyses (Raghuvier Rao & Rao 1964, Whitton *et al.* 2012).

Determining the phylogenetic placement of the type species, *B. concinnum*, is crucial to delineate taxonomic boundaries within this polyphyletic and morphologically heterogeneous genus. In this study, ITS and LSU sequences were analyzed with other taxa in *Tubeufiales* to estimate phylogenetic relationships.

MATERIALS AND METHODS

Sampling and isolation of fungi

Field collections of *Berkleasmium concinnum* were made independently in Gatineau, Quebec, Canada and the Great Smoky Mountains National Park, North Carolina and Tennessee, USA. Single conidium cultures were generated by transferring individual conidia to 6 cm diam Petri dishes containing MEA using an electrolytically sharpened tungsten needle (Brady 1965). Conidial germination was visually verified and cultures were incubated at 16 °C under 12:12

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h dark:light conditions. Specimens were accessioned in the Canadian National Mycological Herbarium (Ottawa, Canada; DAOM) or the Illinois Natural History Survey Fungarium (Champaign, USA; ILLS) and living cultures were deposited in the Canadian Collection of Fungal Cultures (Ottawa, Canada; DAOMC).

Morphological observations

Conidiomata and ascomata from fresh and dried specimens were mounted in deionized water, 5 % KOH, or lactic acid. Observations were made using an Olympus BX50 light microscope (Olympus, Tokyo) and an Olympus SZX12 stereomicroscope and micrographs were captured using an InfinityX-32 camera (Lumenera, Ottawa) and Infinity Analyze (Lumenera) software. Photographic plates were assembled using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from 12-wk-old cultures or directly from conidiomata of *B. concinnum* using the Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) or NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturers' protocol. The entire ITS and the first two domains of LSU were amplified and sequenced following the methods of Promputtha & Miller (2010) and Tanney *et al.* (2015).

Phylogenetic analyses

Sequence contigs were assembled and trimmed using Geneious R8 v. 8.1.5 (Biomatters, Auckland, New Zealand). ITS and LSU sequences were concatenated to create a dataset containing 37 sequences that was aligned using MAFFT v. 7 (Katoh & Standley 2013) and visually inspected in Geneious. The most suitable sequence evolution model (GTR+I+G) was determined based on the optimal Akaike information criterion scores in MrModeltest v. 2.2.6 (Nylander 2004). The ex-type culture of *Botryosphaeria corticis* (CBS119047; NR 111213) was selected as outgroup based on previous analyses (Sanchez *et al.* 2012). Bayesian inference (BI) phylogenetic reconstruction was performed with MrBayes v. 3.2 (Ronquist *et al.* 2012). Three independent Markov Chain Monte Carlo (MCMC) samplings were performed with 12 chains (11 heated and one cold) with sampling every 500 generations until the standard deviation of split frequencies reached a value < 0.01. The first 25 % of trees were discarded as burn-in and the remaining trees kept and combined into one 50 % majority rule consensus tree. Convergence was assessed from the three independent runs using Tracer v. 1.6 (Rambaut *et al.* 2014). Maximum likelihood (ML) analysis was performed using RAxML v. 8.2.4 (Stamatakis 2014) in PAUP v. 4.0b10 (Swofford 2003) starting from a random starting tree with 1000 bootstrap replicates. Consensus trees were visualized in FigTree 1.4.2 (available at <http://tree.bio.ed.ac.uk/software/figtree/>) and exported as SVG vector graphics for assembly in Adobe Illustrator v10 (Adobe Systems, San Jose, CA). All novel sequences used in this study were accessioned in GenBank (Table 1) and taxonomic novelties and associated metadata were deposited in MycoBank (www.mycobank.org).

RESULTS

The concatenated ITS-LSU alignment consisted of 37 sequences and 1277 positions. All *Berkleasium concinnum* specimens shared identical ITS sequences except for a single A-to-G transition (position 441 in alignment) in specimen ILLS 80804. *Berkleasium concinnum* ITS sequences were identical to the ITS sequence of the holotype of *Neoacanthostigma septoconstrictum* (ILLS 59356), except for the single bp difference in ILLS 80804. LSU sequences for all *Berkleasium concinnum* isolates were identical and 99 % similar to the *N. septoconstrictum* type (ILLS 59356; NR_119758), with one A-to-T transversion (position 758) and two C-to-T transitions (positions 806 and 1213).

Phylogenies inferred from BI and ML analyses were generally concordant. However, the backbone topology was polytomous in the BI analysis versus dichotomous in the ML analysis, albeit with low-supported branch nodes in both analyses. The placement of *Acanthostigma filiforme* was also discordant among both analyses, probably a result of the poorly-supported backbone, and PP values were generally higher than BS values. *Berkleasium concinnum* and *N. septoconstrictum*, including the type species *N. fusiforme* (MFLUCC 110510; KF301529) and a specimen identified as *N. septoconstrictum* (MFLUCC 151248; KX454176) but with a distinct ITS sequence, formed a well-supported clade in both analyses (BS = 93; PP = 1) (Fig. 1). The *Berkleasium-Neoacanthostigma* clade was weakly-supported (BS = 37; PP = 0.57) sister to a clade comprising *Helicoma conicodentatum*, *Helicosporium linderi*, *Tubeufia khunkornensis*, and a strain identified as *Chlamydotubeufia cf. huaikangplaensis*.

Sequence similarity and ITS-LSU phylogenetic analyses reveal that *B. concinnum* is the asexual morph of *N. septoconstrictum* (*Tubeufiaceae*, *Tubeufiales*) and that the type species of both genera occur in a well-supported clade (BS = 100; PP = 1). Although not originally reported with the description of *N. septoconstrictum* (Promputtha & Miller 2010), re-examination of the type specimen revealed numerous conidiomata of *B. concinnum* covering the woody substrate along with ascomata of *N. septoconstrictum*. Examining additional reference specimens of *B. concinnum* confirmed the co-occurrence of the ascomatal sexual morph (i.e. *N. septoconstrictum*) among the asexual *B. concinnum* conidiomata (DAOM 24916, DAOM 29377, DAOM 41029, DAOM 75764, DAOM 155883, DAOM 155884, DAOM 210103, ILLS 80802; Fig. 2A–C). Since *Berkleasium* Zobel 1854 is an earlier name than *Neoacanthostigma* Boonmee *et al.* 2014, *N. septoconstrictum* must be synonymized under *B. concinnum*, on the assumption that all priority currently accorded to generic names typified by a sexual morph is ended in July 2017. In addition, the specimen previously identified by Hyde *et al.* (2016) as *N. septoconstrictum* (MFLUCC 15–1248; KX454176) is not conspecific with the type specimen of this species (ILLS59356; NR119758) based on asexual morph dissimilarity (hyaline helicoid conidia) and genetic (13 % ITS sequence divergence) differences and is therefore described as a new species, *N. thailandicum*.

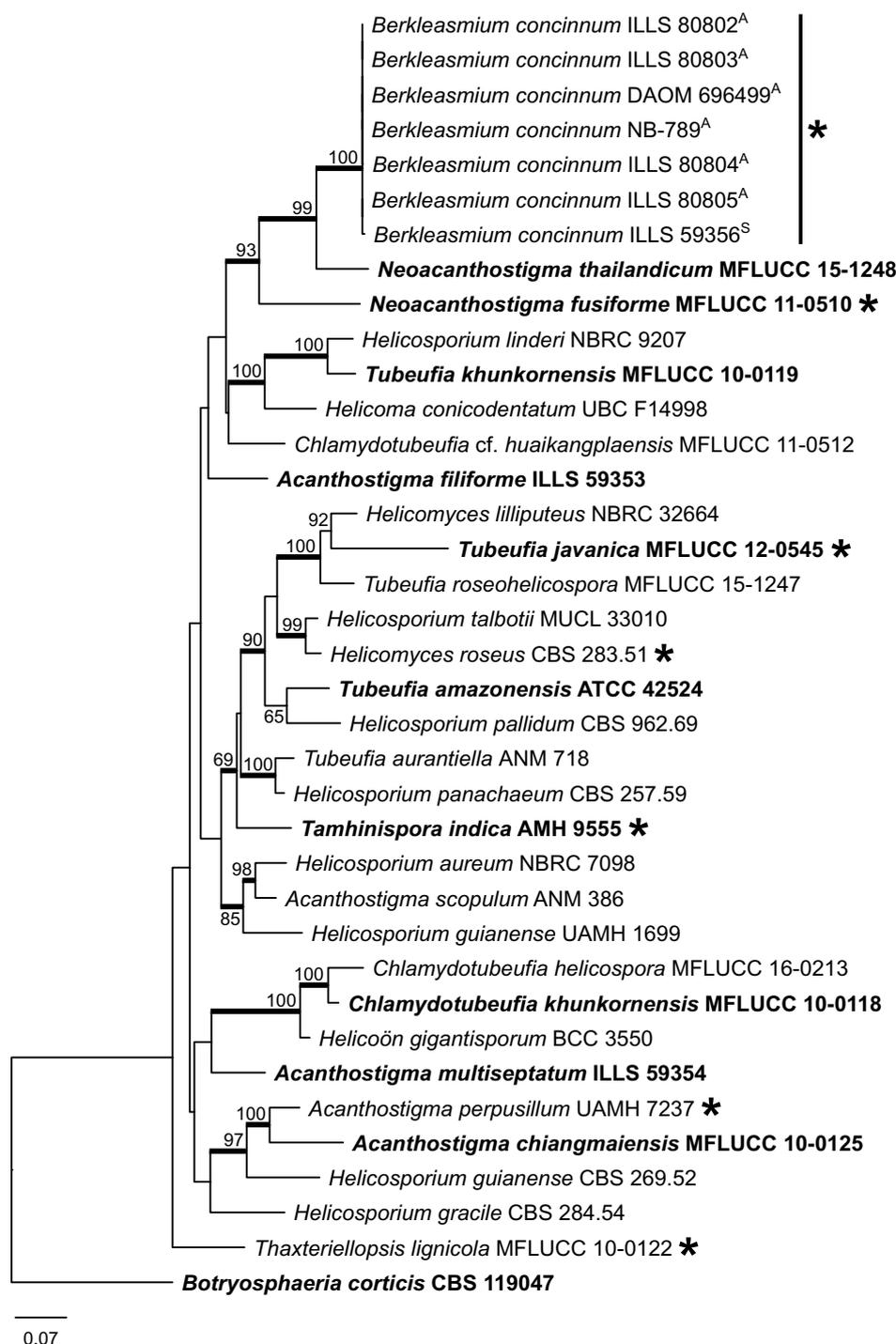


Fig. 1. Most likely tree from a RAxML analysis of ITS-LSU dataset containing representative *Tubeufiales* species. Culture collection accession numbers or specimen identifiers follow the species name, with type or ex-type strains in bold and asterixes (*) denoting generic types. ^A denotes sequences derived from *Berkleasium concinnum* asexual morph and ^S denotes sequences derived from *B. concinnum* sexual morphs. RAxML bootstrap support percentages ≥ 50 from a summary of 1000 replicates are presented at the branch nodes. Thickened branches indicate Bayesian posterior probability values ≥ 0.95 . The tree was rooted with *Botryosphaeria corticis* and the scale bar represents the number of substitutions per site.

TAXONOMY

Berkleasium concinnum (Berk.) S. Hughes, *Canad. J. Bot.* **36**: 740 (1958).

(Fig. 2; Promputtha & Miller 2010: figs 15–22)

Basionym: *Sporidesmium concinnum* Berk., *London J. Bot.* **4**: 309 (1845).

Synonyms: *Berkleasium cordeanum* Zobel, in Corda, *Icon. Fung.* **6**: 4 (1854); nom. illegit. (Art. 52.1).

Neocanthostigma septoconstrictum (Promp. & A.N. Mill.) S. Boonmee & K.D. Hyde, *Fungal Diversity* **68**: 279 (2014).

Acanthostigma septoconstrictum Promp. & A.N. Mill., *Mycologia* **102**: 579 (2010).

Type: **USA**: Ohio: on dead wood, *T.G. Lea 168* (K(M) - holotype, n.v.; K(M)-IMI94327 - slide ex-holotype).

Other specimens examined: **Canada**: Quebec: Gatineau, Aylmer, Boucher Forest, on large, rotten, decorticated hardwood log, 17 Jul. 2015, *J.B. Tanney* (DAOM 696473); *ibid.*, 29 Aug. 2015, *J.B. Tanney* (DAOM 696486); *ibid.*, 10 Jul. 2015, *J.B. Tanney* (DAOM 696499, DAOMC 251512, DAOMC 251513); *ibid.*, fallen hardwood log, 5 Sep. 2016, *J.B. Tanney* (NB-789). – **Russia**: Primorsky Krai, Khasansky District, Kedrovaya Pad Nature Preserve, log of an

unknown deciduous species, 3 Oct. 1987, *I.M. Bacuebeba* (DAOM 210103). – **USA**: Louisiana: St. Tammany Parish, Honey Island Swamp near Pearl River, rotten wood, 6 Jun. 1976, *S.J. Hughes* (DAOM 155883); *ibid.*, 6 Jun. 1976, *W.B. Cooke* (DAOM 155884). Massachusetts: Salem, on *Salix* wood, 1834 (DAOM 43686). Missouri: St Louis County, Benbush, on dead wood, 15 Nov. 1941, *G.D. Darker* (DAOM 75764). New York: Flatbush, 1890, *I.L. Zabriskie* (DAOM 34307); Lloyd-Cornell Preserve, Ringwood, on rotten wood, 6 Sep. 1952, *S.J. Hughes* (DAOM 29052, DAOM 29377); *ibid.*, on decorticated wood (old), 6 Sep. 1952, *W.I. Illman* (DAOM 239154);

Table 1. Sequences used in phylogenetic analyses in this study.

Species	Specimen/strain	GenBank No.	
		ITS	LSU
<i>Acanthostigma chiangmaiense</i>	MFLUCC 10-0125 ^T	JN865209	JN865197
<i>Acanthostigma filiforme</i>	ILLS 59353 ^T	GQ856146	GQ850494
<i>Acanthostigma multiseptatum</i>	ILLS 59354 ^T	NR_119759	GQ850492
<i>Acanthostigma perpusillum</i>	UAMH 7237	AY916492	AY856892
<i>Acanthostigma scopulum</i>	ANM 386	GQ856141	GQ850489
<i>Berkleasium concinnum</i>	ILLS 59356 ^(H)	NR_119758	NG_042520
<i>Berkleasium concinnum</i>	DAOM 696499	KY611397	KY611399
<i>Berkleasium concinnum</i>	NB-789	KY611398	KY611400
<i>Berkleasium concinnum</i>	ILLS 80802		KY582484
<i>Berkleasium concinnum</i>	ILLS 80803		KY582485
<i>Berkleasium concinnum</i>	ILLS 80804		KY582486
<i>Berkleasium concinnum</i>	ILLS 80805		KY582487
<i>Botryosphaeria corticis</i>	CBS 119047 ^T	NR_111213	NG_042457
<i>Chlamydotubeufia</i> cf. <i>huaikangplaensis</i>	MFLUCC 11-0512	KF301528	KF301536
<i>Chlamydotubeufia helicospira</i>	MFLUCC 16-0213 ^T	KX454169	KX454170
<i>Chlamydotubeufia khunkomensis</i>	MFLUCC 100118 ^T	NR_137588	JN865190
<i>Helicoma conicodentatum</i>	UBC F14998	AY916450	AY856869
<i>Helicomycetes lilliputeus</i>	NBRC 32664	AY916483	AY856899
<i>Helicomycetes roseus</i>	CBS 283.51	AY916464	AY856881
<i>Helicoön gigantisporum</i>	BCC 3550	AY916467	AY856904
<i>Helicosporium aureum</i>	NBRC 7098	AY916478	AY856894
<i>Helicosporium gracile</i>	CBS 284.54	AY916485	AY916086
<i>Helicosporium guianense</i>	UAMH 1699	AY916479	AY856891
<i>Helicosporium guianense</i>	CBS 269.52	AY916487	AY856893
<i>Helicosporium linderi</i>	NBRC 9207	AY916454	AY856895
<i>Helicosporium pallidum</i>	CBS 962.69	AY916460	AY856886
<i>Helicosporium panachaeum</i>	CBS 257.59	AY916471	AY916087
<i>Helicosporium talbotii</i>	MUCL 33010	AY916465	AY856874
<i>Neoacanthostigma fusiforme</i>	MFLUCC 11-0510 ^T	KF301529	KF301537
<i>Neoacanthostigma thailandicum</i>	MFLUCC 15-1248 ^T	KX454176	—
<i>Tamhinispora indica</i>	AMH 9555 ^T	NR_137801	KC469283
<i>Thaxteriellopsis lignicola</i>	MFLUCC 10-0122	JN865206	JN865194
<i>Tubeufia amazonensis</i>	ATCC 42524 ^T	AY916458	AY856911
<i>Tubeufia aurantiella</i>	ANM 718	GQ856140	GQ850485
<i>Tubeufia javanica</i>	MFLUCC 12-0545 ^T	KJ880034	KJ880036
<i>Tubeufia khunkomensis</i>	MFLUCC 10-0119 ^T	JN865203	JN865191
<i>Tubeufia roseohelicospira</i>	MFLUCC 15-1247	KX454177	KX454178

New sequences generated in this study are in **bold**, ^T denotes sequence from type or ex-type material, ^(H) refers to the holotype of *Neoacanthostigma septoconstrictum*, not *B. concinnum*.

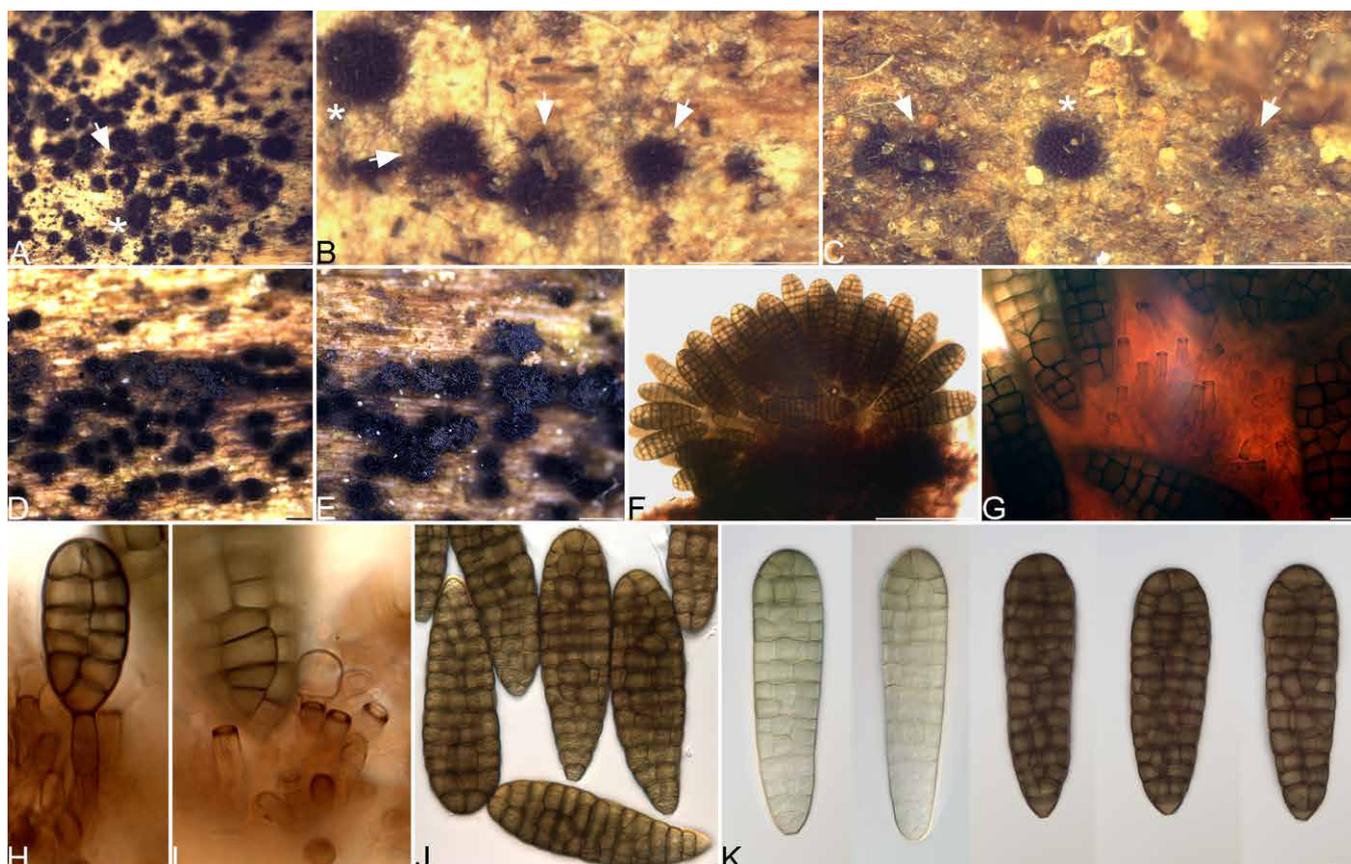


Fig. 2. *Berkleasium concinnum* conidiomata and ascomata. **A–C.** Co-occurring ascomata and sporodochia, arrows point to ascomata and asterisks (*) denote sporodochia. **D–E.** Sporodochia in situ. **F.** Sporodochium. **G–I.** Conidiogenous cells. **J.** Conidia mounted in H₂O. **K.** Developing and mature conidia mounted in lactic acid. **A–C** DAOM 696473, **D–H** NB-789, **I–J** DAOM 155884, **K** DAOM 155883. Bars: **A–E** = 500 μ m, **F** = 100 μ m, **G–K** = 10 μ m.

Buffalo, G.W. *Clinton* (DAOM 34362). *North Carolina*: Haywood Co., Great Smoky Mountains National Park, Big Creek, Baxter Creek Trail, 35.7485° N, -83.1119° W, 610 m elev., decorticated 3 cm diam branch on ground, 16 Jun. 2007, A.N. Miller et al. (ANM 1141, ILLS 80802); Cullowhee, on rotten wood, Jun. 1887, R. Thaxter (DAOM 29026). *South Carolina*: on wood, (DAOM 34361). *Tennessee*: Blount County, Bote Mountain, Great Smoky Mountains National Park, on rotten wood and bark, 23 Aug. 1977, S.J. Hughes (DAOM 188987, DAOM 169302); Cocke County, Great Smoky Mountains National Park, Cosby, Low Gap Trail, 35.7539° N, 83.2071° W, 716 m elev., decorticated wood on ground, 15 Jul. 2005, A.N. Miller & A.M. Stchigel (ANM536.1, ILLS 59356 – holotype of *Acanthostigma septoconstrictum*); *ibid.*, decorticated 5 cm diam. branch on ground, 3 Nov. 2007, A.N. Miller et al. (ANM 1407, ILLS 80805); *ibid.*, Cosby Nature Trail, 35.7538° N, 83.2072° W, 716 m elev., 50 cm diam log on ground, 20 May 2008, A.N. Miller et al. (ANM 1701, ILLS 80806); Sevier Co., Great Smoky Mountains National Park, 5 miles east of Gatlinburg, Greenbrier, Old Settlers Trail, 35.7076° N, 83.3804° W, 457 m elev., decorticated 6 cm diam branch on ground, 19 Jun. 2007, A.N. Miller et al. (ANM 1171, ILLS 80803); Twin Creeks, Twin Creeks Nature Trail, near ATBI plot, 35.6881° N, 83.4999° W, 549 m elev., decorticated 5 cm diam branch on ground, 18 Jun. 2007, A.N. Miller et al. (ANM 1227, ILLS 80804). *West Virginia*: Giles County, Mountain Lake, on well decayed log of deciduous tree, 2 Sep. 1936, D.H. Linder (DAOM 24916); Nuttallburg, Nov. 1893, on rotten wood, L.W. Nuttall (DAOM 34290). *Wisconsin*: Devil's Lake near Madison, 4 Sep. 1953, R.F. Cain (DAOM 41029).

***Neoacanthostigma thailandicum* Tanney & A.N. Mill.
sp. nov.**

MycoBank MB819966

Etymology: Named for the country where the type specimen was collected.

Diagnosis: *Neoacanthostigma thailandicum* is distinguished from all other species in the genus by macronematous conidiophores and larger, pale brown to brown, multi-septate conidia (to 920 μ m long).

Description: For a complete description of this taxon see Hyde et al. (2016: 125; *N. septoconstrictum* MFLU 16-1134).

Type: **Thailand**: Prachuap Khiri Khan, Bang Sapan, Ron Thai, on decaying wood in flowing freshwater stream, 30 Jul. 2015, K.D. Hyde KH02 (MFLU 16-1134 – holotype, n.v.; BBH 41051 – isotype, n.v.; MFLUCC 15-1248, TBRC – cultures ex-type).

Illustration: Hyde et al. (2016: fig. 76).

Notes: *Neoacanthostigma thailandicum* (MFLU 16-1134) was identified as *N. septoconstrictum* in Hyde et al. (2016).

DISCUSSION

Both ITS and LSU sequences confirm that the hyphomycete morph *Berkleasium concinnum* and the pyrenomycete morph *Neoacanthostigma septoconstrictum* are the same species and therefore must be given a single name. Thus, *N. septoconstrictum* is synonymized under *B. concinnum* because of the priority of *Berkleasium* over *Neoacanthostigma*. Re-examination of the *N. septoconstrictum* type specimen and additional *B. concinnum* reference specimens confirmed the frequent co-occurrence of both the asexual and sexual morphs on the same substrate, providing additional circumstantial support for this connection (Fig. 2 A–C).

Neoacanthostigma was proposed to accommodate a new species, *N. fusiforme*, which was chosen as the type species, along with *N. filiforme* and *N. septoconstrictum*, two species previously included in *Acanthostigma* but demonstrated to be phylogenetically distinct from the type species, *A. perpusillum* (Boonmee *et al.* 2014). Based on the ITS–LSU phylogeny presented here, the transfer of *Acanthostigma filiforme* to *Neoacanthostigma* appears unwarranted given its phylogenetic distance from *N. fusiforme* (Fig. 1). However, these two species were weakly clustered together based on the LSU phylogeny from Promputtha & Miller (2010) and the combined ITS and LSU phylogeny from Boonmee *et al.* (2014). The placement of *N. filiforme* was not resolved in this study because of low support. Hyde *et al.* (2016) reported a helicosporous-like asexual morph for *N. septoconstrictum* based on a specimen from decaying wood in a flowing freshwater stream in Thailand; however, the ITS sequence (KX454176) of this specimen (MFLUCC 15–1248) clearly distinguishes it from the *N. septoconstrictum* type specimen [identities = 465/537 (87 %), gaps = 42/537 (7 %)]. The ITS dissimilarity and distinctive asexual morph morphology from *B. concinnum* resulted in our description of this specimen as a novel species, *N. thailandicum*.

Tubeufiaceae (*Tubeufiales*) asexual morphs are morphologically diverse, containing helicosporous genera such as *Helicoma*, *Helicosporium*, *Helicoön*, and *Helicosporium*, and staurosporous genera including *Araneomyces* and *Tetracrium* (Réblová & Barr 2000, Kodsueb *et al.* 2004, Tsui & Berbee 2006). Reports of dictyosporous conidial morphs in *Tubeufiaceae* more reminiscent of *B. concinnum* include *Chlamydotubeufia* spp., *Manoharachariella tectonae*, *Tubeufia amazonensis*, and *T. khunkornensis* (Rossman & Müller 1979, Boonmee *et al.* 2011, 2014, Rajeshkumar & Sharma 2013, Doilom *et al.* 2016). The close phylogenetic relationship between *B. concinnum* and the helicosporous *N. fusiforme* and *N. thailandicum* is unexpected but supported by molecular evidence generated from independently collected and sequenced specimens and isolates. The phylogenetic placement and identification of a sexual morph for *B. concinnum* presented in this study demonstrates the significance of culturing and sequencing named-but-unsequenced dematiaceous hyphomycetes.

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