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Three new species of *Acanthostigma* (Tubeufiaceae, Dothideomycetes) from Great Smoky Mountains National Park

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Abstract: Three new bitunicate ascomycetes belonging to the genus *Acanthostigma* are described from terrestrial decomposing wood collected from Great Smoky Mountains National Park, USA. Phylogenetic analyses of the nuclear ribosomal 28S large subunit and internal transcribed spacer region placed all three species in the Tubeufiaceae and confirmed morphological analyses that these are distinct species. Expanded phylogenetic analyses of 28S large subunit including taxa throughout the Dothideomycetes confirmed the placement of *Acanthostigma* in the Tubeufiaceae. *Acanthostigma filiforme* differs from other *Acanthostigma* species in having longer ascospores with more septa. *Acanthostigma multiseptatum* can be distinguished in having longer asci and longer ascospores with more septa. *Acanthostigma septoconstrictum* differs in having longer setae and asci and broader, asymmetrical ascospores that are constricted at their septa. A dichotomous key to *Acanthostigma* species is provided.

Key words: Ascomycota, ITS, LSU, saprobe, southern Appalachians, systematics, *Tubeufia*

INTRODUCTION

The taxonomic status of *Acanthostigma* de Not. (de Notaris 1863) has had an obscure history mostly due to the perceived loss of the type specimen for its type species, *A. perpusillum* de Not. The recent rediscovery and re-examination of the type specimen of *A. perpusillum* (Réblova and Barr 2000) along with molecular analyses including the type species (Tsui et al. 2006, 2007) has clarified the circumscription of *Acanthostigma* and confirmed its taxonomic placement in the Tubeufiaceae. The taxonomy of *Acanthostigma* has been discussed adequately elsewhere (Müller 1965; von Arx and Müller 1975; Barr 1977, 1980, 1990; Rossman 1987; Erikkson and Hawksworth 1998; Réblova and Barr 2000; Kodsueb et al. 2006). *Acanthostigma* is characterized by having minute, dark, setose ascomata with thick-walled setae, cellular pseudoparaphyses, cylindrical to clavate, bitunicate asci, and cylindrical-fusiform to elongate-fusiform, hyaline, transversely multiseptate ascospores (Réblova and Barr 2000). Members of *Acanthostigma* usually occur as saprobes on terrestrial decomposing wood (Rossman 1987, Réblova and Barr 2000) but also have been found on submerged wood in freshwater (Kodsueb et al. 2006). Some species are associated with helicosporous hyphomycete anamorphs (Barr 1980, Réblova and Barr 2000, Kodsueb et al. 2004, Tsui et al. 2006).

During our ongoing study of fungal diversity in Great Smoky Mountains National Park (GSMNP) five species of *Acanthostigma* were found, three of which do not fit the description of any known species. These three newly discovered species are described, illustrated and compared morphologically and genetically to other known species in the genus. A key to nine accepted species in *Acanthostigma* is provided.

MATERIALS AND METHODS

Morphological characterization.—Eighty-six collections of Tubeufiaceae were made from 2004 to 2007, but morphological examination occurred mostly throughout 2008. Due to the large number of specimens collected during our inventory and because of the delayed morphological analyses cultures were not obtained from these specimens. Thus anamorphic data from cultures are not available for these species. Ascomata were squash-mounted in water and images of micromorphological structures were captured with a QImaging QColor 3 digital camera mounted on either a Leica MZ7.5 dissecting microscope with a Schott KL1500 fiber optics light source or an Olympus BX51 compound microscope with differential interference microscopy. Images were processed with Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View, California). A minimum of 30 measurements was taken for all morphological structures when possible with NIH Image 1.63 (National Institute of Health, Bethesda, Maryland). Means, which are shown in brackets, were calculated for asci and ascospores. Ascomata were sectioned at ca. 5 µm with a modified procedure of Huhndorf (1991). Taxa were compared with published species descriptions of *Acanthostigma* taxa listed on Index Fungorum (www.indexfungorum.org, 1 Feb 2009).

Molecular sequencing.—A DNeasy® Mini Plant Extraction Kit (QIAGEN Inc., Valencia, California) was used to extract DNA from dried ascomata following the manufacturer's protocols, except tissues were not ground in liquid nitrogen and final elution volume was 25 µL. Ascomata initially were

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rehydrated in 50 μL AP1 buffer 3–5 h, followed by freezing at −80 C for 3–14 d before DNA extraction. Due to their relatively small size, 30–50 ascomata were used in the extraction of DNA. The relative quantity of total genomic DNA was observed on a 1% TBE agarose gel stained with ethidium bromide. The entire internal transcribed spacer (ITS) region and the first 600 bp of the 5′ end of 28S large subunit (LSU) was PCR amplified on a Bio-Rad PTC 200 thermal cycler with PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Piscataway, New Jersey) and primers ITS1F/ITS5 and ITS4 or LROR and LR6 respectively (Vilgalys and Hester 1990, White et al. 1990, Gardes and Bruns 1993, Rehner and Samuels 1995). These reagents were added to each PCR reaction: 1–5 μL DNA, 1.0 μL each 10 μM primer and sufficient amount of sterile H_2O to bring the total volume to 25 μL. Occasionally 2.5 μL BSA (bovine serum albumin, New England Biolabs, Ipswich, Massachusetts) and/or 2.5 μL DMSO (dimethyl sulfoxide, Fisher Scientific, Pittsburgh, Pennsylvania) also were added to the PCR reaction. These thermo-cycling parameters were used: initial denaturation at 95 C for 5 min, followed by 35 or 40 cycles of 95 C for 30 s, 41 or 50 C for 15 s, and 72 C for 1 min with a final extension step of 72 C for 10 min. After verification on an ethidium bromide-stained 1% TBE agarose gel, PCR products were purified with either a QIAquick PCR purification kit (QIAGEN Inc.) or ExoSAP-IT® (Affymetrix, Cleveland, Ohio) following the manufacturer's recommendations. A BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, California) was used to sequence both strands with a combination of these primers: ITS = ITS1F, ITS5, ITS1, ITS4; LSU = LROR, LRFF1, LRAM1, LR3, LR3R, LR5, LR6 (Vilgalys and Hester 1990, White et al. 1990, Gardes and Bruns 1993, Rehner and Samuels 1995, Huhndorf et al. 2004). Sequences were generated on an Applied Biosystems 3730XL high-throughput capillary sequencer. Each sequence fragment was subjected to an individual BLAST analysis to verify its identity. Sequences were assembled with Sequencher 4.7 (Gene Codes Corp., Ann Arbor, Michigan).

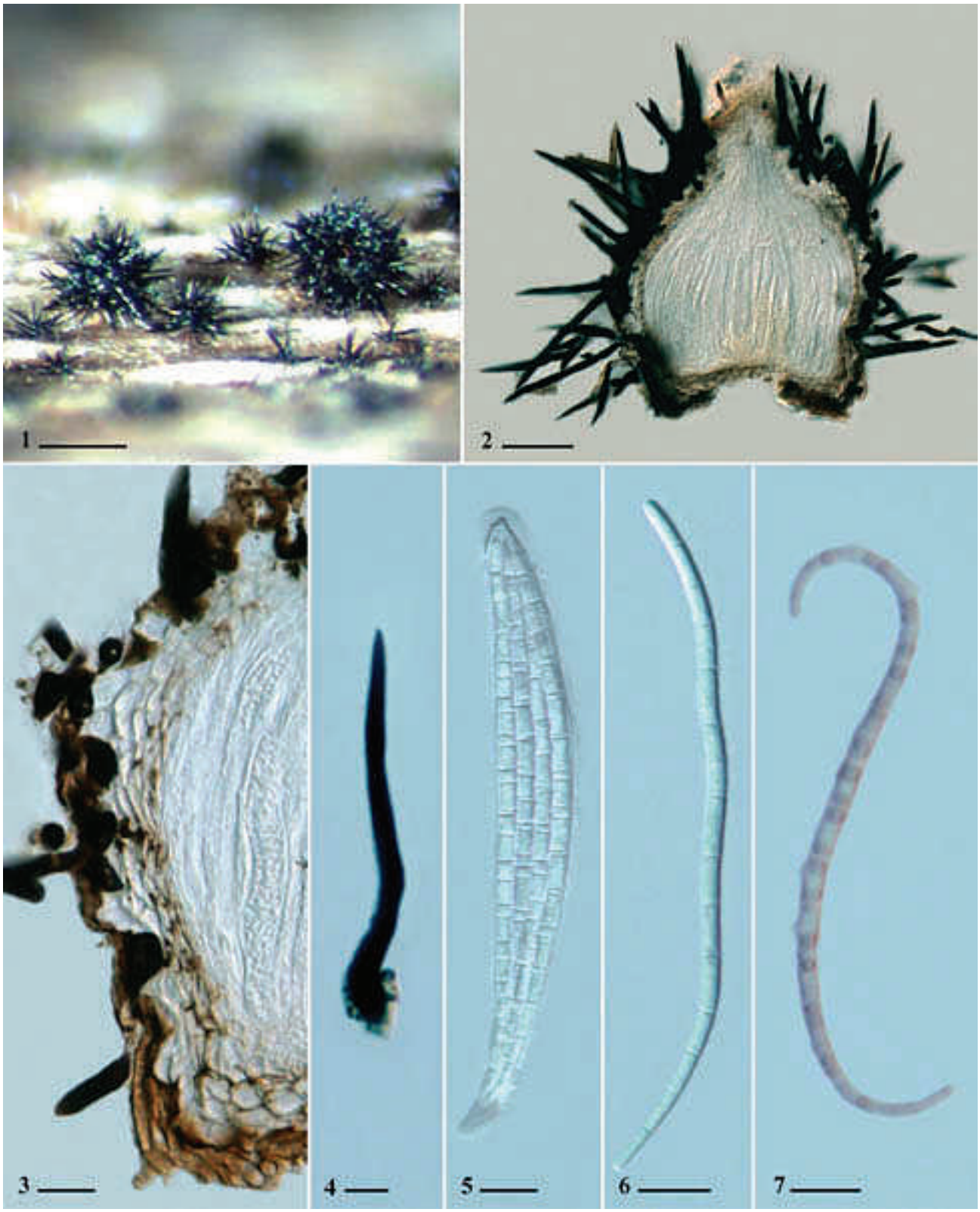
Phylogenetic analyses.—Three datasets were assembled and analyzed: (i) an expanded LSU dataset consisting of nearly all Dothideomycetes taxa included in Schoch et al. (2006) along with representatives of *Acanthostigma*; (ii) a limited LSU dataset consisting of previously published anamorphic and teleomorphic taxa in the Tubeufiaceae along with the newly sequenced taxa generated during this study and (iii) an ITS dataset with the same taxon sampling strategy used in the limited LSU dataset. The LSU datasets were aligned in Sequencher 4.7 and manually corrected by eye when necessary. The ITS dataset was aligned with Clustal X 2 (Larkin et al. 2007), optimized by eye and manually corrected with BioEdit 7.0.9 (Hall 1999). GenBank accession numbers are given after taxon names in the phylogenetic trees.

Maximum parsimony (MP) analyses were performed on all three datasets, while maximum likelihood (ML) analyses were performed on the limited LSU and ITS datasets with PAUP* 4.0b10 (Swofford 2002). Approximately 20–70 bp of the 5′ and 3′ ends of each of the three datasets were excluded from all analyses due to missing data in most taxa. Thirteen, four and nine ambiguously aligned regions were delimited respectively in the expanded LSU, limited LSU and ITS datasets. A portion of the phylogenetic signal was recovered from all four regions in the limited LSU dataset and three of the nine regions in the ITS dataset by recoding them with the program INAASE (Lutzoni et al. 2000). The remaining six ambiguously aligned regions in the ITS dataset and all 13 regions in the expanded LSU could not be recoded due to their size so they were excluded from all analyses. The remaining unambiguously aligned characters in each dataset were subjected to a symmetrical step-matrix to differentially weight nucleotide transformations with STMatrix 2.2 (François Lutzoni and Stefan Zoller, Duke University), which calculates the costs for changes among character states based on the negative natural logarithm of the percentages of reciprocal changes between any two character states. Unequally weighted MP analyses were performed with 1000 stepwise random addition heuristic searches, TBR branch-swapping, MULTREES option in effect, zero-length branches collapsed, constant characters excluded and gaps treated as missing. Branch support was estimated by performing 100 bootstrap replicates (Felsenstein 1985) each consisting of 10 stepwise random addition heuristic searches as above. Modeltest 3.7 (Posada and Crandall 1998) was used to determine the best-fit model of evolution. The model of evolution selected by Modeltest for the LSU datasets was the GTR model (Rodríguez et al. 1990), while the model selected for the ITS dataset was the TrN model (Tamura and Nei 1993). Both models included a proportion of invariable sites with the remaining sites subjected to a gamma distribution shape parameter. ML analyses were performed on the limited LSU and ITS datasets with the above models with 100 stepwise random addition replicates and TBR branch-swapping with a reconnection limit of 12. Constant characters were included and ambiguously aligned characters were excluded from the ML analyses. Bayesian analyses were performed on all datasets with MrBayes 3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) as an additional means of assessing branch support. Constant characters were included, the above models of evolution were implemented and 50 000 000 or 100 000 000 generations were sampled every 1000th generation resulting respectively in 50 000 or 100 000 total trees for the ITS and limited LSU datasets and expanded LSU dataset. The Markov chain always achieved stationarity after the first 100 000 generations, so the first 10 000 trees, which extended well beyond the burn-in phase of each analysis, were discarded. Posterior probabilities were determined from a 95% consensus tree generated with the remaining 40 000 or 90 000 trees. This analysis was repeated twice for all datasets starting from different random trees to ensure trees from the same tree space were ultimately being sampled during each analysis.

TAXONOMY

Acanthostigma filiforme Promputtha & A.N. Mill. sp. nov. FIGS. 1–7

TYPE. UNITED STATES. TENNESSEE: Blount County, Great Smoky Mountains National Park,



FIGS. 1–7. *Acanthostigma filiforme* (all from Holotype). 1. Ascomata on substrate. 2. Longitudinal section through ascoma. 3. Longitudinal section through ascomatal wall. 4. Seta. 5. Ascus. 6–7. Ascospores. Bars: 1 = 200 μm; 2 = 50 μm; 3 = 30 μm; 4–7= 10 μm.

vicinity of Townsend, Cades Cove, Willie Myers, 35°33′43.31″N, 83°50′48.97″W, 602 m, decorticated branch on ground, 5 cm diam, 14 Jul 2004, *A.N. Miller, S.M. Huhndorf, G.K. Mugambi, ANM101* (HOLOTYPE designated here, ILLS 59352).

Etymology. referring to the thread-like ascospores.

Ascomata superficialia, sparsa vel gregaria, globosa ad subglobosa, 150–300 μm diam, 150–300 μm alta, nigra, setosa, non collabens. Seta brunea ad atrobrunea, acutata ad apicem, (45–)70–130 μm longa, 5–7 μm lata. Asci bitunicati, cylindrici ad cylindro-clavati, octaspori, 100–115 × 11.5–13 μm. Ascosporae longae fusiformes-filiformes vel longae cylindro-filiformes, symmetricae, 12–16 septatae, non-constrictae, hyalinae, (75–)85–135(–150) × 2.5–4 μm.

Ascomata superficial, scattered or gregarious, globose to subglobose, 150–300 μm diam, 150–300 μm high, shiny black when dry, densely setose, non-collapsing. *Setae* one-celled or rarely one-septate, thick-walled, brown to dark brown, straight to slightly curved, with acute tip, (45–)70–130 μm long, 5–7 μm wide in the middle. *Ascomatal wall* two-layered in longitudinal section, inner layer composed of 3–5 rows of light brown, thin-walled, flattened to angular cells; outer layer composed of 1–2 rows of dark brown, angular to elongated cells which sometimes produce setae. *Pseudoparaphyses* numerous among asci, branched, anastomosing, hyaline, septate, up to 2 μm wide. *Asci* bitunicate, cylindrical to cylindrical-clavate, broadly rounded and thickened at the apex, short-stipitate, eight-spored, 100–115 × 11.5–13 μm [109 × 12.5 μm, n = 10]. *Ascospores* long fusiform-filiform to cylindrical-filiform, straight or curved or sometimes sigmoidal, tapering and narrowly rounded at both ends, ends symmetrical, 12–16-septate, not constricted at the septa, hyaline, (75–)85–135(–150) × 2.5–4 μm [105 × 3.5 μm, n = 190].

Habitat. Scattered to gregarious on decorticated wood in mixed coniferous-deciduous forest.

Specimens examined: USA. NORTH CAROLINA: Haywood County, Great Smoky Mountains National Park, Big Creek, Big Creek Trail and behind campgrounds, 35°45′2.9″N, 83°6′34.8″W, 533 m, on branch on ground, 5 cm diam, 9 Sep 2005, *A.N. Miller, S.M. Huhndorf, G.K. Mugambi, H.A. Raja, ANM702*; Swain County, Great Smoky Mountains National Park, Smokemont, Bradley Fork Trail, 35°33′46.4″N, 83°18′38.3″W, 686 m, on log on ground, 13 cm diam, 6 Oct 2004, *A.N. Miller, D.M. Ketzner, ANM205*; TENNESSEE: Blount County, Great Smoky Mountains National Park, vicinity of Townsend, Cades Cove, Willie Myers, 35°33′43.31″N, 83°50′48.97″W, 602 m, on branch on ground, 3 cm diam, 14 Jul 2004, *A.N. Miller, S.M. Huhndorf, G.K. Mugambi, ANM102*; 5 miles east of Cades Cove, Tremont, West Prong Trail, 35°38′27.4″N, 83°41′28.6″W, 427 m, on branch on ground, 2 cm diam, 12 Oct 2006, *A.N. Miller, E.B. Lickey, P. Chaudhary, ANM1021*; on branch on ground, 2.5 cm diam, 18 Jul 2007, *T.J. Atkinson, P. Chaudhary, ANM1328*; on wood on ground, 6 Nov 2007, *A.N. Miller, S.M. Huhndorf, J.L. Crane, T.J. Atkinson, I. Promputtha, M. Grief, G.K. Mugambi, P. Chaudhary, ANM1533*; Cocke County, Great Smoky Mountains National Park, Cosby, Gabes Mountain Trail, 35°45′25.6″N, 83°12′32.9″W, 686 m, on wood fragment on ground, 17 Sep 2005, *A.N. Miller, L. Vasilyeva, ANM746*; Low Gap Trail, 35°45′14.1″N, 83°12′25.7″W, 716 m, on branch on ground, 7 cm diam, 15 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM541*; Lower Mount Cammerer Trail, 35°45′25.6″N, 83°12′32.9″W, 686 m, on branch on ground, 3 cm diam, 17 Sep 2005, *A.N. Miller, L. Vasilyeva, ANM756*; Maddron Bald Trail, 35°46′9.6″N, 83°16′.5″W, 579 m, on branch on ground, 3 cm diam, 21 Sep 2005, *A.N. Miller, L. Vasilyeva, ANM784*; Sevier County, Great Smoky Mountains National Park, 5 miles east of Gatlinburg, Greenbrier, Old Settlers Trail, 35°42′27.4″N, 83°22′49.5″W, 457 m, on branch on ground, 3.5 cm diam, 7 Nov 2007, *A.N. Miller, S.M. Huhndorf, J.L. Crane, T.J. Atkinson, I. Promputtha, M. Grief, G.K. Mugambi, P. Chaudhary, ANM1546*; Porters Creek Trail, 35°41′49.7″N, 83°23′18.5″W, 518 m, on wood fragment on ground, 10 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM447*; on branch on ground, 3 cm diam, 10 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM456*; alternative side trail to Whaley Cemetery, 35°42′ 27.1″N, 83°22′55.8″W, 549 m, on branch on ground, 3 cm diam, 10 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM471*; near Gatlinburg, Grotto Falls, Trillium Gap Trail, 35°40′51.6″N, 83°27′45.1″W, 945 m, on wood fragment on ground, 12 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM514* (PARATYPE designated here, ILLS 59353); Rainbow Falls Trail, 35°40′32.6″N, 83°29′8.4″W, 792 m, on hemlock branch on ground, 3 cm diam, 15 May 2006, *A.N. Miller, G.K. Mugambi, L. Vasilyeva, ANM864*; Sugarlands Visitor Center, Old Sugarlands Trail, 35°41′16.8″N, 83°32′7.9″W, 442 m, on branch on ground, 4 cm diam, 13 Sep 2005, *A.N. Miller, L. Vasilyeva, ANM733*; Twin Creeks, Twin Creeks Nature Trail, near ATBI plot, 35°41′17″N, 83°29′59.8″W, 549 m, on branch on ground, 2 cm diam, 11 Oct 2006, *A.N. Miller, P. Chaudhary, ANM1042.*

Acanthostigma multiseptatum Promputtha & A.N. Mill. sp. nov. FIGS. 8–14

TYPE. UNITED STATES. TENNESSEE: Sevier County, Great Smoky Mountains National Park, Greenbrier, alternative side trail to Whaley Cemetery, 35°42′27.1″N, 83°22′55.8″W, 549 m, decorticated branch on ground, 2 cm diam, 10 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM475* (HOLOTYPE designated here, ILLS 59354).

Etymology. referring to the ascospores having many septa.

Ascomata superficialia, sparsa vel gregaria, globosa ad subglobosa, 130–380 μm diam, 130–380 μm alta, nigra, setosa, non collabens. Seta brunea ad atrobrunea, acutata ad apicem, 45–85(–100) μm longa, 4–6.5 μm lata. Asci bitunicati, cylindri-clavati vel clavati, octaspori, (90–)

FIGS. 8–14. *Acanthostigma multiseptatum* (all from Holotype). 8–9. Ascomata on substrate. 10. Longitudinal section of ascoma. 11. Longitudinal section through ascomatal wall. 12. Seta. 13. Asci. 14. Ascospore. Bars: 8–9 = 150 μm; 10 = 40 μm; 11, 13 = 20 μm; 12, 14 = 10 μm.

105–145 × 20–25 μm. Ascosporae fusiformes, symmetricae, 14–18 septatae, non-constrictae, hyalinae, 60–90 × 5–7.5 μm.

Ascomata superficial, scattered or gregarious, globose to subglobose, 130–380 μm diam, 130–380 μm high, shiny black when dry, densely setose, non-collapsing. *Setae* one-celled or septate, thick-walled, brown to dark brown, straight to slightly curved, with acute tip, 45–85(–100) μm long, 4–6.5 μm wide in the middle. *Ascomatal wall* single-layered in longitudinal section, composed of 3–5 rows of brown, thick-walled, polygonal to angular or flattened cells. *Pseudoparaphyses* numerous among asci, branched, hyaline. *Asci* bitunicate, cylindrical-clavate to clavate, broadly rounded and thickened at the apex, short-stipitate, eight-spored, (90–)105–145 × 20–25 μm [120 × 22.5 μm, n = 20]. *Ascospores* fusiform, straight or slightly curved, tapering and narrowly rounded at both ends, ends symmetrical, 14–18-septate, not constricted at the septa, hyaline, 60–90 × 5–7.5 μm [72 × 6.5 μm, n = 146].

Habitat. Scattered to gregarious on decorticated wood in mixed coniferous-deciduous forest.

Specimens examined: USA. NORTH CAROLINA: Haywood County, Great Smoky Mountains National Park, Big Creek, Baxter Creek Trail, 35°45′7″N, 83°6′37″W, 518 m, on branch on ground, 3 cm diam, 7 Mar 2006, *A.N. Miller, A. Kruys, ANM809*; on branch on ground, 3 Nov 2007, *A.N. Miller, S.M. Huhndorf, J.L. Crane, T.J. Atkinson, I. Promputtha, M. Grief, G.K. Mugambi, P. Chaudhary, ANM1400*; around parking lot and Baxter Creek Trail, 35°44′54.5″N, 83°6′42.8″W, on branch on ground, 3 cm diam, 19 Mar 2007, *A.N. Miller, P. Chaudhary, H.A. Raja, ANM1064*; TENNESSEE: Blount County, Great Smoky Mountains National Park, 5 miles east of Cades Cove, Dorsey Trail, 35°38′27.4″N, 83°41′28.6″W, 213 to 610 m, on branch on ground, 4 cm diam, 3 Oct 2004, *A.N. Miller, D.M. Ketzner, ANM157*; Cades Cove, Willie Meyer, 35°33′44.5″N, 83°50′47.2″W, 594 m, on branch near stream, 3 cm diam, 21 Jun 2005, *A.N. Miller, H.A. Raja, V.R. Hustad, E.B. Lickey, ANM401*; Cocke County, Great Smoky Mountains National Park, Cosby, Lower Mount Cammerer Trail, 35°45′25.6″N, 83°12′32.9″W, 686 m, on branch on ground, 2 cm diam, 17 Sep 2005, *A.N. Miller, L. Vasilyeva, ANM751*; near Cosby, Cosby Nature Trail, 35°45′13.8″N, 83°12′25.8″W, 716 m, on branch in stream, 1.5 cm diam, 19 Jun 2007, *A.N. Miller, T.J. Atkinson, I. Promputtha, P. Chaudhary, ANM1155*; on branch on ground, 1 cm diam, 19 Jun 2007, *A.N. Miller, T.J. Atkinson, I. Promputtha, P. Chaudhary, ANM1239*; on wood fragment on ground near stream, 19 Jul 2007, *T.J. Atkinson, P. Chaudhary, ANM1251*; Low Gap Trail and Cosby Nature Trail, on branch on ground, 2 cm diam, 3 Nov 2007, *A.N. Miller, S.M. Huhndorf, J.L. Crane, T.J. Atkinson, I. Promputtha, M. Grief, G.K. Mugambi, I. Promputtha, ANM1405*; Marion County, Tennessee River Gorge Trust, 20 miles W Chattanooga, off US 41, by small animal rehabilitation facility, 35°1′33.3″N, 85°26′6.4″W, 213 m, on branch on ground, 1 cm diam, 6 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM421*; Sevier County, Great Smoky Mountains National Park, off US 441, Alum Cave Trail, 35°37′43.3″N, 83°27′3.2″W, 1189 m, on branch on ground, 1 cm diam, 8 Sep 2005, *A.N. Miller, S.M. Huhndorf, G.K. Mugambi, H.A. Raja, ANM665* (PARATYPE designated here, ILLS 59355); Roaring Fork Motor Nature Trail, Rainbow Falls Trail, 35°40′32.6″N, 83°29′8.4″W, 792 m, branch on ground, 3 cm diam, 20 Jun 2007, *A.N. Miller, E.B. Lickey, T.J. Atkinson, I. Promputtha, P. Chaudhary, ANM1184.*

Acanthostigma septoconstrictum Promputtha & A.N. Mill. sp. nov. FIGS. 15–22

TYPE. UNITED STATES. TENNESSEE: Cocke County, Great Smoky Mountains National Park, Cosby, Low Gap Trail, 35°45′14.1″N, 83°12′25.7″W, 716 m, decorticated wood on ground, 15 Jul 2005, *A.N. Miller, A.M. Stchigel, ANM536.1* (HOLOTYPE designated here, ILLS 59356).

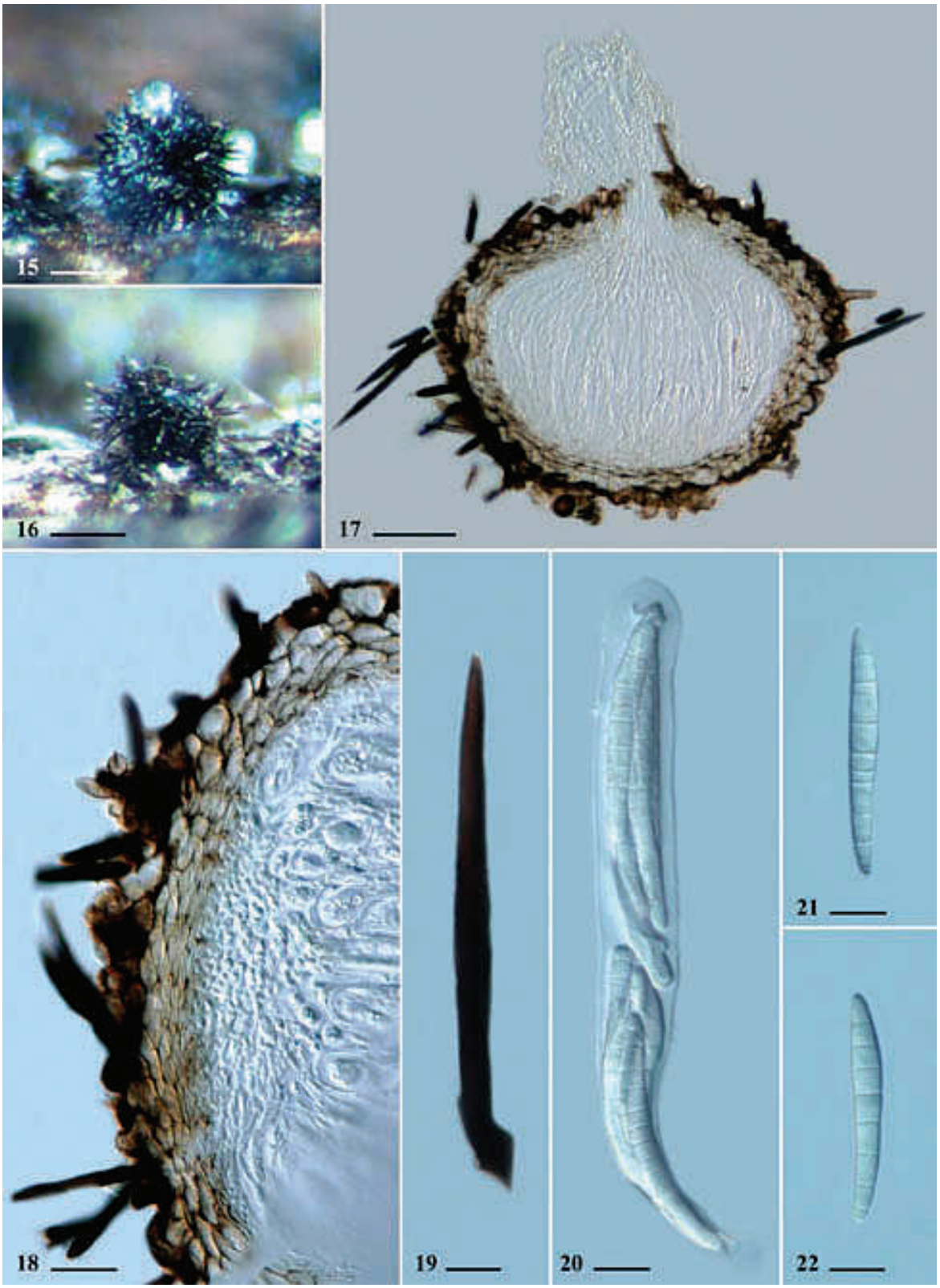
Etymology. refers to the ascospores having constricted septa.

Ascomata superficialia, sparsa vel gregaria, globosa ad subglobosa, 200–250 μm diam, 150–200 μm alta, nigra, setosa, non collabens. Seta brunea ad atrobrunea, acutata ad apicem, 45–120 μm longa, 5–7 μm lata. Asci bitunicati, cylindri-clavati vel clavati, octaspori, 110–140 × 17–22 μm. Ascosporae fusiformes, non-symmetricae, 7–10 septatae, constrictae, hyalinae, 40–50 × 5–6.5 μm.

Ascomata superficial, scattered or gregarious, globose to subglobose, 200–250 μm diam, 150–200 μm high, shiny black when dry, densely setose, non-collapsing. *Setae* one-celled, thick-walled, brown to dark brown, straight to slightly curved, with acute tip, 45–120 μm long, 5–7 μm wide in the middle. *Ascomatal wall* two-layered in longitudinal section, inner layer composed of 4–6 rows of brown, thin-walled, flattened to angular cells; outer layer composed of 1–2 rows of dark brown, angular to elongated cells which sometimes produce setae. *Pseudoparaphyses* numerous among asci, hyaline, up to 4.5 μm wide. *Asci* bitunicate, cylindrical-clavate to clavate, broadly rounded and thickened at the apex, short stipitate, eight-spored, 110–140 × 17–22 μm [126 × 20 μm, n = 10]. *Ascospores* fusiform, straight or curved, ends asymmetrical, tapering at both ends with basal end more narrow than apical end, 2–3 inner cells near the apical end slightly broader than the others, 7–10-septate, constricted at the septa, hyaline, 40–50 × 5–6.5 μm [45.5 × 5.5 μm, n = 20].

Habitat. Scattered to gregarious on decorticated wood in mixed coniferous-deciduous forest.

Specimens examined: known only from type specimen.



FIGS. 15–22. *Acanthostigma septoconstrictum* (all from Holotype). 15–16. Ascomata on substrate. 17. Longitudinal section of ascoma. 18. Longitudinal section through ascomatal wall. 19. Seta. 20. Ascus. 21–22. Ascospores. Bars: 15–16 = 150 μm, 17 = 50 μm, 18 = 20 μm, 19–22 = 10 μm.

KEY TO SPECIES OF *ACANTHOSTIGMA*[2]

1.1 Ascospores less than 5 µm wide 2
1.2 Ascospores ≥5 µm wide 6
2.1 Ascospores up to 4 µm wide 3
2.2 Ascospores 4–5 µm wide 5
3.1 Ascospores 22–27 × 3–3.5 µm, 5(–9)-septate . . . . . *A. revocatum*
3.2 Ascospores more than 30 µm long 4
4.1 Ascospores (40–)56–78(–95) × (2–)2.5–3(–3.5) µm, 10–14-septate . . . . . *A. scopulum*
4.2 Ascospores (75–)85–135(–150) × 2.5–4 µm, 12–16-septate . . . . . *A. filiforme*
5.1 Ascospores (29–)38–51 × 4–4.5(–5), (4–)6–8-septate . . . . . *A. longisporum*
5.2 Ascospores (38–)48–64.5 × 4–4.5 µm, 6–12-septate . . . . . *A. ellisii*
6.1 Ascospores not or slightly constricted at the septa . 7
6.2 Ascospores constricted at the septa, 40–50 × 5–6.5 µm, 7–10-septate . . . . . *A. septoconstrictum*
7.1 Ascospores 30.5–35.5(–42) × 5–6 µm, (5–)6–7(–8)-septate . . . . . *A. perpusillum*
7.2 Ascospores longer, ≥10-septate 8
8.1 Ascospores 40–55(–63) × (5–)6–7(–7.5) µm, 10–14-septate . . . . . *A. minutum*
8.2 Ascospores 60–90 × 5–7.5 µm, 14–18-septate . . . . . *A. multiseptatum*

RESULTS

The expanded LSU dataset consisted of 114 taxa and 3008 characters of which 2496 were excluded. Thirteen ambiguously aligned regions or introns were delimited and excluded from all analyses resulting in 383 parsimony informative characters. A single most parsimonious tree was generated in the MP analysis (FIG. 23). All major taxonomic clades presented in the multigene phylogeny of Schoch et al. (2006) also were recovered in these LSU analyses. All species of *Acanthostigma* occurred in the well supported Tubeufiaceae clade including the correct LSU sequence of the type species, *A. perpusillum* (AY856892, culture = UAMH7237), generated by Tsui et al. (2006). This sequence should not be confused with the incorrectly identified LSU sequence in GenBank purported to be *A. perpusillum* (DQ296556, culture = MUCL41721) generated by Kodsueb et al. (2006), which is actually *Capronia parasitica* (Ellis & Everh.) E. Müll., Petrini, P.J. Fisher, Samuels & Rossman (Tsui et al. 2007). This incorrectly identified sequence was included in these analyses where it grouped with *Capronia parasitica*, which was expected because there are no base pair differences between these two sequences (FIG. 23). Both the GenBank sequence record and the MUCL culture record, the latter of which is currently referenced online as *Tubeufia clintonii* (Peck) M.E. Barr, a synonym of *A. perpusillum* (Réblova and Barr 2000), should be updated to reflect accurate taxonomy and avoid further confusion.

The limited LSU dataset consisted of 75 taxa and 643 characters of which 499 were excluded. Four ambiguously aligned regions were delimited and recoded, resulting in 115 parsimony informative characters. The MP analysis generated 33 most parsimonious trees, which differed only slightly in topology due to equally parsimonious rearrangements among taxa with little or no bp differences (e.g. within *Tubeufia paludosa* clade). (One of these most parsimonious trees is shown in FIG. 24.) The ML analysis generated three most likely trees, which did not differ significantly from one another or from the most parsimonious trees (data not shown). The ITS dataset consisted of 56 taxa and 698 characters of which 582 were excluded. Three of the nine ambiguously aligned regions were recoded, resulting in 87 parsimony informative characters. A MP analysis generated 111 most parsimonious trees. The large number of most parsimonious trees was due primarily to equally parsimonious rearrangements among taxa with little or no bp differences (e.g. within *Helicosporium griseum* clade). (One of these most parsimonious trees is shown in FIG. 25.) The ML analysis generated a single most likely tree, which did not differ significantly from the most parsimonious trees (data not shown). All *Acanthostigma* species occurred in the Tubeufiaceae but were scattered throughout the family. In the LSU analyses *A. filiforme* clustered with *A. septoconstrictum* and *Tubeufia amazonensis* in an unsupported clade while *A. multiseptatum* clustered with *Helicosporium gracile* in an unsupported clade. In the ITS analyses *Acanthostigma filiforme* and *A. multiseptatum* occurred on lone branches as separate lineages while *A. septoconstrictum* clustered with *Helicosporium aureum*.

DISCUSSION

The taxonomic placement of *Acanthostigma* has been disputed for many years due to the uncertainly surrounding its type species, the emphasis placed on different morphological characters and the lack of molecular studies that included species in the genus. The genus has been transferred in and out of the Tubeufiaceae numerous times by various authors (TABLE I in Kodsueb et al. 2006). Recent phylogenetic studies including only the type species showed that *Acanthostigma* belongs in the Tubeufiaceae (Tsui et al. 2006). Our analyses included five species of

[2]An online interactive key to several species in the Tubeufiaceae also is available at http://www.discoverlife.org/mp/20q?guide=Tubeufiaceae

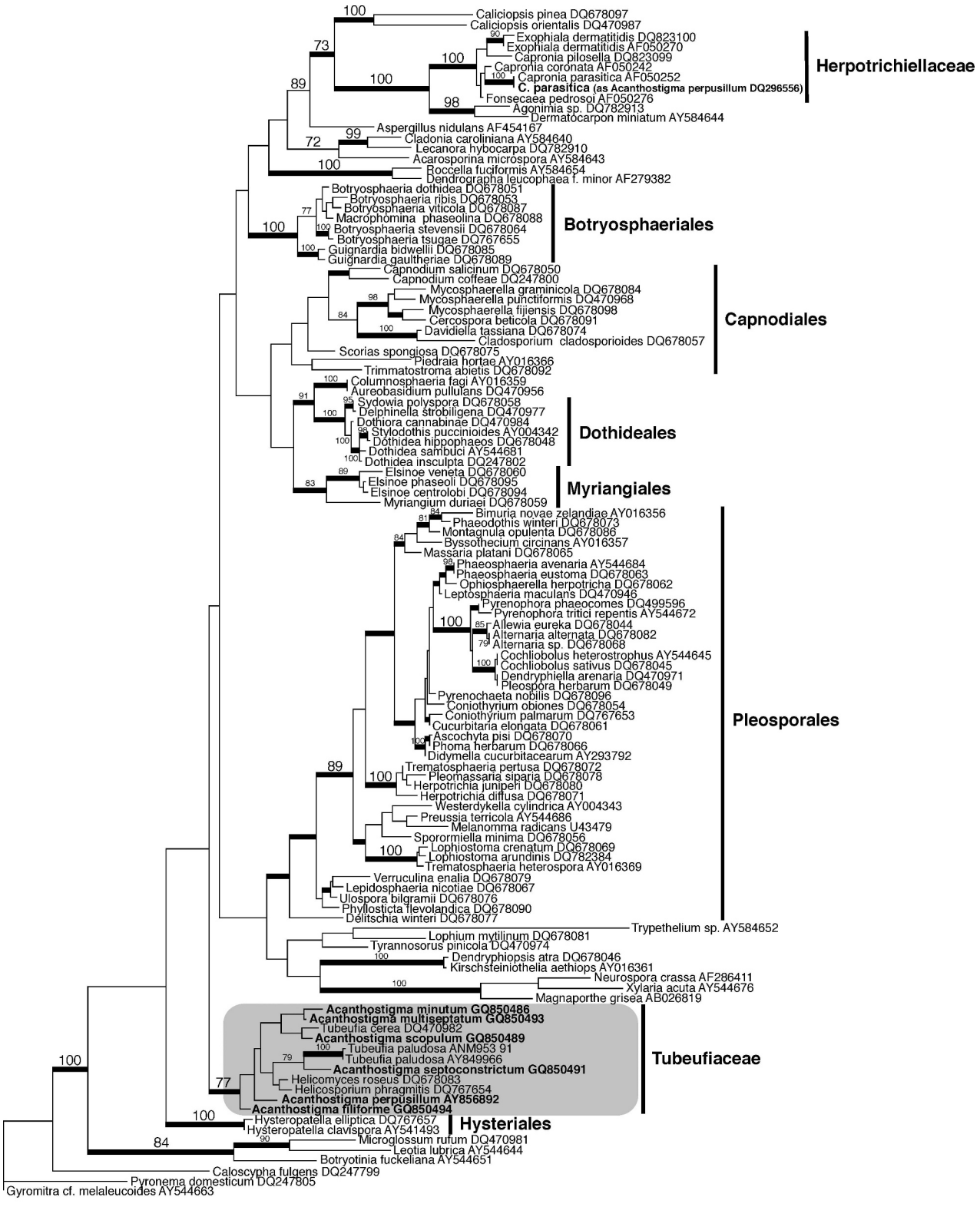


FIG. 23. Dothideomycetes phylogeny of the single most parsimonious tree generated from a MP analysis of LSU sequence data for 114 taxa showing the phylogenetic placement of six *Acanthostigma* species (in boldface) in the Tubeufiaceae (in shaded box) and one misidentified sequence (in boldface) that is actually *Capronia parasitica* (L = 4267.88 steps, CI = 0.320, RI = 0.715, RCI = 0.229). Thickened branches indicate Bayesian posterior probabilities ≥95%, while numbers above or below branches refer to MP bootstrap values ≥70%. Three species in the Pezizomycetes are outgroups.

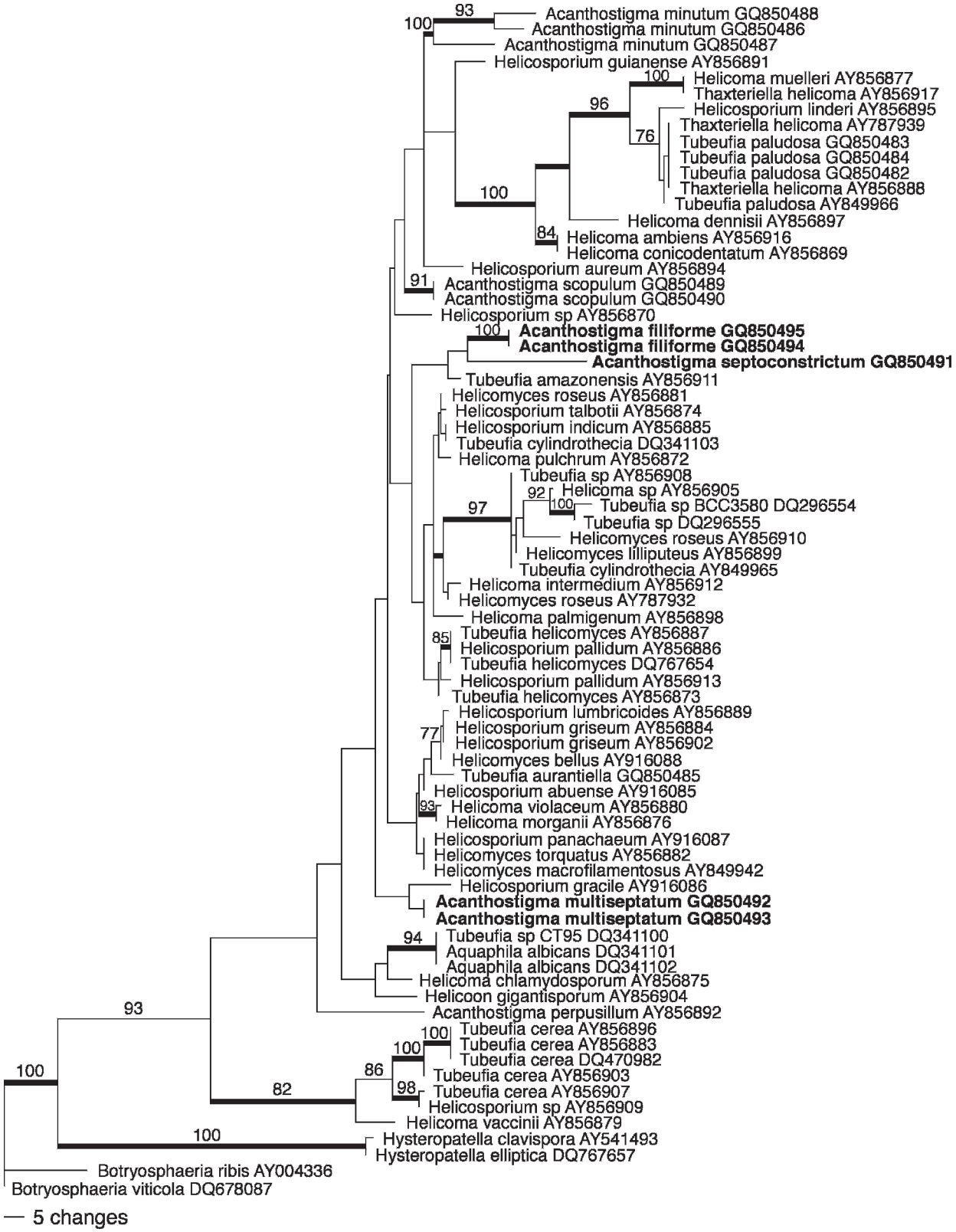


FIG. 24. Tubeufiaceae phylogeny of one of 33 most parsimonious trees generated from a MP analysis of LSU sequence data for 75 taxa showing the phylogenetic placement of six *Acanthostigma* species within the family (L = 590.91 steps, CI = 0.550, RI = 0.840, RCI = 0.462). The three newly described species are in boldface. Thickened branches indicate Bayesian posterior probabilities ≥95%, while numbers above or below branches refer to MP bootstrap values ≥70%. Two species of *Hysteropatella* and two species of *Botryosphaeria* are outgroups.

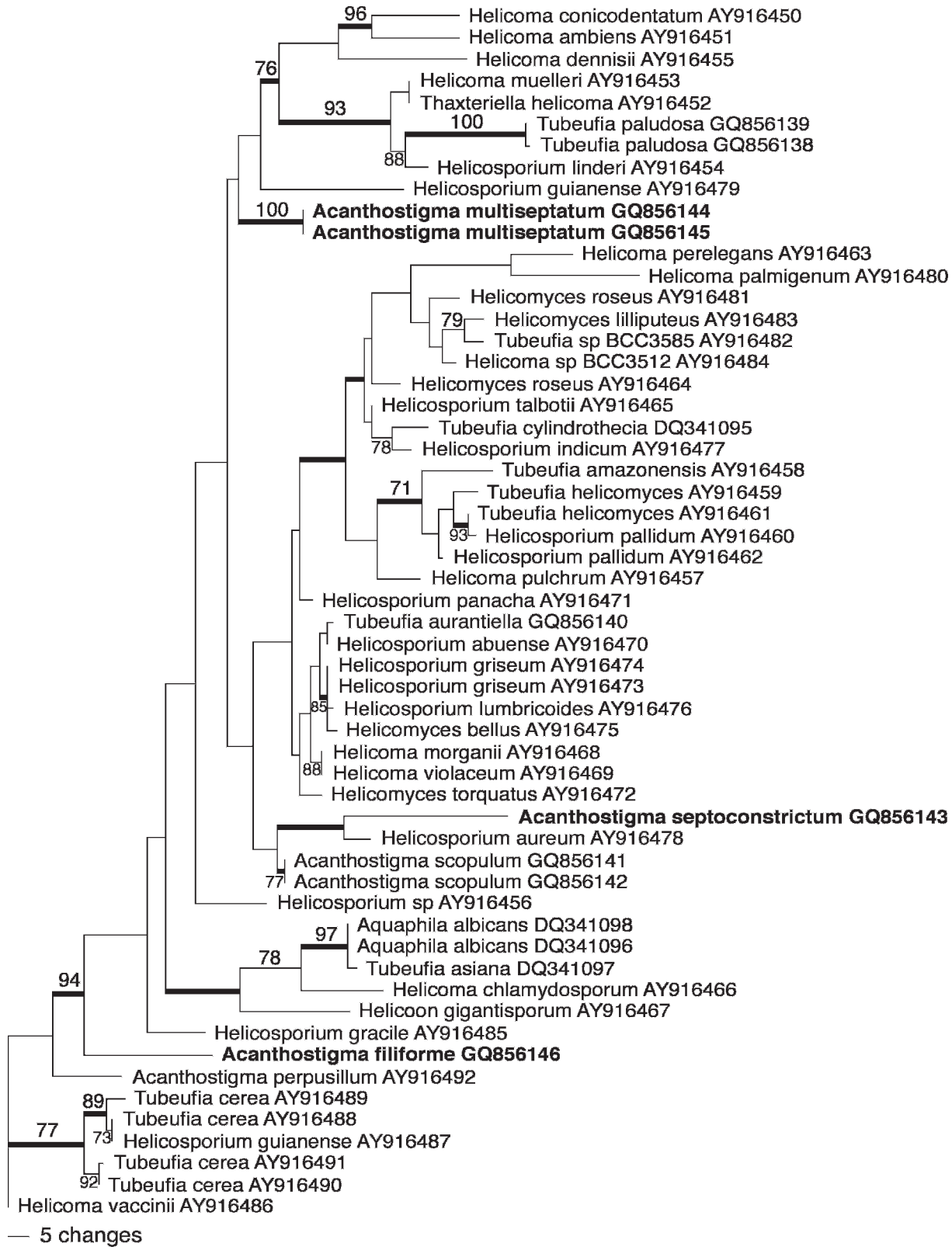


FIG. 25. Tubeufiaceae phylogeny of one of 111 most parsimonious trees generated from a MP analysis of ITS sequence data for 56 taxa showing the phylogenetic placement of five *Acanthostigma* species within the family (L = 705.76 steps, CI = 0.456, RI = 0.738, RCI = 0.337). The three newly described species are in boldface. Thickened branches indicate Bayesian posterior probabilities ≥95%, while numbers above or below branches refer to MP bootstrap values ≥70%. *Helicoma vaccinii* is used as outgroup.

TABLE I. Comparison of *Acanthostigma filiforme*, *A. multiseptatum* and *A. septoconstrictum* with six other known species in the genus (Réblova and Barr 2000)

Taxa	Ascomata diam × height (μm)	Setae length × width (μm)	Asci length × width (μm)	Ascospore ends	Ascospore shape	Ascospore septa	Ascospore size length × width (μm)
A. ellisii	non-collapsing 150–230 × 160–210	acute tip 20–85 × 4–4.5	Clavate (63–)84–90 × 14–15	asymmetric	cylindrical-fusiform to long fusiform, rounded at apical end, tapering at basal end, 1–2 middle cells near apical end slightly broader than the others	6–12-septate, not constricted at septa	(38–)48–64.5 × 4–4.5
A. filiforme	non-collapsing 150–300 × 150–300	acute tip (45–)70–130 × 5–7	cylindrical to cylindrical-clavate 100–115 × 11.5–13	symmetric	long fusiform-filiform to cylindrical-filiform, tapering and narrowly rounded at both ends	12–16-septate, not constricted at septa	(75–)85–135 (–150) × 2.5–4
A. longisporum	non-collapsing 150–260 × 160–250	obtuse tip 30–90 × 3.5–4	cylindrical-clavate 73–85(–94) × 10–15	asymmetric	long fusiform, broadly rounded at apical end, tapering and narrowly rounded and often curved at basal end	(4–)6–8-septate, not or slightly constricted at septa	(29–)38–51 × 4–4.5(–5)
A. minutum	non-collapsing 120–230 × 150–250	acute tip 28–100 × 4.5–5	cylindrical-clavate 75–95(–126) × 18–23	symmetric	long fusiform, tapering at both ends, narrowly rounded, one of middle cells slightly broader	10–14-septate, not or slightly constricted at septa	40–55(–63) × (5–) 6–7(–7.5)
A. perpusillum	collapsing 150–155 × 100–110	acute tip (10–)28–97 × 5–6	clavate 77–79 × 14–16	symmetric	fusiform, narrowly rounded at both ends, one of middle cells often broader than the others	(5–)6–7(–8)-septate, not or slightly constricted at septa	30.5–35.5(–42) × 5–6
A. multiseptatum	non-collapsing 130–380 × 130–380	acute tip 45–85(–100) × 4–6.5	cylindrical-clavate to clavate (90–)105–145 × 20–25	symmetric	fusiform, tapering and narrowly rounded at both ends	14–18-septate, not constricted at septa	60–90 × 5–7.5
A. revocatum	non-collapsing 120–150 × 120–140	acute tip 15–20 × 2.5–3.5	cylindrical-clavate 40–50 × 13–15	symmetric	cylindrical to fusiform, broadly rounded at both ends	5(–9)-septate, not constricted at septa	22–27 × 3–3.5
A. scopulum	non-collapsing 165–310 × 180–300	acute tip 37–90 × 4.5–5	cylindrical-clavate (67–)86–110(–130) × 12–15(–22)	symmetric	long fusiform to cylindrical-fusiform, tapering and narrowly rounded at both ends	10–14-septate, not constricted at septa	(40–)56–78(–95) × (2–)2.5–3 (–3.5)
A. septoconstrictum	non-collapsing 200–250 × 150–200	acute tip 45–120 × 5–7	cylindrical-clavate to clavate 110–140 × 17–22	asymmetric	fusiform, tapering at both ends but more narrow at basal end, 2–3 inner cells near apical end slightly broader than the others	7–10-septate, constricted at septa	40–50 × 5–6.5

Acanthostigma in addition to the type species and confirmed the taxonomic placement of the genus in the family (FIGS. 23–25).

Acanthostigma shares several morphological characters with *Tubeufia*, and some have suggested recently that *Acanthostigma* should be synonymized under *Tubeufia* (Tsui et al. 2007) while others have maintained them as distinct genera (Réblova and Barr 2000). *Acanthostigma* has been distinguished from *Tubeufia* in having dark (vinaceous, reddish-brown, brown or dark brown), setose ascomata, whereas *Tubeufia* has light (hyaline, white, yellowish, pinkish) ascomata that darken and may be glabrous or with thick-walled, short hyphal appendages or thick-walled short setae (Réblova and Barr 2000). *Helicosporium* and *Helicomyces* anamorphs have been reported in both genera (Barr 1980, Réblova and Barr 2000, Kodsueb et al. 2004, Tsui et al. 2006). This is the first molecular study to include multiple species of *Acanthostigma* and reveals the polyphyletic nature of the genus. In fact none of the sampled anamorphic or teleomorphic genera in the Tubeufiaceae are monophyletic (FIGS. 23–25). While we agree with Tsui et al. (2007) that *Acanthostigma* probably should be synonymized under *Tubeufia* at least 17 additional genera might belong in the Tubeufiaceae (Kodsueb et al. 2006) and must be included in more rigorous phylogenetic analyses, which incorporate additional genes other than nuclear ribosomal genes, before genera within the family are recircumscribed. Thus the three new species, which fit the traditional morphological circumscription of *Acanthostigma*, are placed within the genus until a thorough revision of Tubeufiaceae is completed.

Within *Acanthostigma* species can be distinguished based on the morphology of the setae, size of asci and shape and size of the ascospores. The three newly described species were compared with six other recently accepted species in the genus (TABLE I). *Acanthostigma filiforme* is unique in that it possesses the longest ascospores in the genus. It is most similar to *A. scopulum* (Cook & Peck) Peck but differs in having wider setae and longer ascospores with more septa. *Acanthostigma multiseptatum* possesses ascospores with the greatest number of septa in the genus. It is most similar to *A. minutum* (Fuckel) Sacc. but differs in having longer asci and longer ascospores with more septa. *Acanthostigma septoconstrictum* is most similar to *A. ellisii* Sacc. & Syd. but differs in having wider setae, larger asci and broader ascospores that are constricted at the septa. Although helicosporous-type anamorphs occur throughout the Tubuefiaceae and occasionally are observed on or near ascomata on the substrate, no anamorphs were found associated with these three new species.

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