A new Thai species of *Astrosphaeriella* (Dothideomycetes, Ascomycota) from submerged wood in freshwater

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A new species of *Astrosphaeriella* was collected from submerged wood in Thailand. Identification based on morphological characters and a comparison of sequence data from partial regions of the 28 large subunit ribosomal (LSU) RNA and 18 small subunit ribosomal (SSU) RNA genes supported its novel status. *Astrosphaeriella thailandensis,* sp. nov., is described and illustrated. It is distinguished from other similar species in size and ornamentation of ascospores.

Keywords: taxonomy, phylogeny.

Astrosphaeriella was originally introduced with the type species *Astrosphaeriella fusispora* Syd. & P. Syd., recorded from bamboo stems in Japan in 1912 (Sydow & Sydow 1913). Hawksworth (1981) re-introduced and emended the genus by accommodating four species of loculoascomycetous fungi with characteristic hemispherical to conical ascomata and treated *A. stellata* (Pat.) Sacc, as an earlier name for *A. fusispora*; he also circumscribed *Astrosphaeriella* as an exclusively tropical genus occurring on bamboo or palms (Liu *et al.* 2011). The generic concept was subsequently extended to include six additional species and a key to ten known species was provided (Hawksworth & Boise 1985). Presently 57 taxa are listed under *Astrosphaeriella* (Chen & Hsieh 2004, Chen & Huang 2006, Fröhlich & Hyde 1995, Hyde *et al.* 2000, Hyde & Fröhlich 1998, Rogers & Barr 2003, Tanaka & Harada 2005, Tsui *et al.* 2001, Zhou *et al.* 2003), and some are also reported from freshwater (Hyde 1994, Tsui *et al.* 2001, Cai *et al.* 2003) or brackish water habitats (Hyde 1992).

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In a continuing survey of Dothideomycetes on wood in Thailand (Boonmee *et al.* 2011), an *Astrosphaeriella* species was collected and isolated from submerged wood in a freshwater stream. The species has conical, carbonaceous ascomata with a non-papillate apex and most closely resembles *A. stellata* (Saccardo 1928). The new taxon is illustrated with light micrographs and its uniqueness is confirmed by morphological comparison and a phylogenetic analysis of combined LSU and SSU RNA genes sequence data.

Materials and methods

Morphological and cultural studies

The species was collected from wood submerged in a freshwater stream in Phisanulok Province, Thailand, in November 2011, and returned to the laboratory in paper envelopes. Observations and photomicrographs were made from material mounted in water using a Nikon ECLIPSE 80i microscope (Nikon Corporation, Japan). Measurements were made with the Tarosoft (R) Image Frame Work (Liu *et al.* 2010). Isolations were made from single ascospores using the method of Chomnunti *et al.* (2011). Ascomata were cut horizontally and the contents transferred to a drop of sterile water on a flamed microscope slide. A portion was subsequently taken and spread over a few square centimeters of a Petri dish containing 2 % water agar (WA) and then incubated at 25 °C overnight. The next day, individual germinating spores were transferred to potato dextrose agar (PDA) media. The holotype and ex-type culture are deposited in Mae Fah Luang University Culture Collection (MFLU) with an isotype in the Plant Pathology Herbarium of Guizhou University (HGUP). Cultures are also deposited in HGUP and MFLU.

DNA sequencing and alignment

Fungal isolates were grown on PDA for 30 days at 28 °C in the darkness. Genomic DNA was extracted from the fresh mycelium using the Biomiga EZgene™ Fungal gDNA Miniprep Kit (Biomiga, San Diego, USA). DNA sequences were amplified by polymerase chain reaction (PCR). Primer pairs NS1 and NS4 were used to amplify a region spanning the small subunit rDNA (White *et al.* 1990). LROR and LR5 primer pairs, as defined by Vilgalys & Hester (1990), were used to amplify a segment of the large subunit rDNA. The amplifications were performed in a 50 µl reaction volume containing 1 × PCR buffer, 0.2 mM d'NTP, 0.3 µM of each primer; 1.5 mM $MgCl_2$, 0.8 units Taq Polymerase and 5–10 ng DNA. The PCR amplified DNA fragments were fractionated in 1 % agarose gels in 0.5 × TBE buffer, and DNA was visualized by ethidium bromide staining and UV illumination. The reference nucleotide sequences of LSU and SSU regions of various taxa were obtained from GenBank (Tab. 1; Liu *et al.* 2011). Clustal X 1.81 (Thompson *et al.* 1997) was used to align the sequences, and then the alignments were refined by hand. Alignments files are available in TreeBASE (www.treebase.org/treebase–web/

home.html) with study ID 13346. Phylogenetic analyses were performed by using PAUP v. 4.0b10 (Swofford 2002) for Maximum-parsimony (MP) and MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003) for Bayesian analyses.

Tab. 1. Strains used in phylogenetic analyses and their corresponding GenBank accession numbers. The new species is in bold.

Taxon	Strain	GenBank Accession numbers	
		LSU	SSU
Aigialus grandis	BCC 18419	GU479774	GU479738
A. grandis	BCC 20000	GU479775	GU479739
A. grandis	JK 5244A	GU301793	GU296131
A. mangrovis	BCC 33563	GU479776	GU479741
A. mangrovis	BCC 33564	GU479777	GU479742
A. parvus	BCC 18403	GU479778	GU479743
A. parvus	BCC 32558	GU479779	GU479744
A. rhizophorae	BCC 33572	GU479780	GU479745
A. rhizophorae	BCC 33573	GU479781	GU479746
Ascocratera manglicola	HHUF 30032	GU479783	GU479748
A. manglicola	BCC 09270	GU479782	GU479747
A. manglicola	JK 5262C	GU301799	GU296136
Astrosphaeriella africana	MFLUCC10-0553	JN846721	JN846731
A. bakeriana	MFLUCC11-0027	JN846730	JN846740
A. lophiostomopsis	HKUCC2984	GU205215	GU205232
Astrosphaeriella sp.	A70	GU205213	GU205233
A. stellata	KT 998	AB524592	AB524451
A. stellata	MFLUCC 10-0555	JN846723	JN846733
A. thailandensis	**MFLUCC 11-0596**	**JX546576**	**JX546575**
Delitschia didyma	UME 31411	DQ384090	AF242264
D. winteri	CBS 225.62	DQ678077	DQ678026
Dothidea sambuci	DAOM 231303	AY544681	AY544722
Fissuroma (Astrosphaeriella) aggregata	KT 767	AB524590	AB524449
F. (A.) aggregata	KT 984	AB524591	AB524450
F. (A.) aggregata	MFLUCC 10-0554	JN846722	JN846732
F. (A.) maculans	MFLUCC 10-0886	JN846724	JN846734
F. (A.) maculans	MFLUCC 10-0887	JN846725	JN846736
F. (A.) maculans	MFLUCC 10-0888	JN846726	JN846737
F. (A.) maculans	MFLUCC 11-0023	JN846728	JN846738
Polyplosphaeria fusca	KT 1616	AB524604	AB524463
Pseudotetraploa curviappendiculata	HC 4930	AB524608	AB524467
Quadricrura septentrionalis	CBS 125429	AB524615	AB524474

Taxon	Strain	GenBank Accession numbers	
		LSU	SSU
Rimora mangrovei	JK 5246A	GU301868	GU296193
R. mangrovei	JK 5437B	GU479798	GU479765
Tetraplosphaeria sasicola	MAFF 239677	AB524631	AB524490
Triplosphaeria maxima	MAFF 239682	AB524637	AB524496

Abbreviations of isolates and culture collections: BCC: Belgian Coordinated Collections of Microorganisms; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; HHUF: Herbarium of Hirosaki University, Japan; MAFF: Ministry of Agriculture, Forestry and Fisheries, Japan; MFLUCC: Mae Fah Luang University Culture Collection, Thailand; UME: Herbarium of the University of Umeå, Umeå, Sweden; Culture and specimen abbreviations; JK: J. Kohlmeyer; KT: K. Tanaka.

Phylogenetic analyses

The combined 28S (LSU) and 18S (SSU) rDNA data set consists of 36 taxa with *Dothidea sambuci* as outgroup. A partition homogeneity test (Farris *et al.* 1994) was applied to evaluate the feasibility of combining the datasets.

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa (Hillis & Bull 1993).

The model of evolution in Bayesian analysis was TrN+G estimated by jModelTest 0.0.1 (Posada 2008). A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v3.1.1 for the SSU and LSU sequence datasets. Two independent runs of four MCMC chains were run simultaneously from random trees for 1000000 generations and sampled every 100 generations for the combined analysis of the gene partitions. Both runs converged on the same likelihood score and tree topology, and the first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from the remaining 7500 trees.

Results

Phylogenetic analysis

The aligned sequence data matrix contained 36 taxa, including the outgroup taxon and 1470 characters. LSU has 882 characters and SSU 588 characters (instead of the usual ca. 1200 base pairs, sequencing only gave about 600 bp fragments several times), with 259 being parsimony informa-

tive. Partition homogeneity tests for combinations of the two gene regions used, yielded a P-value of 0.001. Based on the tree topologies and a P-value of 0.001 (Cunningham 1997, Dettman *et al*. 2003), the gene regions were combined. Ten most parsimonious trees were obtained, and one that represented the topology of the strict consensus tree was selected for presentation (Fig. 1). The tree is described as follows; Tree Length (TL) = 1146, Consistency Index (CI) = 0.650, Retention Index (RI) = 0.739, Homoplasy Index (HI) = 0.350 and Rescaled Consistency Index (RC) = 0.480. In Fig. 1, 35 species of Pleosporales resided in a large strongly supported monophyletic clade. All *Astrosphaeriella* strains here are shown to be polyphyletic. Among them, only *A. thailandensis*, along with *A. bakeriana, A. lophiostomopsis* and *A. stellata* clustered together with a 53 % bootstrap value. *Astrosphaeriella africana* and *Astrosphaeriella* sp. (A70) showed a close relationship with the Aigialaceae group (Liu *et al*. 2011) supported by a moderate value (54 % BT), which was a sister group to the *Astrosphaeriella* group. We selected two isolates of *A. stellata* (MFLUCC 10-0555 and KT998) in this study (Fig. 1). Our new taxon and MFLUCC strain 10-0555 (*Astrosphaeriella stellata*) grouped into one branch with a high statistical support (97 % BT), but showed a relatively distinct relationship with KT998.

The topology in Bayesian analysis was nearly identical to that of the MP analyses. The Bayesian trees are not shown, but the statistically supported clades (posterior probabilities ≥0.95) are marked with a thickened line in the parsimony tree (Fig. 1).

Taxonomy

Astrosphaeriella thailandensis Jun Ren, Chun-Yu Jie, Y. L. Jiang, K. D. Hyde & Yong Wang bis, **sp. nov.** – Fig. 2.
MycoBank no.: MB 801309

Typus. – THAILAND, Phisanulok Province, San Janpu Phadan, on wood submerged in a freshwater stream, November 2011, *leg*. J. Ren (MFLUCC 11-0596 **holotype**), ex-type living culture MFLUCC 11-0596 and HGUP3008.

Description. – Ascomata 600–850 µm in diam., 350–550 µm high, black, scattered, rarely clustered, superficial on host tissue, as subglobose, carbonaceous domes, base applanate, apex non-papillate. Peridium 40–60 µm thick, carbonaceous, uneven in thickness, composed of thick-walled cells. Pseudoparaphyses 0.5–1.2 µm wide, trabeculate, filiform, hyaline, persistent, numerous, septate, anastomosing and branched, embedded in a gelatinous matrix. Asci 78–144 × 5–7 µm (M = 100 × 6 µm, n = 20), 8–spored, bitunicate, fissitunicate, cylindric-clavate, with a long, thin pedicel, apex wide and rounded. Ascospores 22–25 × 3–4.5 µm (M = 23 × 3.5 µm, n = 20), fusiform, hyaline, smooth-walled, containing refractive globules, 1-septate, upper cell slightly shorter and wider, deeply constricted at the middle septum.

Etymology. – The epitheton *thailandensis* refers to the country, where the holotype specimen was collected.

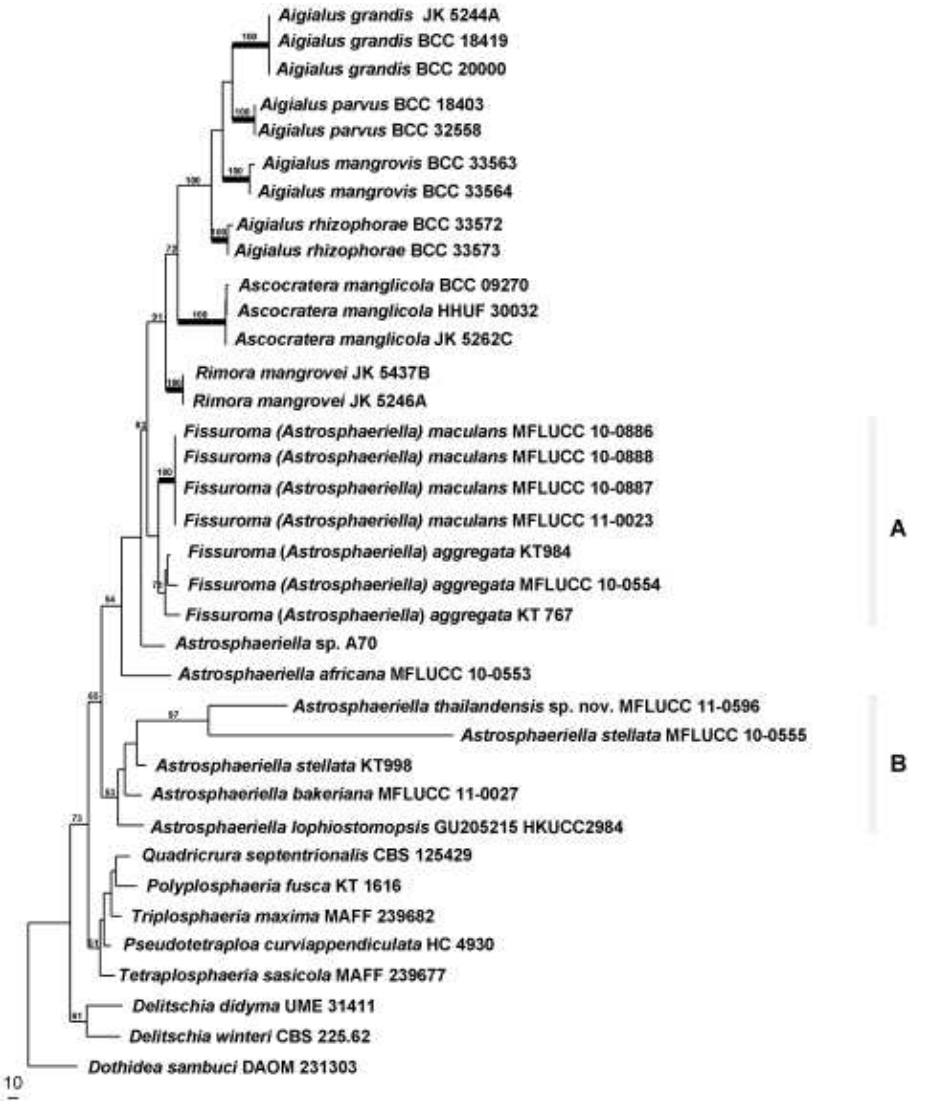

Fig. 1. Parsimonious tree based on combined 28S (LSU) and 18S (SSU) rDNA gene regions for *Astrosphaeriella thailandensis* and 34 other species downloaded from GenBank. *Dothidea sambuci* is the outgroup taxon. The detailed phylogenetic relationships between *A. thailandensis, A. stellata, A. bakeriana, A. lophiostomopsis* are shown in the tree. Bootstrap values ≥ 50 % are shown above branches. Statistically supported clades in the Bayesian analysis (posterior probability ≥ 90 %) are indicated by thickened lines.

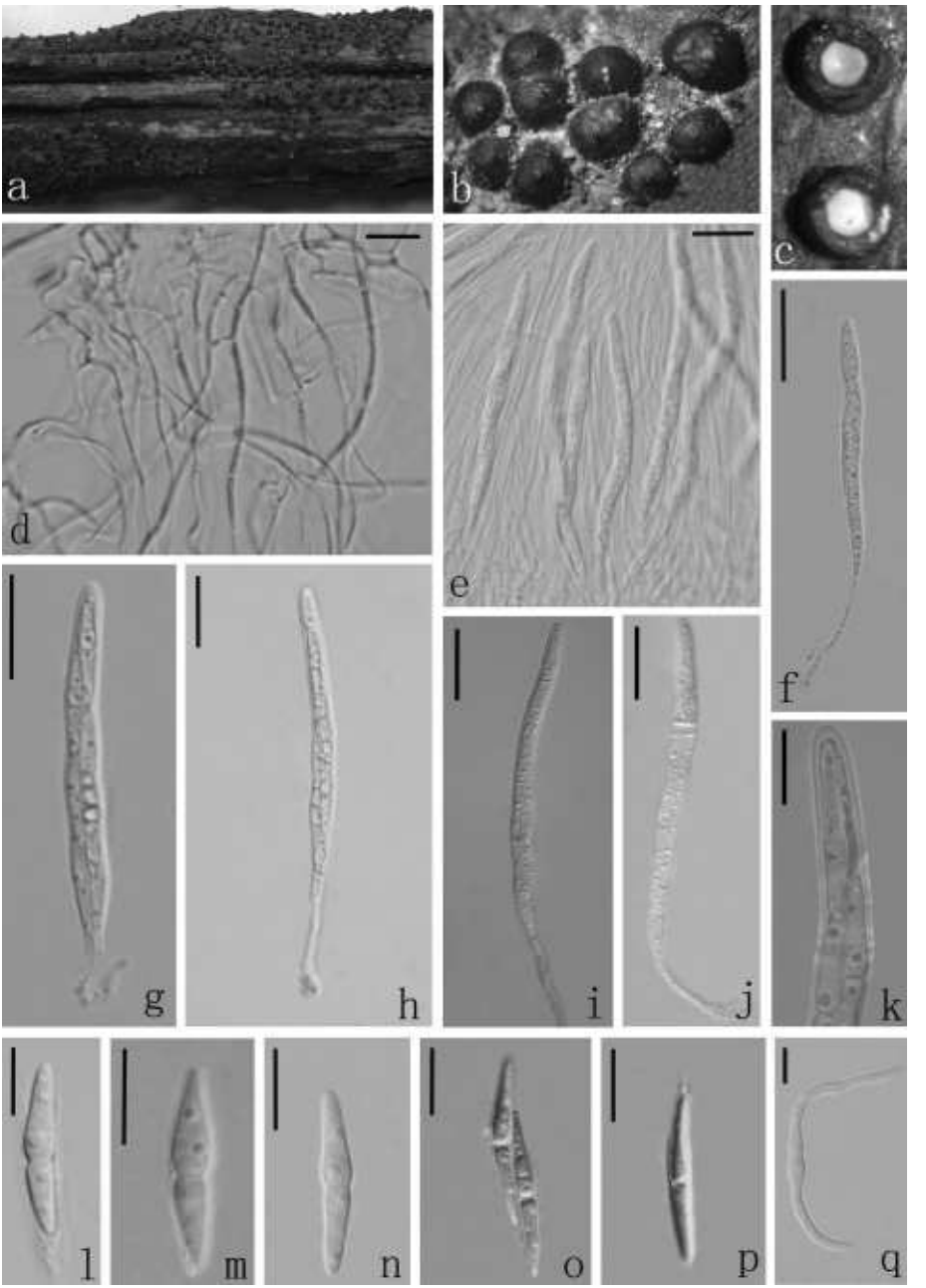

Fig. 2. *Astrosphaeriella thailandensis*. **a, b.** Appearance of ascomata on the host surface. **c.** Horizontal section of ascomata. **d.** Pseudoparaphyses stained in lactophenol cotton blue. **e-k.** Asci. **l–p.** Ascospores. **q.** Germinating ascospore. Bars: d 5 µm; e–k 20 µm; l–q 10 µm.

Discussion

Astrosphaeriella thailandensis is morphologically similar to *A. stellata* as both produce black ascomata, 1-septate ascospores which are deeply constricted at the septum. However, the ascospores of *A. thailandensis* are obviously smaller than those of *A. stellata* (36–50 × 5–7.5 µm) in Liu *et al.* (2011) and (40–56 × 5.5–7 µm) in Chen & Hsieh (2004). The ascospores of *A. thailandensis* contain refractive globules, a character not seen in *A. stellata*. In addition, our present taxon produces smaller asci than those of *A. stellata* (120-201 × 8.5–14.5 µm) in Liu *et al.* (2011) and (170–240 × 11–13 µm) in Chen & Hsieh (2004). Asci of *A. thailandensis* have a long and thin pedicel, but in the two descriptions of *A. stellata* the asci have shorter and thicker pedicells. Until now, there are seven *Astrosphaeriella* species (*A. aquatica*, *A. bakeriana*, *A. lophiostomopsis*, *A. papillata*, *A. papuana*, *A. stellata* and *A. tornata,* Tab. 2) from freshwater (Aptroot 1995, Hawksworth 1981, Hawksworth & Boise 1985, Hyde 1994, Hyde & Fröhlich 1998). Ascospores of these seven species are bigger than those of *A. thailandensis*. *Astrosphaeriella tornata* has 3-septate ascospores, ascospores of *A. thailandensis* are only 1-septate. The ascospores of *A. thailandensis* lack a sheath and thereby obviously differ from *A. aquatica*, *A. bakeriana*, *A. lophiostomopsis*, *A. papillata*, *A. stellata* and *A. tornata,* which have a sheath. *Astrosphaeriella thailandensis* has smooth ascospores, while *A. papuana* has ascospores with distinct striation. A detailed comparison of morphological differences between *A. thailandensis* and the other seven *Astrosphaeriella* species known from freshwater is shown in Tab. 2.

Tab. 2. Morphological comparison of *Astrosphaeriella* species from freshwater.

Species	Ascospore size	Ascospore shape	Reference
Astrosphaeriella aquatica K. D. Hyde	30–42 × 7–8 µm	Ascospores fusiform, surrounded by a wide, distinctive mucilaginous sheath, 1-septate	Hyde (1994)
A. bakeriana K. D. Hyde & J. Fröhl.	36–44 × 5–7(–8) µm	Ascospores fusiform, with an inconspicuous mucilaginous sheath, 1–septate	Hyde & Fröhlich (1998)
A.lophiostomopsis K. D. Hyde & J. Fröhl.	48–52 × 8–10 µm	Ascospores fusiform, surrounded by a wide, irregular mucilaginous sheath, 1–septate	Hyde & Fröhlich (1998)
A. papillata K. D. Hyde & J. Fröhl.	31–45 × 7–8 µm	Ascospores minutely striate, fusiform, surrounded by an irregular mucilaginous sheath, 1–septate	Hyde & Fröhlich (1998)

Species	Ascospore size	Ascospore shape	Reference
A. papuana Aptroot	34–42 × 6–6.5 µm	Ascospores wall distinctly striate, fusiform, lacking a sheath, 1–septate	Aptroot (1995)
A. stellata (Pat.) Sacc.	42–58 × 5.5–7 µm	Ascospores fusiform, with a thin sheath which is obtuse at the ends, 1–septate	Hawksworth (1981)
A. thailandensis J. Ren, C.Y. Jie, Y. L. Jiang, K. D. Hyde & Y. Wang bis	22–25 × 3–4.5 µm	Ascospores fusiform, smooth-walled, lacking a sheath, 1-septate,	This paper
A. tornata (Berk. & Curtis) D. Hawksworth & Boise	46–56 × 6–8 µm	Ascospores fusiform, with a thin sheath, 3-septate	Hawksworth & Boise (1985)

The phylogenetic analyses based on LSU and SSU regions showed that *A. thailandensis* clustered together with *A. stellata* (MFLUCC 10-0555) with 97% bootstrap support, which is in accordance with the morphological characters. Surprisingly, the two *A. stellata* isolates referred to in this study did not display a close relationship. However, we did not have the opportunity to study the herbarium specimen of KT998 and thus to observe the morphological characters. In Tanaka *et al.* (2009), there is only a photo of the ascomata; specimen identification should be reconsidered. Hyde & Fröhlich (1998) suggested that there is a wide range of ascospore size in *A. stellata*, but the ascospore sheath with truncated ends is a striking character of this species. We discriminate *A. stellata* and *A. thailandensis* by ascospore size and sheath and by the phylogenetic analyses supporting the morphological characters. It is proposed that obvious differences in ascospores dimensions are valuable in identifying different *Astrosphaeriella* species (Tab. 2). In summary, combining morphology and phylogeny, we conclude that *A. thailandensis* is a novel taxon.

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