



Ligninsphaeria jonesii gen. et. sp. nov., a remarkable bamboo inhabiting ascomycete

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Abstract

A new ascomycete species, inhabiting decaying bamboo culms, is introduced in this paper based on morphology and molecular evidence. Analyses of partial LSU sequence data place the taxon in a monophyletic clade within the order *Pleosporales*. Morphological characters of the taxon are also distinct from other members of *Pleosporales*. *Ligninsphaeria jonesii* gen. et sp. nov. is therefore introduced to accommodate the new taxon, which is characterized by its clypeate, scattered, deeply immersed ascomata, with a slit-like opening, long ostioles, cellular pseudoparaphyses, clavate asci containing broad-fusiform and 1-septate ascospores surrounded by a gelatinous cap at both ends. The phylogenetic analyses show that the new taxon is phylogenetically close but distinct from the families *Amniculicolaceae* and *Testudinaceae*, and forms a distinct clade from other taxa in *Pleosporales*. The new taxon is described and compared with similar taxa. A new genus *Ligninsphaeria* (*Pleosporales*, *incertae sedis*) is introduced to accommodate this distinct lineage.

Key words: Dothideomycetes, New genus, Phylogeny, Pleosporales, Taxonomy

Introduction

The *Dothideomycetes* is the largest class of Ascomycota with approximately 23 orders, 105 families and 19000 species (Kirk *et al.* 2008, Ruibal *et al.* 2009, Hyde *et al.* 2013, Liu *et al.* 2014, McKenzie *et al.* 2014). This group is ecologically highly diverse, comprising saprobes, pathogens, endophytes, epiphytes, lichenized and rock-inhabiting fungi (Sterflinger *et al.* 1999, Selbmann *et al.* 2008, Schoch *et al.* 2009, Hyde *et al.* 2013). These fungi are characterized mainly by bitunicate asci with fissitunicate dehiscence and occur in terrestrial, freshwater and marine habitats worldwide (Kirk *et al.* 2008, Lumbsch & Huhndorf 2010, Hyde *et al.* 2013, McKenzie *et al.* 2014, Wijayawardene *et al.* 2014).

The *Pleosporales* is the largest order in the class *Dothideomycetes*, comprising 43 families, 332 genera and more than 4700 species (Kirk *et al.* 2008, Zhang *et al.* 2012, Hyde *et al.* 2013, Ariyawansa *et al.* 2013, Liu *et al.* 2013, 2014). Previous classification has posed major challenges in this complicated group, because of lack of molecular sequence data. In recent years, a great amount of molecular data has become available, with many molecular studies revising and classifying taxa (Mugambi & Huhndorf 2009, Zhang *et al.* 2009, Phookamsak *et al.* 2014, Liu *et al.* 2015). However, some families lack sequence data and collections are needed to resolve these groups (Liu *et al.* 2014).

Studies on the taxonomy and biodiversity of microfungi inhabiting bamboo have revealed several new taxa over the past 15 years (Hyde *et al.* 2002, Cai *et al.* 2003, Zhou *et al.* 2003, Tanaka *et al.* 2009, Dai *et al.* 2012, 2014, 2015, Liu *et al.* 2013). Collection of fungi from decaying wood in Thailand, yielded a new taxon from bamboo culms collected on limestone outcrops near the Mae Chang Hot Spring in Chiang Rai Province, in northern Thailand. The morphological identification could not place the species in any existing genus or even family. Molecular analysis of LSU sequence data suggested this taxon as a monophyletic clade in the order *Pleosporales*. In this study we introduce *Ligninsphaeria jonesii* gen. et sp. nov. to accommodate this new taxon, which is placed in the order *Pleosporales*, genera *incertae sedis*.

Materials and Methods

Collection, examination and isolation of specimens

Materials were collected in Chiang Rai Province in November 2014, and taken back to the laboratory in paper envelopes. The samples were processed and examined following the methods described by Liu *et al.* (2010) under a stereomicroscope (Motic SMZ 168). Hand sections of fruiting bodies were made and mounted in a drop of water for microscopic studies and photomicrography. Micro-morphological images were captured using a Nikon ECLIPSE 80i compound microscope fixed with a Cannon EOS 600D digital camera. Melzer's reagent and India ink were added to water mounts to detect the presence of the apical structures of the asci and the gelatinous sheath or appendages of ascospores. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures were processed by Adobe Photoshop CS5 (Adobe Systems, USA).

Isolates were made from single ascospores following the method described by Chomnunti *et al.* (2014). The single germinated ascospores were picked up and transferred to potato-dextrose agar (PDA) media. The holotype is deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand. Isolates are deposited in the Mae Fah Luang University Culture Collection (MFLUCC) Chiang Rai, Thailand, and (GZCC) Gui Yang, China. Facesoffungi and Index Fungorum numbers are provided as explained in Jayasiri *et al.* (2015) and Index Fungorum (2015). Attempts were made to obtain the asexual morph in culture following the method of Pooksamak *et al.* (2015).

DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA media at 25–30 °C for two weeks. Total genomic DNA was extracted from the fresh mycelium, which was scraped from the edge of the growing culture (Wu *et al.* 2001), with the CTAB method established by Lee *et al.* (1988) and optimized as in Cubero *et al.* (1999).

DNA amplification procedure was carried on by Polymerase Chain Reaction (PCR) in a 25 µl reaction volume containing 5–10 ng DNA, 1X PCR buffer, 0.8 units Taq polymerase, 0.3 µM of each primer, 0.2 mM dNTP and 1.5 mM MgCl₂. LSU was used in this study and the primers of LROR and LR5 (White *et al.* 1990) were used for amplification it under the PCR thermal cycle program as follows: initially 94 °C for 3 mins, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 50 s, elongation at 72 °C for 1 min and a final extension at 72 °C for 10 mins. PCR products were then purified and sequenced by Invitrogen Biotechnology Co., Ltd (Shanghai, P. R. China).

Phylogenetic analysis

Sequences were analyzed using the BLAST search engine of the National Center for Biotechnology Information (NCBI) for revealing the closest matches with taxa in *Pleosporales*. The consensus sequences were aligned using Bioedit v.7.1.3 (Hall 1999) and ClustalX v. 1.83 (Thompson *et al.* 1997). The alignments were checked visually and optimized manually where necessary.

A maximum likelihood analysis was performed at the CIPRES web portal (Miller *et al.* 2010) using RAxML v.7.2.8 as part of the "RAxML-HPC2 on TG" tool (Stamatakis 2006). A general time reversible model (GTR) was applied with a discrete gamma distribution and four rate classes. Fifty thorough maximum likelihood (ML) tree searches were done in RAxML v. 7.2.7 under the same model. One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution. The resulting replicates were plotted on to the best scoring tree obtained previously. The phylogram with bootstrap values above the branches is presented in FIG. 1 by using graphical options available in TreeView (Page 1996).

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Hillis & Bull 1993). The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1000000 generations and trees were sampled every 100th generation (resulting in 10000 total trees). The first 2000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 8000 trees used for calculating posterior probabilities (PP) in the majority rule consensus tree. Phylogenetic trees were drawn using Treeview (Page 1996) and MEGA5 (Tamura *et al.* 2011). Sequences derived in this study are deposited in GenBank (Table 1).

TABLE 1.—Isolates used in this study.

Taxon	Strain No. ¹	GenBank Accession No. ²
		LSU
<i>Aigialus grandis</i>	BCC 18419	GU479774
<i>Alternaria alternata</i>	CBS 916.96	DQ678082
<i>Amniculicola immersa</i>	CBS 123083	FJ795498
<i>Anteaglonium abbreviatum</i>	ANM 925a	GQ221877
<i>Anteaglonium globosum</i>	ANM 925.2	GQ221879
<i>Anteaglonium parvulum</i>	SMH 5223	GQ221909
<i>Ascocratera manglicola</i>	BCC 09270	GU479782
<i>Astrosphaeriella bakeriana</i>	CBS 115556	GU301801
<i>Astrosphaeriella fusispora</i>	MFLUCC 10_0555	KP057604
<i>Bambusicola massarinia</i>	MFLUCC 11_0389	JX442037
<i>Bambusicola splendida</i>	MFLUCC 11_0439	JX442038
<i>Bimuria novae zelandiae</i>	CBS 107.79	AY016356
<i>Curreya grandicipis</i>	CPC 1852	JN712520
<i>Delitschia chaetomioides</i>	SMH 3253.2	GU390656
<i>Delitschia winteri</i>	AFTOL ID1599	DQ678077
<i>Dothidotthia aspera</i>	CPC 12933	EU673276
<i>Dothidotthia symphoricarpi</i>	CBS 119687	EU673273
<i>Floricola striata</i>	JK 5603K	GU479785
<i>Helicascus nypae</i>	BCC 36752	GU479789
<i>Herpotrichia diffusa</i>	CBS 250.62	DQ678071
<i>Hysterium angustatum</i>	CBS 236.34	FJ161180
<i>Lentithecium fluviatile</i>	CBS 122367	GU301825
<i>Lepidosphaeria nicotiae</i>	AFTOL ID1576	DQ678067
<i>Leptosphaeria maculans</i>	DAOM 229267	DQ470946
<i>Lignosphaeria jonesii</i>	GZCC 15_0080	KU221308
<i>Lignosphaeria jonesii</i>	MFLUCC 15_0641	KU221307
<i>Lindgomyces breviappendiculatus</i>	KT 1215	AB521748
<i>Lindgomyces ingoldianus</i>	ATCC 200398	NG_042321
<i>Lophiostoma caulium</i>	KT 573	AB619005
<i>Lophiostoma macrostomum</i>	KT 508	AB619010
<i>Lophiostoma vaginatispora</i>	D 43	KJ591576
<i>Lophiotrema lignicola</i>	CBS 122364	GU301836
<i>Lophiotrema neoarundinaria</i>	KT 856	AB524596
<i>Lophiotrema nucula</i>	CBS 627.86	GU301837
<i>Massariosphaeria grandispora</i>	CBS 613_86	GU301842
<i>Massariosphaeria typhicola</i>	CBS 123126	GU301844
<i>Melanomma pulvis pyris</i>	CBS 124080	GU456323
<i>Montagnula opulenta</i>	CBS 168.34	NG_027581
<i>Morosphaeria ramunculicola</i>	JK 5304B	GU479794
<i>Phaeosphaeria oryzae</i>	CBS 110110	KF251689
<i>Phoma exigua</i>	CBS 431.74	EU754183
<i>Phoma herbarum</i>	CBS 276.37	DQ678066
<i>Pleomassaria siparia</i>	CBS 279.74	AB553759
<i>Pleospora herbarum</i>	CBS 191.86	DQ247804
<i>Preussia lignicola</i>	CBS 264.69	GU301872
<i>Pseudoastrosphaeriella thailandensis</i>	MFLUCC 10_0553	KT955477
<i>Pseudoastrosphaeriella thailandensis</i>	MFLUCC 11_0144	KT955478
<i>Pseudotetraploa curviappendiculata</i>	CBS 125426	AB524610
<i>Pteridiospora chiangraiensis</i>	MFLUCC 11_0162	KT955480
<i>Repetophragma ontariense</i>	HKUCC 10830	DQ408575
<i>Sporormiella minima</i>	CBS 524.5	DQ678056
<i>Tetraplophaeria sasicola</i>	MAFF 239677	AB524631
<i>Trematosphaeria pertusa</i>	CBS 122368	FJ201990
<i>Trematosphaeria pertusa</i>	CBS 122371	FJ201992
<i>Ulospora bilgramii</i>	CBS 101364	DQ678076
<i>Verruculina enalia</i>	BCC 18401	GU479802

¹**AFTOL**: Assembling the Fungal Tree of Life; **ATCC**: American Type Culture Collection, Virginia, USA; **BCC**: BIOTEC Culture Collection, Bangkok, Thailand; **CBS**: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CPC**: Collection of Pedro Crous housed at CBS; **DAOM**: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; **GZCC**: Guizhou Culture Collection, Guiyang, China; **MAFF**: Ministry of Agriculture, Forestry and Fisheries, Japan; **MFLUCC**: Mae Fah Luang University Culture Collection, ChiangRai, Thailand; **ANM**: A.N. Miller; **JK**: J. Kohlmeier; **KT**: K. Tanaka; **SMH**: S.M. Huhndorf.

²Sequence accession numbers in bold were obtained in the present study. All others were retrieved from GenBank.

Results

Phylogenetic analysis

The LSU data set comprises 57 taxa with *Hysterium angustatum* CBS236.34 as the outgroup taxon. The data comprises 1265 characters after alignment, 915 characters were constant, and 257 characters were parsimony informative, while 93 variable characters are parsimony-uninformative. Bayesian, RAxML and Maximum parsimony analysis of the LSU dataset resulted in phylogenetic reconstructions with similar topologies, and the best scoring RAxML tree is shown in FIG. 1.

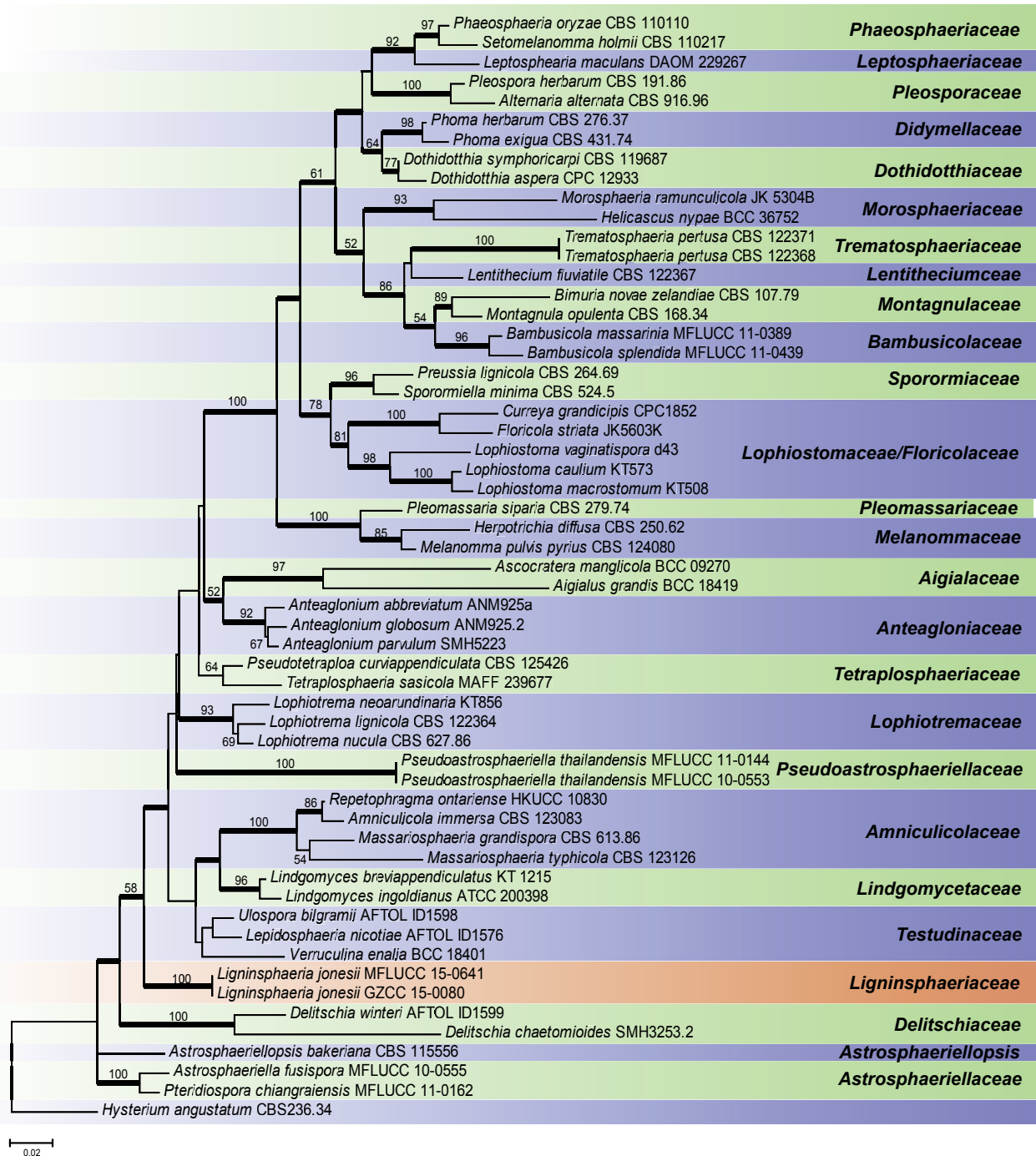


FIGURE 1. RAxML tree based on LSU sequences alignment. ML bootstrap proportion (BP) greater than 50% is presented above the nodes. The branch with Bayesian posterior probabilities (PP) above 0.95 are thickened. The original isolate numbers are noted after the species name. The scale bar shows 0.02 changes and the tree was rooted to *Hysterium angustatum* (CBS 236.34)

The core families of *Pleosporales* (Hyde *et al.* 2013) are included in our phylogenetic analysis (FIG. 1), and two suborders *Massarineae* and *Pleosporineae* are represented with well-supported clades. The new genus showed the closest phylogenetic relationship with *Testudinaceae* and *Delitschiaceae*. The genus *Ligninsphaeria* is introduced to accommodate the unusual member of *Pleosporales* that occurs on bamboo from limestone outcrops. Although this distinct lineage is unstable in the tree, we follow Jaklitsch *et al.* (2015) in introducing this new genus, which has strong molecular support and is morphologically distinct. The BLAST search of LSU gene in NCBI showed that *Ligninsphaeria* is related to taxa in the above families, as well as *Astrosphaeriella*-like taxa. The recently introduced families *Astrosphaeriellaceae* and *Pseudoastrosphaeriellaceae* (Phookamsak *et al.* 2015) were included in the phylogenetic analysis to understand their phylogenetic relationships. The results show that the new fungus is phylogenetically distinct from other existing groups in *Pleosporales* and is near to the base of the order. We therefore introduce a new genus to accommodate this distinct lineage. The new genus has both molecular support as well as distinct morphology.

Taxonomy

Ligninsphaeria J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu, *gen. nov.*

Index Fungorum number: IF551757, Facesoffungi number: FoF 01662

Etymology: From the Latin *lignin* refers to wood and *sphaeria* meaning fruiting body, in reference to the fruiting bodies on wood.

Type species: *Ligninsphaeria jonesii*

Saprobic on decaying bamboo culms. **Sexual morph**: *Ascomata* scattered, solitary, immersed under the host tissue, black, subglobose or obpyriform, clypeate, ostiole at the centre, with a slit-like opening. *Peridium* coriaceous to carbonaceous, composed of two strata, an outer stratum of thick-walled and brown cells arranged in a *textura angularis* and fusing with host cells, and inner layer of hyaline cells of *textura angularis*. *Hamathecium* comprising numerous, cellular pseudoparaphyses, embedded in a gelatinous matrix. *Asci* 8-spored, broad-clavate, bitunicate, fissitunicate, with tapering pedicel, apically rounded with a distinct ocular chamber. *Ascospores* 3–4-seriate, hyaline, broad-fusiform, with tapering apices, indistinctly 1-septate when mature, pointed at both ends with a gelatinous cap. **Asexual morph**: Undetermined.

Ligninsphaeria jonesii J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu, *sp. nov.*

Index Fungorum number: IF551758, Facesoffungi number: FoF 01636, FIG. 2.

Etymology: Named in honour of E. B. Gareth Jones for his contributions to tropical mycology.

Saprobic on decaying bamboo culms, forming black spots on the host surface. **Sexual morph**: *Ascomata* 353–541 μm high (including neck), 298–317 μm diam, scattered, solitary, immersed under the host tissue, black, clypeate, subglobose to obpyriform, visible on host surface as raised, dark spots. *Ostiole* central, long, slit-like opening, lacking periphyses in ostiolar canal. *Peridium* up to 19–37 μm wide, coriaceous to carbonaceous, composed of two strata, an outer stratum of thick-walled and brown cells arranged in a *textura angularis* and fusing with host cells, and inner layer of hyaline cells of *textura angularis*. *Hamathecium* up to 3–6 μm wide, comprising dense, cellular pseudoparaphyses, anastomosing above and between the asci, embedded in a gelatinous matrix. *Asci* 163–243 \times 38–57 μm (\bar{x} = 208 \times 48 μm , n = 20), 8-spored, bitunicate, fissitunicate, broad-clavate with tapering pedicel, apically rounded, with a distinct ocular chamber. *Ascospores* 79–121 \times 14–23 μm (\bar{x} = 97 \times 18 μm , n = 40), 3–4-seriate, hyaline, indistinctly 1-septate when mature, broadly fusiform, slightly curved in the center, pointed at both ends with a 3 μm long, 2.5 μm wide, gelatinous cap. **Asexual morph**: Undetermined.

Culture characters: Ascospores germinating on WA within 24 h and germ tubes produced from ends. Colonies growing fast on PDA, reaching 23 mm in 2 weeks at 25 °C, flat or raised at the center, circular, irregular at the margin, grayish from above, dark brown from below. Mycelium immersed in the media, composed of branched, septate, smooth, grayish hyphae.

Specimen examined. THAILAND, Chiang Rai, Muang District, on limestone outcrops, Mae Chang Hot Spring, on decaying bamboo culms, 25 Nov 2014, J. F. Zhang (holotype MFLU 15-2718, ex-type living culture, MFLUCC 15-0641); *Ibid.*, 25 Nov 2014, J.F Zhang (GZAAS 15-0115); living culture GZCC 15-0080.

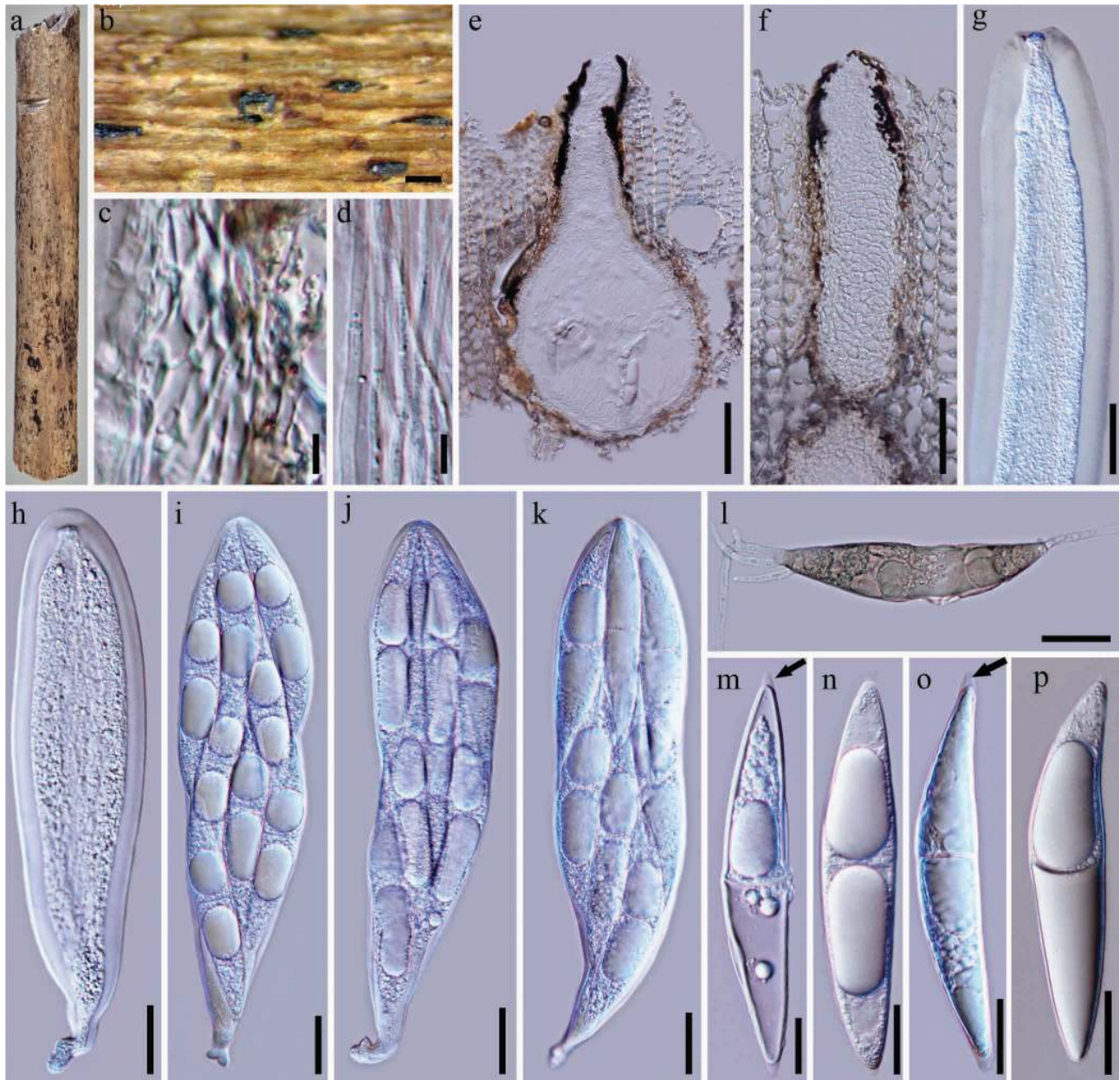


FIGURE 2. *Ligninsphaeria jonesii* (MFLU 15-2718) a The host, a decaying bamboo culm. b Ascomata immersed in bamboo host. c Section of peridium. d Pseudoparaphyses. e Vertical section through ascoma. f Long immersed ostiole. g Asci with fission-like dehiscence. h–k Asci. l Germinating ascospore. m–p Ascospores. Note the arrowheads indicate the gelatinous cap on both ends in m and o. Scale bars: b = 200 μ m, c, d = 5 μ m, e = 100 μ m, f = 50 μ m, g–h, n–p = 20 μ m, i–k, m = 10 μ m, l = 30 μ m

Notes: *Ligninsphaeria* is clearly different from other groups within the order of *Pleosporales* based on molecular data and morphological characters. This monotypic genus is introduced to accommodate those taxa characterized by having deeply immersed ascomata, surrounded by a large blackened clypeus, with long immersed ostiole canal, and broad-clavate asci with fusiform ascospores surrounded by a gelatinous cap at both ends. *Ligninsphaeria jonesii* is similar to *Pseudotrisha guatupoensis* in having immersed, obpyriform ascomata and clavate asci. However, the former is distinct in pseudoparaphyses type (cellular vs. trabeculate), ascospore appendages (gelatinous cap at both ends vs. 5–6 μ m wide gelatinous sheath) and ascospore septation (1-septate vs. 3–5-septate) (Huhndorf *et al.* 1994). The new fungus is also similar with *Lophiotremataceae*, but differs by its broad-clavate ascus and ascospores surrounded by a gelatinous cap at both ends, while the ascus in the latter taxon is cylindrical and ascospores lack gelatinous appendages (Hirayama & Tanaka 2011). In addition, the molecular analysis showed that this new fungus is phylogenetically close with *Astrosphaeriella*-like taxa, as well as the families *Delistchiaceae* and *Testudinaceae*. However, they have different morphological characters.

Morphological characters of this new fungus is obviously different from other genus even families within in the *Pleosporales*, but the phylogenetic placement of these groups is not stable, which may be caused by the lack of molecular data for taxa in these groups. Moreover, only one collection was obtained in this study, which cannot commendably illustrate the phylogenetic relationship with close groups. Therefore, we introduced a new genus *Ligninsphaeria* to accommodate the new fungus and future studies in tropical regions are likely to collect and provide data for this undersampled group. The recent study on *Astrosphaeriellaceae* and *Pseudoastrosphaeriellaceae* is a good example (Phookamsak *et al.* 2015).

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