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Lentithecium cangshanense sp. nov. (Lentitheciaceae) from freshwater habitats in Yunnan Province, China

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Abstract

Lentithecium cangshanense sp. nov. (Lentitheciaceae, Dothideomycetes), was found on submerged decaying wood in a freshwater stream in Yunnan Province, China. The species is characterized by its black, semi-immersed to superficial, globose ascomata, cylindrical or obclavate, short pedicellate, bitunicate asci and bi-seriate, fusiform, 1-septate, yellowish to brown ascospores. Phylogenetic analyses of combined LSU, SSU and RPB2 sequence data show that *L. cangshanense* belongs in the family Lentitheciaceae, order Pleosporales and is a distinct species in the genus. The new species is introduced with an illustrated account and compared with morphologically and phylogenetically similar species.

Key words: aquatic fungi, Lentitheciaceae, phylogeny, taxonomy

Introduction

Freshwater lignicolous fungi, are mostly ascomycetes, and have been collected and described from wood submerged in streams (Wong *et al.* 1998, Cai *et al.* 2003a, b, Jones & Choeyklin 2008, Hyde *et al*. 1998a, 1999, 2015) and lakes (Hyde *et al*. 1998b, Luo *et al*. 2004), and in surveys in Australia, China, Malaysia, Philippines and Thailand (Nawawi 1985, Kuthubutheen 1987, Hyde 1995, Sivichai *et al.* 2000, Cai *et al.* 2003, Jones *et al*. 2007, Kurniawati *et al*. 2010, Luo *et al.* 2015, 2016). The results of some of these studies have been summarized by Hyde *et al*. (2016). Two hundred and fifty-six freshwater ascomycetes have been reported from China including 57 Dothideomycetes with many species are from Yunnan Province (Hu *et al.* 2013). Although there have been several investigations on freshwater fungi in Yunnan Province (Cai *et al.* 2002, Jeewon *et al*. 2003, Luo *et al*. 2004, Shen & Ye 2006, Hu *et al*. 2013), studies in new streams generally reveal novel taxa (Liu *et al.* 2015, Su *et al*. 2015).

Zhang *et al.* (2012) introduced the family Lentitheciaceae to accommodate massarina-like species in the suborder Massarineae (Zhang *et al.* 2009, 2012, Hyde *et al.* 2013). Presently, there are eight genera included in Lentitheciaceae *viz. Darksidea, Katumotoa*, *Keissleriella, Lentithecium, Murilentithecium, Poaceascoma, Setoseptoria* and *Tingoldiago*, with *Lentithecium fluviatile* (Aptroot & Van Ryck.) K.D. Hyde *et al*. being the type (Hyde *et al.* 2013, Phookamsak *et al.* 2015, Knapp *et al.* 2015). Members of Lentitheciaceae were reported as being saprobes on herbaceous and woody plants and produce coelomycetous asexual morphs.

Lentithecium was introduced by Zhang *et al.* (2009) to accommodate the species which share similar morphological characters in having immersed to erumpent lenticular ascomata, clavate asci, anastomosing cellular pseudoparaphyses and hyaline 1-septate ascospores. There are seven *Lentithecium* names in Index Fungorum (2016).

In this paper, we introduce a new ascomycetes from Cangshan Mountain, western of Yunnan Province, with an illustrated account and its novelty is supported by morphological and molecular data.

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Materials and methods

Isolation and morphology

Submerged decaying wood samples were collected from a stream in Cangshan Mountain, Yunnan Province, China in July 2013 and returned to the laboratory in plastic bags. The samples were incubated in plastic boxes lined with moistened tissue paper at room temperature for one week. The samples were processed and examined following the methods described by Taylor & Hyde (2003). Morphological observations were made using a Motic SMZ 168 Series stereomicroscope and photographed using an OLYMPUS BX51 microscope-camera system. The fungal structures were measured using Image-Pro-Express software.

Single spore isolations were made to obtain pure cultures as described in Chomnunti *et al*. (2014). Herbarium specimens are deposited in the Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS), Yunnan, China. The pure cultures are deposited in Kunming Institute of Botany Culture Collection (KUMCC) and Dali University Culture Collection (DLUCC). Facesoffungi numbers were obtained as in Jayasiri *et al*. (2015).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh fungal mycelium grown on PDA at 25 °C. The EZ gene™ Fungal gDNA Kit (GD2416) was used to extract DNA according to the manufacturer's instructions. The gene regions of the large subunit of the nuclear ribosomal LSU, SSU and protein coding RPB2 were amplified using the primer pairs LROR/LR7 (Vigalys & Hester 1990), NS1/NS4 (White *et al*. 1990) and fRPB2-5F/fRPB2-7cR (Liu *et al.* 1999) respectively. Polymerase chain reaction (PCR) was carried out using the following protocol: The final volume of the PCR reaction was 25 µL and contained 12.5 µL of 2×Power Taq PCR MasterMix (a premix and ready to use solution, including 0.1 Units/µl Taq DNA Polymerase, 500 µm dNTP Mixture each (dATP, dCTP, dGTP, dTTP), 20 mM Tris–HCl pH 8.3, 100MmKCl, 3 mMMgCl2, stabilizer and enhancer), 1 µL of each primer (10 µM), 1 µL genomic DNA extract and 9.5 µL deionised water. The PCR thermal cycle program for LSU and SSU amplifications were followed as: initially 95 °C for 3 mins, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 40 seconds, elongation at 72 °C for 90 seconds, and final extension at 72 °C for 10 mins. The PCR thermal cycle program for the partial RNA polymerase second largest subunit (RPB2) was followed as initially 95 °C for 5 mins, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 mins, elongation at 72 °C for 90 seconds, and final extension at 72 °C for 10 mins. PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amershamproduct code: 27–9602–01). The PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and sequencing of PCR products were conducted at Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, P.R. China).

Phylogenetic analysis

Sequences generated from this study were analyzed with reference sequences from GenBank and those derived from Knapp *et al.* (2015), Phookamsak *et al.* (2015), and Luo *et al.* (2016). The consensus sequences were initially aligned using MAFFTv.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) and further improved using Bioedit v.5.0.6 (Hall 2001) and ClustalX v. 1.83 (Thompson *et al*. 1997) to allow maximum alignment and maximum sequence similarity.

A maximum likelihood analysis was performed using RAxMLGUI v. 1.3 (Silvestro & Michalak 2011). The optimal ML tree search was conducted with 1000 separate runs, using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR+GAMMA substitution model with a final ln value of -20356.150570.

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).

Bayesian analyses were performed by using PAUP v.4.0b10 (Swofford 2002) and MrBayes v3.0b4 (Ronquist & Huelsenbeck 2003). The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996) were performed by Markov Chain Monte Carlo Sampling (BMCMC) in MrBayes v. 3.0b4 (Liu *et al.* 2012). Six simultaneous Markov Chains were run for 1 million generations and trees were sampled every 100th generations (Resulting 10000 total trees) (Cai *et al.* 2006). The first 2000 trees representing the

burn-in phase of the analyses were discarded and the remaining 8000 (post burning) trees used for calculating posterior probabilities (PP) in the majority rule consensus tree (Cai *et al.* 2006, Liu *et al.* 2012).

Trees were viewed in Treeview (Page 1996). Sequences derived in this study are deposited in GenBank (Table 1).

TABLE 1. Isolates used in this study and their GenBank accession numbers, newly generated sequences are indicated in red.

Taxon	Culture/voucher	GenBank Accession Number		
		LSU	SSU	*RPB2*
Bambusicola bambusae	MFLUCC 11-0614	JX442035	JX442039	KP761718
Bambusicola irregulispora	MFLUCC 11-0437	JX442036	JX442040	KP761719
Bambusicola massarinia	MFLUCC 11-0389	JX442037	JX442041	KP761716
Bambusicola splendida	MFLUCC 11-0439	JX442038	JX442042	KP761717
Corynespora cassiicola	CBS 100822	GU301808	GU296144	GU371742
Corynespora smithii	CABI 5649b	GU323201	–	GU371783
Darksidea zeta	CBS 135640	KP184013	KP184071	–
Darksidea alpha	CBS 135650	KP184019	KP184049	–
Darksidea beta	CBS 135637	KP184023	KP184074	–
Darksidea epsilon	CBS 135658	KP184029	KP184070	–
Deniquelata barringtoniae	MFLUCC 11-0422	JX254655	JX254656	–
Falciformispora lignatilis	BCC 21117	GU371826	GU371834	–
Falciformispora lignatilis	BCC 21118	GU371827	GU371835	–
Helicascus nypae	BCC 36752	GU479789	GU479755	GU479827
Kalmusia brevispora	KT 1466	AB524600	AB524459	AB539099
Kalmusia brevispora	KT 2313	AB524601	AB524460	AB539100
Karstenula rhodostoma	CBS 690.94	GU301821	GU296154	GU371788
Katumotoa bambusicola	MAFF 239641	AB524595	AB524454	AB539095
Keissleriella cladophila	CBS 104.55	GU301822	GU296155	–
Keissleriella dactylis	MFUCC 13-0751	KP197668	KP197666	–
Keissleriella poagena	CBS 136767	KJ869170	–	–
Keissleriella trichophoricola	CBS 136770	KJ869171	–	–
Lentithecium aquaticum	CBS 123099	GU301823	GU296156	–
Lentithecium arundinaceum	CBS 619.86	GU301824	GU296157	–
Lentithecium cangshanense	DLUCC 0143	KU991149	KU991150	KU991151
Lentithecium fluviatile	CBS 122367	GU301825	GU296158	–
Massarina cisti	CBS 266.62	FJ795447	FJ795490	FJ795464
Massarina eburnea	CBS 473.64	GU301840	GU296170	GU371732
Melanomma pulvis-pyrius	CBS 124080	GU456323	GU456302	GU456350
Montagnula opulenta	CBS 168.34	DQ678086	AF164370	DQ677984
Morosphaeria ramunculicola	JK 5304B	GU479794	GU479760	GU479831
Murilentithecium clematidis	MFLUCC 14-0562	KM408760	KM408761	KM454447
Murilentithecium clematidis	MFLUCC 14-0561	KM408758	KM408760	KM454446
Neottiosporina paspali	CBS 331.37	EU754172	EU754073	GU371779
Ophiosphaerella sasicola	MAFF 239644	AB524599	AB524458	–
Palmiascoma gregariascomum	MFLUCC 11-0175	KP744495	KP753958	–

...Continued on next page

TABLE 1. (Continued)

Taxon	Culture/voucher	GenBank Accession Number		
		LSU	SSU	*RPB2*
Poaceascoma helicoides	MFLUCC 11-0136	KP998462	KP998463	KP998460
Poaceascoma aquaticum	MFLUCC 14-0048	KT324690	KT324691	KT373846
Setoseptoria phragmitis	CBS 114802	KF251752	–	–
Stagonospora macropycnidia	CBS 114202	GU301873	GU296198	–
Stagonospora paludosa	CBS135088	KF251760	–	KF252262
Tingoldiago graminicola	JCM 16485	AB521743	AB521726	–
Trematosphaeria pertusa	CBS 122368	FJ201990	FJ201991	FJ795476
Trematosphaeria pertusa	CBS 122371	FJ201992	FJ201993	GU371801

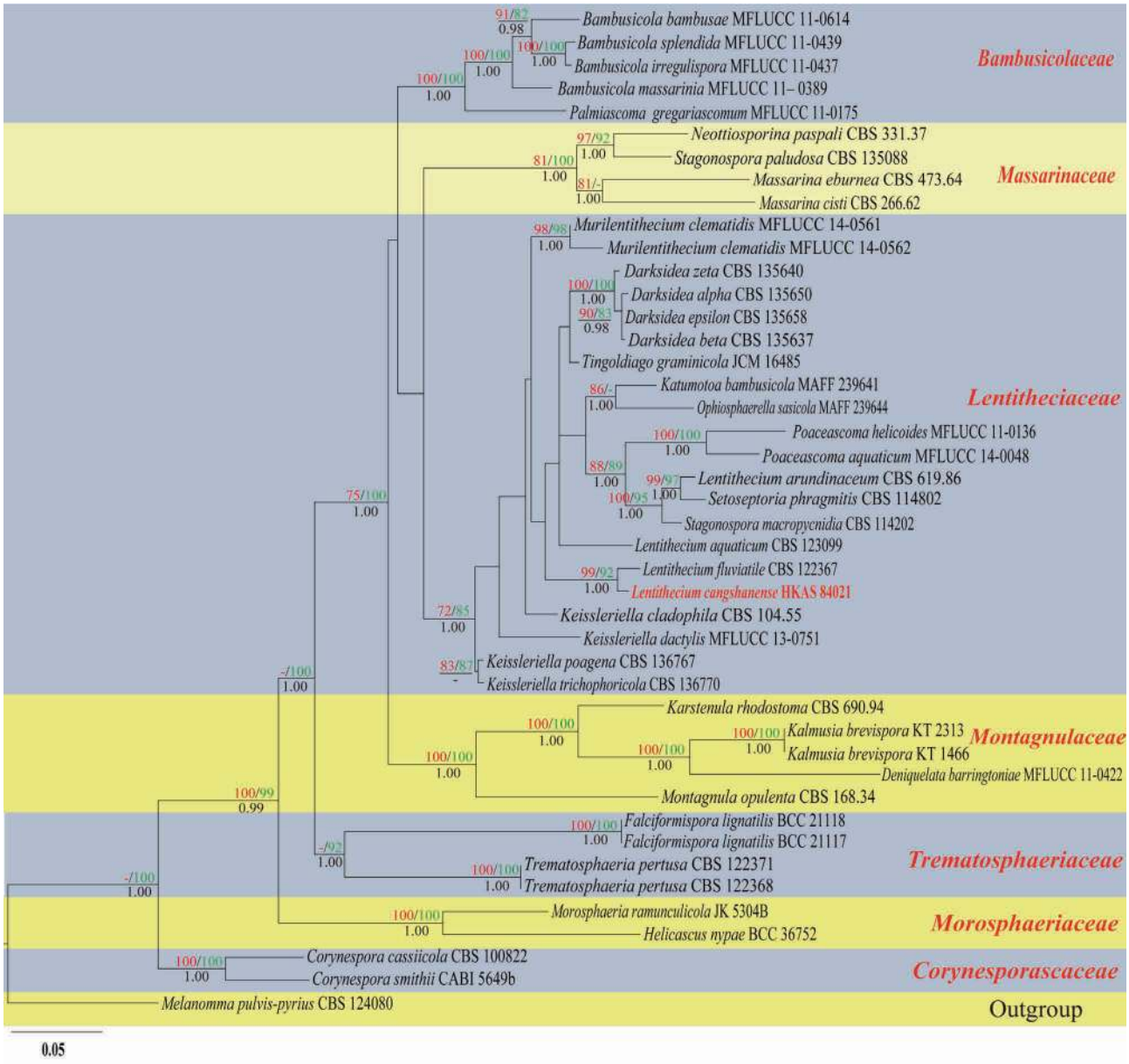

FIGURE 1. Phylogram generated from maximum likelihood analysis (RAxML) based on combined LSU, SSU and RPB2 sequenced data of the families in the suborder Massarineae. Bootstrap support values for maximum likelihood (ML, red) and maximum parsimony (MP, green) equal to or greater than 70% are given above the nodes. The values of the Bayesian posterior probabilities from MCMC analyses (BYPP, black) equal or higher than 95% are given below the nodes. The tree is rooted to *Melanomma pulvis-pyrius* (CBS 124080). Newly generated sequences are indicated in red.

Results

Phylogeny

Analysis of combined LSU, SSU and RPB2 sequence data were used to determine the taxonomic placement of our strain. The dataset comprised 44 taxa included all of genera of Lentitheciaceae, with *Melanomma pulvis-pyrius* (Pers.) Fuckel (CBS 124080) as the outgroup taxon. The phylogenetic trees generated by Maximum likelihood (ML), Maximum-parsimony (MP) and Bayesian analyses of combined LSU, SSU, RPB2 sequence data shown our strain to cluster in the family Lentitheciaceae (FIG. 1). The best scoring RAxML tree was selected to represent the relationships among the taxa and is shown in FIG. 1. The phylogenetic trees generated by Maximum likelihood (ML), Maximum-parsimony (MP) and Bayesian analyses of combined LSU, SSU, RPB2 gene regions showed that our strains clustered with *Lentithecium fluviatile* in the genus *Lentithecium* (FIG. 1).

Taxonomy

Lentithecium cangshanense Z.L. Luo, X.J. Su & K.D. Hyde, *sp. nov.* **FIGURE 2.**

Index Fungorum: IF 552050; Facesoffungi number: FoF 02079.

Etymology: With reference to the collecting location, Cangshan Mountain.

Holotype: HKAS 84021.

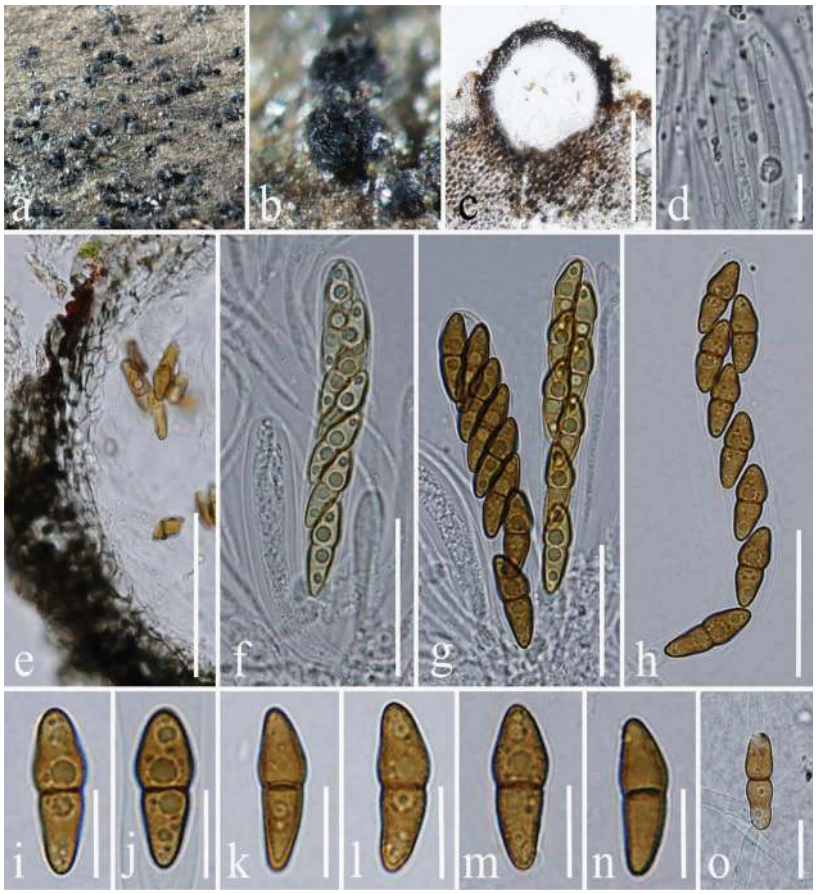

FIGURE 2. *Lentithecium cangshanense* (HKAS 84021, **holotype**). **a**, **b**. Ascomata on submerged bamboo. **c**. Section through ascoma. **d.** Pseudoparaphyses. **e.** Section of peridium. **f–h.** Asci. **i–n.** Ascospores. **o.** Germinating ascospore. Scale bars: c = 150 µm, e = 100 µm, f–h = 25 µm, o = 20 µm, d, i–n = 10 µm.

Saprobic on decaying wood submerged in freshwater. ***Sexual morph***: *Ascomata* 210−310 µm high, 220−320 µm in diam, scattered, black, immersed to partially erumpent, globose to subglobose, glabrous, with papilla visible as raised, dark spots on host surface, ostiole central. *Peridium* 20−29 µm wide, of equal thickness, composed of several layers of

pseudoparenchymatous cells, arranged in a *textura angularis*, pigmented at the outer part. *Hamathecium* comprising numerous, 2.5−3.5 µm wide, septate pseudoparaphyses, embedded in a mucilaginous matrix, rarely anastomosing between and above the asci. *Asci* 65–78× 11–13 µm ($\overline{x}$ = 71 × 12µm, n = 20), cylindric-clavate, pedicellate, apically rounded, with an ocular chamber. *Ascospores* 16.5–17.5 × 6–7 µm ($\overline{x}$ = 17 × 6.5 µm, n = 20), bi-seriate, hyaline to subhyaline when young and pale brown to brown when mature, broadly fusiform, inequilateral in side view,1-septate, slightly constricted at the septum, upper cell wider, smooth-walled, with four refractive globules. ***Asexual morph***: Undetermined.

Material examined:—CHINA, Yunnan Province (N 25°50'52.39", E 100°05'59.87"), saprobic on decaying wood submerged in a stream, July 2013, Z.L. Luo, S-143 (HKAS 84021, **holotype**), ex-type culture, DLUCC 0143 = KUMCC; *ibid*. (DLU14–143, **isotype**).

Notes: *Lentithecium cangshanense* is typical of *Lentithecium* being characterized by scattered, semi-immersed to superficial, ascomata, septate pseudoparaphyses, bitunicate, pedicellate asci, bi-seriate, pale brown to brown ascospores which are slightly constricted at the septum. *Lentithecium cangshanense* resembles *L. aquaticum* in having scattered ascomata, a peridium of *textura prismatica* and broadly fusiform, ascospores which are in equilateral in side view, and have four refractive globules and slightly constricted at the septum. However, *L. cangshanense* differs from *L. aquaticum* in having a thicker, pale black to hyaline peridium, narrower and shorter asci ($\overline{x}$ = 71.5 × 12 µm, versus 175 × 21 µm) and smaller ascospores ($\overline{x}$ = 17 × 6.5 µm, versus 28 × 10.5 µm). In addition, the molecular analysis showed that this new fungus is clusters with *L. fluviatile* with strong support (FIG. 1). However, they have different morphological characters, *L. cangshanense* differs from *L. fluviatile* in having pale brown to brown, smaller sacospores (16.5–17.5 × 6–7 µm, versus 24–31 × 7–10 µm).

Discussion

Zhang *et al*. (2012) introduced the family Lentitheciaceae in the order Pleosporales, typified by *Lentithecium*, with *L. fluviatile* as the type species. Species of the Lentitheciaceae occur on herbaceous plants such as *Phragmites* (*Lentithecium fluviatile*, *L. arundinaceum* (Sowerby) K.D. Hyde *et al*., *Tingoldiago graminicola* K. Hirayama & Kaz. Tanaka) and on submerged wood (*Lentithecium aquaticum* Ying Zhang *et al*.) in freshwater environments (Zhang *et al*. 2012). Phookamsak *et al.* (2015) introduced a new genus *Poaceascoma* to accommodate the ophiosphaeriella-like taxa in Lentitheciaceae and mentioned that the scolecosporous taxa were polyphyletic in the class Dothideomycetes. Several clades in Lentitheciaceae are not well-resolved, future studies in this family is likely to collect and provide data for this undersampled group.

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