



Bambusicola loculata sp. nov. (*Bambusicolaceae*) from bamboo

DONG-QIN DAI^{1,3,4,5}, ALI H. BAHKALI², WEN-JING LI^{4,5}, D. JAYARAMA BHAT^{4,6}, RUI-LIN ZHAO⁷
& KEVIN D. HYDE^{*1,3,4,5}

¹School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand.

²Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, KSA.

³World Agroforestry Centre, East and Central Asia, Kunming 650201, Yunnan, China.

⁴Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Science, Kunming 650201, Yunnan, China.

⁵Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand.

⁶No. 128/1-J, Azad Housing Society, Curca, P.O. Goa Velha-403108, India.

⁷The State key lab of Mycology, Institute of Microbiology, Chinese Academic of Science, Beijing 100101, China.

*Corresponding author: Kevin D. Hyde, email: kdhyde3@gmail.com

Abstract

A new ascomycete species, *Bambusicola loculata*, inhabiting decaying bamboo, is introduced based on morpho-molecular studies. *Bambusicola loculata* is characterized by immersed, dark, stromatic and loculate ascostromata, bitunicate, cylindrical-clavate asci and 1-septate, hyaline, narrowly fusiform ascospores, surrounded by an inconspicuous mucilaginous sheath. Maximum likelihood and Bayesian analyses of combined LSU, SSU, RPB2 and TEF1 gene sequence data as well as morphological characters show that our new taxon belongs to *Bambusicola*, *Bambusicolaceae*. The new species is compared with other morphologically and phylogenetically similar species.

Key words: *Dothideomycetes*, multi-gene, phylogeny, taxonomy

Introduction

Dai *et al.* (2012) introduced the genus *Bambusicola* D.Q. Dai & K.D. Hyde with four new species, typified by *B. massarinia* D.Q. Dai & K.D. Hyde. The genus is known from both its asexual and sexual morphs; one species (*B. bambusae*) having the sexual morph only and two species (*B. irregulispora* Hyde and *B. splendida*) known from its asexual morph only. *Bambusicola* was provisionally placed in family *Trematosphaeriaceae* based on Maximum parsimony analyses of a single LSU gene data set (Dai *et al.* 2012). Hyde *et al.* (2013) provided a combined phylogenetic analysis of a LSU, SSU, RPB2 and TEF1 dataset for families of Dothideomycetes. Species of *Bambusicola* aggregated into a separate clade from other families in the sub-order *Massarineae* for which Hyde *et al.* (2013) introduced the new family *Bambusicolaceae* to accommodate the genus *Bambusicola*. However, only LSU and SSU genes of *Bambusicola* species are available in that paper. Liu *et al.* (2015) isolated a fungus from dead frond of palm, and introduced a new genus *Palmiascoma* Phookamsak & K.D. Hyde in this family based on morphology and phylogenetic analysis.

From our on-going studies on the diversity and taxonomy of microfungi inhabiting bamboo (*Poaceae*, *Bambusoideae*) in Thailand and China, some new taxa have already been described (Dai *et al.* 2012, 2014a–c, Liu *et al.* 2011, 2012, 2014, 2015, Wijayawardene *et al.* 2014). In this paper, we introduce a new ascomycetous species which belongs to the genus *Bambusicola* by natural classification, and re-sequenced protein coding genes (RPB2 and TEF1) of the four species of *Bambusicola*.

Materials & methods

Collection and isolation of fungi

Dead bamboo culms were collected from Doi Mae Salong in Chiang Rai Province, Thailand. The samples were placed in plastic Ziplock bags and brought to the laboratory. The specimen was examined under stereo and compound microscopes and the taxon isolated by single spore isolation following the method of Chomnunti *et al.* (2014). The growing colonies were transferred to 1.5 ml microcentrifuge tubes with 2% potato-dextrose agar (PDA) to deposit at 4°C and suspended in 2 ml screw cap microcentrifuge tubes with 15% Glycerol to store at –20°C. Microscopic observations and photomicrographs were made as described in Boonmee *et al.* (2011). Type materials are deposited at the herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU) and Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN). The living cultures are deposited at Mae Fah Luang University Culture Collection (MFLUCC) and the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands (CBS).

DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA for 30 d at 25°C and genomic DNA was extracted from fresh mycelia, following the specification of Biospin Fungus Genomic DNA Extraction Kit (BioFlux®). ITS5 and ITS4, NS1 and NS4 (White *et al.* 1990) and LROR and LR5 (Vilgalys & Hester 1990) primers were used for the amplification of internal transcribed spacers (ITS), small subunit rDNA (SSU) and large subunit rDNA (LSU) respectively. Translation elongation factor 1- α gene region (*TEF 1-alpha*) and RNA polymerase II subunit 2 (RPB2) was amplified by using EF1-983F and EF1-2218R primers (Rehner 2001), fRPB2-5f and fRPB2-7cr primers (Liu *et al.* 1999) respectively. Polymerase chain reaction (PCR) amplification was carried out following the method of Phillips *et al.* (2008). Amplified PCR fragments were sequenced at Kunming Shuo Yang Technology Company, P.R. China. Generated new sequences of ITS, LSU, SSU, RPB2 and TEF1 regions are deposited in GenBank.

Sequence alignment and phylogenetic analyses

Blast searches at GenBank were carried out for both LSU and SSU rDNA sequences in order to reveal the closest taxa to our strain. Sequence data for *Bambusicolaceae* were selected from Hyde *et al.* (2013) (Table 1). Further, we included other families from the sub-order *Massarinae* of *Pleosporales* i.e. *Didymosphaeriaceae*, *Massarinaceae*, *Morosphaeriaceae*, *Lentitheciaceae* and *Trematosphaeriaceae* (Hyde *et al.* 2013, Lui *et al.* 2014, Wijayawardene *et al.* 2014). The tree was rooted to *Halojulella avicenniae* (BCC 18422). Sequences were aligned using Bioedit (Hall 2001) and ClustalX (Kohli & Bachhawat 2003). Alignments were checked and manual adjustments were carried out when necessary. In the analyses, gaps were treated as missing data, and all characters were unordered and of equal weight (Begoude *et al.* 2010, Liu *et al.* 2011, 2012).

TABLE 1. DNA sequences used for the phylogenetic tree in Fig 1

Species	Strain	GenBank accession numbers			
		LSU	SSU	RPB2	TEF1
<i>Bambusicola bambusae</i>	MFLUCC 11-0614*	JX442035	JX442039	KP761718	KP761722
<i>Bambusicola irregulispora</i>	MFLUCC 11-0437*	JX442036	JX442040	KP761719	KP761723
<i>Bambusicola loculata</i>	MFLUCC 13-0856*	KP761729	KP761735	KP761715	KP761724
<i>Bambusicola massarinia</i>	MFLUCC 11-0389*	JX442037	KP761736	KP761716	KP761725
<i>Bambusicola splendida</i>	MFLUCC 11-0439*	JX442038	JX442042	KP761717	KP761726
<i>Bimuria novae-zelandiae</i>	CBS 107.79*	AY016356	AY016338	DQ470917	DQ471087
<i>Didymosphaeria rubi-ulmifolii</i>	MFLUCC 14-0023*	KJ436586	KJ436588		
<i>Falciformispora lignatilis</i>	BCC 21118	GU371827	GU371835		GU371820
<i>Falciformispora lignatilis</i>	BCC 21117	GU371826	GU371834		GU371819
<i>Halojulella avicenniae</i>	BCC 18422	GU371823	GU371831	GU371787	GU371816
<i>Helicascus nypae</i>	BCC 36752	GU479789	GU479755	GU479827	GU479855
<i>Katumotoa bambusicola</i>	MAFF 239641	AB524595	AB524454	AB539095	AB539108

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TABLE 1. (Continued)

Species	Strain	GenBank accession numbers			
		LSU	SSU	RPB2	TEF1
<i>Lentithecium aquaticum</i>	CBS 123099*	GU301823	GU296156	GU371789	GU349068
<i>Lentithecium fluviatile</i>	CBS 122367	GU301825	GU296158		GU349074
<i>Massarina cisti</i>	CBS 266.62*	FJ795447	FJ795490	FJ795464	
<i>Massarina eburnea</i>	CBS 473.64	GU301840	GU296170	GU371732	GU349040
<i>Massarina igniaria</i>	CBS 845.96	FJ795452	GU296171	GU371793	
<i>Montagnula opulenta</i>	CBS 168.34	DQ678086	AF164370	DQ677984	
<i>Morosphaeria ramunculicola</i>	JK 5304B	GU479794	GU479760	GU479831	
<i>Murilentithecium clematidis</i>	MFLUCC 14-0561*	KM408758	KM408760	KM454446	KM454444
<i>Neokalmusia brevispora</i>	CBS 120248	AB524600	AB524459	AB539099	AB539112
<i>Neokalmusia scabrisspora</i>	CBS 120246	AB524593	AB524452	AB539093	AB539106
<i>Neottiosporina paspali</i>	CBS 331.37	EU754172	EU754073	GU371779	GU349079
<i>Palmiascoma gregariascomum</i>	MFLUCC 11-0175*	KP744495	KP753958		KP998466
<i>Pseudodidymosphaeria spartii</i>	MFLUCC 13-0273*	KP325436	KP325438		
<i>Stagonospora paludosa</i>	CBS 135088*	KF251760		KF252262	KF253207
<i>Stagonospora perfecta</i>	CBS 135099*	KF251761		KF252263	KF253208
<i>Trematosphaeria pertusa</i>	CBS 122368*	FJ201990	FJ201991	FJ795476	GU456276
<i>Trematosphaeria pertusa</i>	CBS 122371	FJ201992	FJ201993	GU371801	GU349085

The ex-types or ex-epitype strains are with asterisk. Newly obtained sequences in this study are printed in **bold**. Abbreviations: **BCC**: BIOTEC Culture Collection, Bangkok, Thailand; **CBS**: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **MAFF**: Ministry of Agriculture, Forestry and Fisheries, Japan; **MFLUCC**: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; **JK**: J. Kohlmeyer.

Single gene is not well support to investigate the phylogenetic relationships of *Dothideomycetes* taxa, however, multi-gene analyses are usually considered to be used by mycologists (Zhang *et al.* 2009, Hyde *et al.* 2013, Lui *et al.* 2014, Wijayawardene *et al.* 2014). Therefore, in this study combined LSU, SSU, RPB2 and TEF1 gene sequence data was used in the analyses. All sequences obtained from GenBank and used by Dai *et al.* (2012), Zhang *et al.* (2012), Hyde *et al.* (2013), Liu *et al.* (2014) and Wijayawardene *et al.* (2014) are listed in Table 1. Regions containing many leading or trailing gaps were removed from the alignments prior to tree building. The alignments were checked visually and manually improved wherever necessary.

Maximum likelihood analyses including 1000 bootstrap replicates were run using RAxML v. 7.2.6 (Stamatakis 2006, Stamatakis *et al.* 2008). The online tool Findmodel (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) was used to determine the best nucleotide substitution model for each partition. The best scoring tree was selected with a final likelihood value of -2854.594913. The resulting replicates were plotted on to the best scoring tree obtained previously.

Bayesian analyses were performed by using PAUP v. 4.0b10 (Swofford 2002) and MrBayes v. 3.0b4 (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). Bayesian analyses of 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 (Liu *et al.* 2012).

Trees were visualized with TreeView (Page 1996) and MEGA5 (Tamura *et al.* 2011).

Results

Phylogeny of the combined LSU, SSU, RPB2 and TEF1 gene data set

Partial nucleotide combined sequences of LSU, SSU, RPB2 and TEF1 were used to determine the taxonomic placement of our strain. The dataset comprised 30 strains including one newly sequenced taxon plus one outgroup taxon (Table

1). The best tree is presented in Fig. 1. The phylogenetic trees generated by Maximum likelihood (ML) and Bayesian analyses of combined LSU, SSU, RPB2 and TEF1 gene regions have shown that new taxon clusters within the family *Bambusicolaceae* and same clade within genus *Bambusicola* with high bootstrap support (99%/1.00, MLBS/BYPP) (Fig. 1). Bootstrap support (BS) values of ML and the Bayesian posterior probabilities (PP) from MCMC analyses are shown in Fig 1.

New sequences ITS (KP761732), LSU (KP761729), SSU (KP761735), RPB2 (KP761715) and TEF1 (KP761724) are deposited in GenBank for further studies. All the strains used in this paper together with their GenBank associate numbers are listed in Table 1.

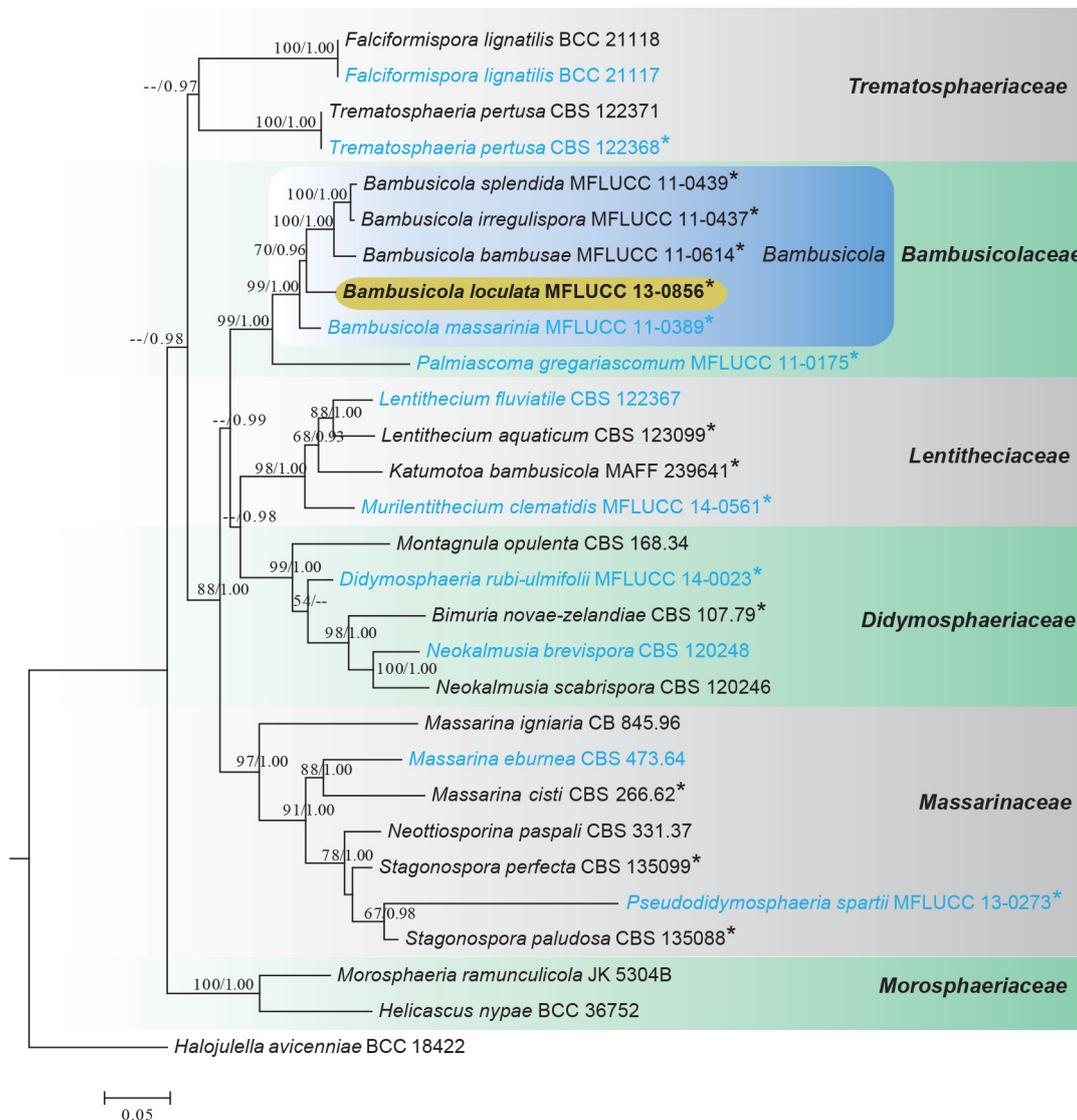


FIGURE 1. Maximum likelihood (RAxML) tree based on combined dataset of LSU, SSU, RPB2 and TEF sequence. Bootstrap support (BS) values for RAxML above 50% and Bayesian posterior probabilities greater than 0.95 are shown near nodes. Hyphen (“--”) indicates a value lower than 50% (BS) or 0.90 (BYPP). The original strain numbers are noted after the species names. Ex-type or ex-epitype strains are with asterisk, and the type species are indicated in blue. Newly species obtained in this study is in bold and highlight. The tree is rooted with *Halojulella avicenniae* (BCC 18422).

Taxonomy

Bambusicolaceae D.Q. Dai & K.D. Hyde, in Hyde *et al.*, Fungal Diversity 63: 49 (2013)

Notes:—The family *Bambusicolaceae* was introduced for bitunicate, ascomycetous fungi from bamboo with hyaline ascospores and oblong, septate conidia (Dai *et al.* 2012, Hyde *et al.* 2013). Liu *et al.* (2015) introduced *Palmiascoma*

from palms, which have brown ascospores and ellipsoidal conidia without septa, thus widening the family concept. The genus *Palmiascoma* is morphologically similar to *Didymosphaeria* and *Verruculina* in having brown, echinulate, 1-septate ascospores (Liu *et al.* 2015). However, *Palmiascoma* is phylogenetically separate from the above genera and clusters within the *Bambusicolaceae* clade.

Bambusicolaceae shares similar morphological characters with some families in the order *Pleosporales*, in having cylindrical to clavate asci and fusiform to ellipsoidal, hyaline to brown, 1-septate ascospores, i.e. *Didymosphaeriaceae*, *Massarinaceae* and *Tetraplosphaeriaceae* (Tanaka *et al.* 2009, Zhang *et al.* 2009, Dai *et al.* 2012, Hyde *et al.* 2013). However, the family *Bambusicolaceae* differs from other families by the asexual morphs. Phylogenetic analyses also show that *Bambusicolaceae* is distinct in the sub-order *Massarineae* (Hyde *et al.* 2013, Liu *et al.* 2014).

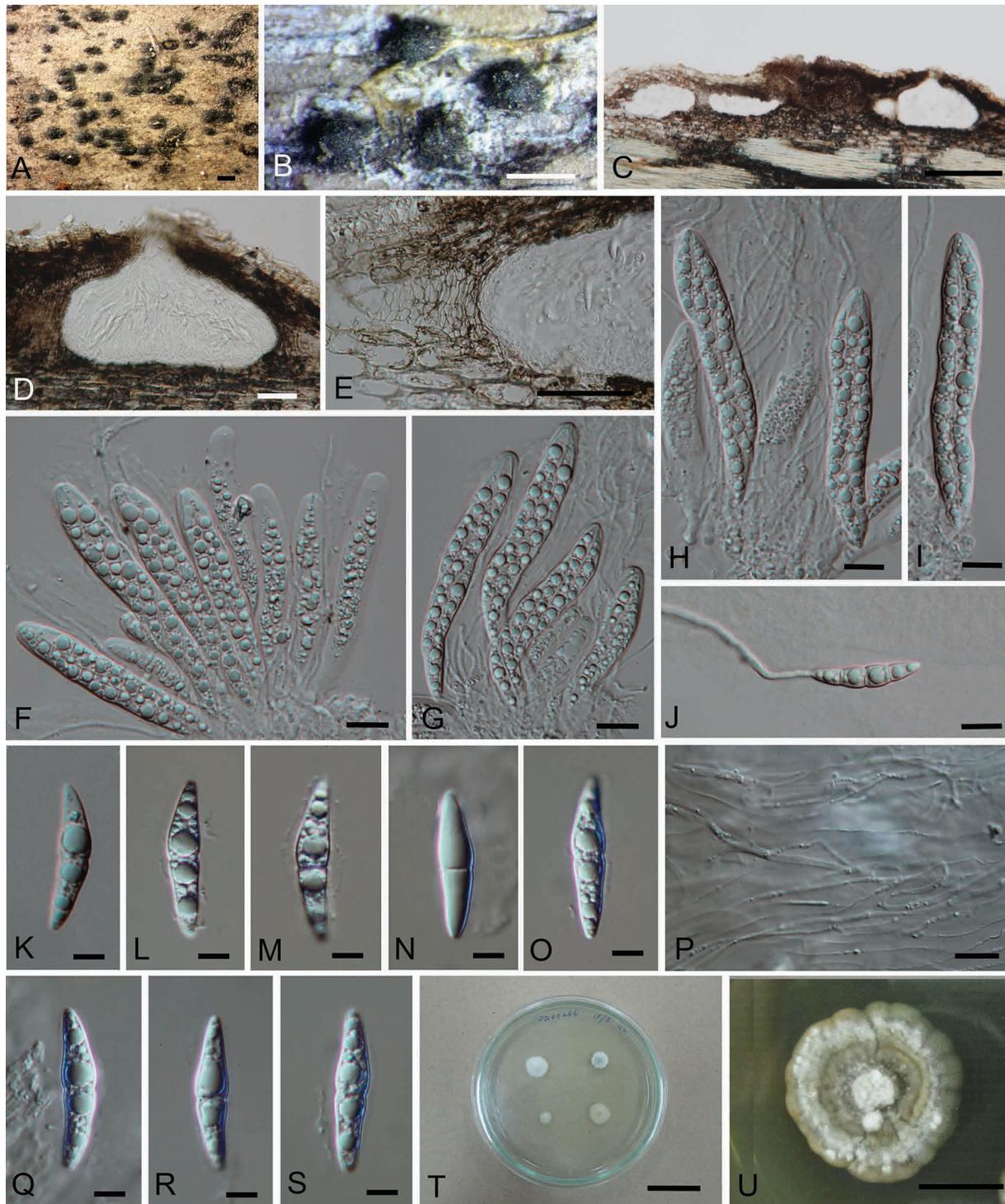


FIGURE 2. *Bambusicola loculata* (MFLU 15-0056, holotype). A, B. Ascomata developing on bamboo culm. C, D. Section of ascoma. E. Peridium of ascoma. H–G, P. Asci and pseudoparaphyses. J. Germinating ascospore. K–O, Q–S. Hyaline ascospores. T, U. Culture on PDA. Scale bars: A, B = 500 μ m, C = 200 μ m, D, E = 50 μ m, F–J = 10 μ m, K–S = 5 μ m, T = 25 mm, U = 15 mm.

Bambusicola loculata D.Q. Dai & K.D. Hyde, *sp. nov.* Figure 2

Index Fungorum number: IF551064

Facesoffungi number: FoF 00587

Etymology: In reference to the loculate ascostromata.

Diagnosis: this new species differs from other species in the genus as it has multi-loculate ascostromata and is phylogenetically resolved from species where the asexual morph is only known.

Holotype: MFLU 15–0056.

Saprobic on dead bamboo culms. **Sexual morph:** *Ascostromata* 150–200 µm high, 350–600 µm diam., solitary to clustered, or two in groups, becoming erumpent, but remaining immersed under host tissue at maturity, with a central ostiolar opening when mature, with 1–3 locules; individual *locules* 150–180 µm high, 200–300 µm diam., immersed in stromata, subglobose, with centrally located ostiole lined with periphyses. *Peridium* comprising host and fungal tissues, 5–10 µm thick at upper side, composed of brown and thick-walled cells of *textura angularis*, with basal part composed of thinner, hyaline, smaller cells, with side wall composed of 30–80 µm wide cells of *textura prismatica*. *Hamathecium* of dense, long and up to 1 µm wide pseudoparaphyses, branching and anastomosing above the asci. *Asci* 80–105 × 8–13 µm (\bar{x} = 90.1 × 11.8 µm, n = 20), 8-spored, bitunicate, cylindrical-clavate, with a short furcate pedicel, with a shallow ocular chamber. *Ascospores* 22–26.5 × 5–6 µm (\bar{x} = 24.5 × 5.2 µm, n = 20), 2–3-seriate, narrowly fusiform, 1-septate, constricted at the septum, slightly curved, smooth-walled, narrowly rounded at both ends, hyaline, surrounded by an inconspicuous mucilaginous sheath. **Asexual morph:** Undetermined.

Culture on PDA:—Ascospores germinating on PDA within 24 h and germ tubes produced at both ends. *Colonies* slow growing, 40 mm diam. in 45 days at 25–32°C, circular, with uneven margin, light-colored at the periphery, floccose and hyaline to light greenish brown from above; pure yellow at the centre from reverse.

Material examined:—THAILAND. Chiang Rai: Doi Mae Salong, temple side, on dead culm of bamboo, 15 August 2013, *Dong-Qin Dai DDQ00266* (MFLU 15–0056, **holotype**), *ibid.* (KUN! HKAS 86441, **isotype**); ex-type living culture at MFLUCC 13–0856 and CBS 139961.

Notes:—*Bambusicola loculata* differs from the uniloculate *B. massarinia* and *B. bambusae* in having multi-loculate ascostromata. In addition, the asci of the new species are longer than those of two latter (90.1 × 11.8 µm vs. 70.1 × 12 µm and 56.6 × 6.6 µm). *Bambusicola irregulispora* and *B. splendida* are only known from their asexual morphs. Moreover the new taxon is distinguished from the other species in the phylogenetic analyses (Fig. 1). The new species can be morphologically and phylogenetically distinguish from *Palmiascoma gregariascomum* (99%/1.00 MLBS/BYPP support) (Fig. 1) which has brown ascospores (Liu *et al.* 2015).

Key to species of *Bambusicolaceae*

1.	Fruiting bodies ascostromata	2
1.	Fruiting bodies conidiomata	5
2.	Ascospores dark brown	<i>Palmiascoma gregariascomum</i>
2.	Ascospores hyaline	3
3.	Ascostromata containing more than one locule	<i>Bambusicola loculata</i>
3.	Ascostromata containing a single locule	4
4.	Ascostromata forming dark rounded erumpent spots on host surface, with a black halo around the ostiolar opening	<i>B. massarinia</i>
4.	Ascostromata forming dark brown spots on the host surface, without a black halo around the ostiolar opening	<i>B. bambusae</i>
5.	Conidiomata acerose, conidia 15–18 × 1.5–3 µm	<i>B. irregulispora</i>
5.	Conidiomata subglobose, conidia 20–30 × 3.5–5 µm	<i>B. splendida</i>

Discussion

Based on the recent studies (Dai *et al.* 2012, Hyde *et al.* 2013, Liu *et al.* 2015), we compared all five known species in the genera of *Bambusicolaceae*, viz. *Bambusicola* and *Palmiascoma*, and include all strains of species in this family in the phylogenetic analyses to determine the placement of our new taxon. The dataset of combined LSU, SSU, RPB2 and TEF1 genes were used in the Maximum likelihood (RAxML) and Bayesian analyses. *Bambusicola loculata* is embedded within the family *Bambusicolaceae* based on the phylogenetic analyses (Fig. 1) and morphologic character.

This new taxon can be phylogenetically distinguished from other species of *Bambusicolaceae* (70%/0.96 MLBS/BYPP support) (Fig. 1).

Zhang *et al.* (2012) suggested that the protein coding genes, RPB2 and TEF1, can well resolve the relationships of taxa of the order *Pleosporales*. Thus we sequenced RPB2 and TEF1 genes of the known species of *Bambusicola* published in Dai *et al.* (2012) with only ITS, LSU and SSU genes. In our phylogenetic analyses, the new taxon together with four species of *Bambusicola*, as well as *Palmiascoma* are accommodated within the family *Bambusicolaceae* (Fig.1) and are resolved as distinct genera.

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