Fungi from Asian Karst formations II. Two new species of *Occultibambusa* *(Occultibambusaceae, Dothideomycetes)* from karst landforms of China

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Abstract

During an investigation of saprobic ascomycetes from karst landforms in southwest China, two new species were isolated from dead bamboo culms collected from Maolan Town in Guizhou Province. The new taxa share similar morphological characters as known *Occultibambusa* species in having immersed, papillate ascoma, broadly-cylindrical to clavate asci and fusiform, hyaline to brown ascospores. Phylogenetic analysis of combined LSU, SSU, TEF1-α and RPB2 sequence data also placed the new taxa within the genus *Occultibambusa* in the family *Occultibambusaceae* with good support. The new taxa can be distinguished from other species by septation and different-sized ascospores and the present or absence of sheaths. The new species, *Occultibambusa jonesii* and *O. maolanensis* are introduced here, with descriptions, illustrations and molecular data.

Key words — Dothideomycetes — phylogeny — Pleosporales — taxonomy

Introduction

We are carrying out the fungal diversity survey in the Karst formations of the Asian region and this is the second in a series of papers (Chen et al. 2017). The family of *Occultibambusaceae* D.Q. Dai & K.D. Hyde was introduced by Dai et al. (2017) and assigned to the order Pleosporales. The family is typified by *Occultibambusa* and characterized by immersed, solitary to gregarious ascoma, cylindrical to clavate, bitunicate asci and fusiform, hyaline to brown, septate ascospores and rather diverse asexual morphs. The family presently comprises four genera: *Neooccultibambusa* Doilom & K.D. Hyde (Doilom et al. 2017), *Occultibambusa* D.Q. Dai, *Seriascoma* Phookamsak., D.Q. Dai & K.D. Hyde (Dai et al. 2017) and *Versicolorisporium* Sat. Hatak., Kaz. Tanaka & Y. Harada (Hatakeyama et al. 2008).

Species in this family occur on monocotyledons and hardwood trees, and share similar morphology with species of the genera *Bambusicola*, *Lophiostoma* and *Massarina* in having clavate asci and fusiform ascospores, however they can be distinguished readily via phylogenetic analysis.
(Zhang et al. 2009, Dai et al. 2012, 2015, 2017). Dai et al. (2017) indicated that the family Occultibambusaceae is phylogenetically close to Biatriosporaceae, but differs from members of the latter, which usually having dark brown ascospores with hyaline, rounded, swollen ends which release mucilage (Hyde et al. 1986, 2013, Dai et al. 2017). There is confusion surrounding Biatriosporaceae and until Biatriospora marina is epitypified (sensu Ariyawansa et al. 2014), we follow the classification of Wijayawardene et al. (2014).

In the course of an ongoing survey of saprobic ascomycetes from Karst landforms, two new taxa were isolated from dead bamboo culms collected in Guizhou Province, southwest China. Molecular analysis of combined LSU, SSU, TEF1-α and RPB2 sequence data placed the new taxa within the family of Occultibambusaceae where they cluster with Occultibambusa species with good support. The taxa also share similar morphological characters with existing Occultibambusa species. Therefore, O. jonesii and O. maolanensis are introduced to accommodate the new taxa.

Materials & Methods

Collection, examination and isolation of specimens

Samples were collected from Maolan Town in Guizhou Province, and taken back to laboratory in envelopes. Examination and vertical sections of samples were processed under a stereomicroscope (Nikon SMZ 745) and a compound microscope (Nikon E100). Micro-morphological characters were observed under the Nikon ECLIPSE Ni compound microscope and captured by using the Canon EOS 70D digital camera with DIC microscopy. The Tarosoft (R) Image Frame Work version 0.9.7 program was used to measure micro-morphological characters, and photographic plates were edit by using Adobe Photoshop CS6 (Adobe Systems Inc., USA).

Isolates were made from single ascospore following the method by Chomnunti et al. (2014). The single germinated ascospore was individually transferred to potato dextrose agar (PDA; 39 g/l distilled water, Difco potato dextrose) and incubated at 25 °C in the dark for recording growth rates and culture characters. The holotypes are deposited at the herbarium of Guizhou Academy of Agricultural Sciences (GZAAS), Guiyang, China and duplicated at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS), Kunming, China. Isolates are deposited at Guizhou Culture Collection (GZCC), Gui Yang, China and duplicated at Kunming Culture Collection (KUMCC), Kunming, China. Facesoffungi and Index Fungorum numbers are provided as explained in Jayasiri et al. (2015) and Index Fungorum (2017).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the fresh mycelia, and the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, P. R. China) was used to processed it following the manufacturer’s instructions.

Primers of LR0R and LR5 (Vilgalys & Hester 1990), NS1 and NS4 (White et al. 1990) were used for the amplification of large subunit rDNA (LSU) and small subunit rDNA (SSU) respectively. Translation elongation factor 1-α gene (TEF 1-α) and RNA polymerase II second largest subunit gene (RPB2) were amplified by the primers of EF1-983F and EF1-2218R (Rehner 2001), fRPB2-5f and fRPB2-7cr (Liu et al. 1999) respectively.

DNA amplification procedure was performed by Polymerase Chain Reaction (PCR) in a 50 μl reaction volume, which contains 19 μl Distilled-Deionized-water, 25 μl of 2 × Power Taq PCR Master Mix (TIANGEN Co., China), 2 μl of DNA template and 2 μl of each forward and reverse primers. The PCR thermal cycle program of LSU, SSU and TEF1-α gene amplifications were provided as: initially 94 °C for 3 minutes, followed by 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 56 °C for 50 seconds, elongation at 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. The PCR thermal cycle program for RPB2 genes was provided as: initially 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 1 minute, annealing at 52 °C for 2 minutes, elongation at 72 °C for 90 seconds, and a final extension at 72 °C for 10 minutes. The quality of PCR products was checked by using 1.2% agarose gel electrophoresis stained with
ethidium bromide and then sent to sequence at Invitrogen Biotechnology Co., Ltd (Shanghai, P. R. China). Newly generated sequences have been submitted to GenBank.

### Sequence alignment and phylogenetic analyses

Newly generated sequences were checked and combined in the program of BioEdit v.7.1.3 (Hall 1999). Then, a BLAST search with the LSU sequence in GenBank was performed to reveal the preliminary identification, and additional sequences were downloaded based on their identities and related publications. Single gene sequence alignments were processed in MAFFT v. 7.215 (Katoh & Standley 2013: [http://mafft.cbrc.jp/alignment/server/index.html](http://mafft.cbrc.jp/alignment/server/index.html)) respectively and edited manually where necessary to minimize the number of uninformative gaps in BioEdit v.7.2. The program of MEGA v.6.6 (Tamura et al. 2013) was used to concatenate the individual datasets into a combined dataset. And the data were converted from fasta to nexus format for Bayesian analysis in ClustalX2 v.1.83 (Thompson et al. 1997) or PHYLIP format for RAxML analysis in the online program ALTER ([http://sing.ei.uvigo.es/ALTER/](http://sing.ei.uvigo.es/ALTER/)).

Maximum likelihood (ML) analysis with 1000 bootstrap replicates was run in the RAxMLGUI v. 1.5b1 program (Silvestro & Michalak, 2012), and the default algorithm was used from a random starting tree for each replicate. The number of replications was inferred using the stopping criterion. Branches of bootstrap values greater than 75% were shown in the tree. The final tree was selected among suboptimal trees from each replicate by comparing likelihood scores under the GTR+GAMMA substitution model.

Bayesian analysis was performed by using MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). The best-fit model of evolution was estimated in MrModeltest 2.3 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4. 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. The branches, which posterior probabilities with those equal or greater than 0.95, were thickened in Fig. 1.

The final layout of phylogenetic tree was visualized with TreeView v. 1.6.6 (Page 1996), and edit by using Adobe Illustrator CS5 (Adobe Systems Inc., USA).

### Results

#### Phylogenetic analysis

The LSU, SSU, TEF1-α and RBP2 dataset was combined and comprised 25 taxa with *Westerdykella ornata* (CBS 379.55) as the outgroup taxon. The dataset comprised 3,677 characters (LSU-851, SSU-982, TEF1-α-919, RBP2-916) after alignment, of which 2,866 characters are constant, and 644 characters are parsimony-informative, while 167 variable characters are parsimony-uninformative in the maximum parsimony (MP) analysis. The best scoring RAxML tree is shown in Fig. 1. The optimal tree (not shown) generated by Bayesian analysis had a similar topology with the RAxML tree.

Most of sequence data for this study are selected from Dai et al. (2017) and Hyde et al. (2016), and most of other families, which are phylogenetically close to this group, are selected from previous studies (Hyde et al. 2013, Liu et al. 2014). The results show that the two new taxa are placed in the genus *Occultibambusa*. *Occultibambusa jonesii* is phylogenetically close to *O. aquatica* with high support (MLBS 97/ BIPP 1.0), and *O. maolanensis* clusters with *O. fusispora* with good support (MLBS 85/ BIPP 0.99). Moreover, all *Occultibambusa* species formed a well-supported (MLBS 83/ BIPP 1.0) clade in the family of *Occultibambusaceae*.
Table 1 GenBank accession numbers of sequences used in phylogenetic analyses. New sequences from this study are in bold.

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Abbreviation: CBS: Centraalbureau voor Schimmelcultures, The Netherlands; CPC: Collection of Pedro Crous housed at CBS; GZCC: Guizhou culture collection, Guizhou, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; KT: K. Tanaka.

Taxonomy

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

Index Fungorum number: IF 552743
Faces of fungi number: FoF 02873

Etymology – Named in honour of E.B. Gareth Jones for his contributions to tropical mycology. Holotype – GZAAS 16-0162
Saprobic on dead bamboo culms, forming dark, raised spots on the host surface. Sexual morph *Ascostromata* 196–236 µm high, 200–260 µm diam, immersed to erumpent, solitary to gregarious, subglobose, ostiolate, papillate, coriaceous, flattened at the base. *Peridium* up to 10–52

![Image of phylogenetic tree]

Fig. 1 – Maximum likelihood phylogenetic tree by RAxML (GTR+G model) analysis based on combined LSU, SSU, TEF1-α and RPB2 sequence data. ML values (≥ 75%) resulting from 1000 bootstrap replicates are shown near the nodes and branches with Bayesian posterior probabilities (PP) greater than 0.95 are in bold. The original isolate numbers are noted after the species names. The tree is rooted to *Westerdykella ornata* (CBS 379.55), and the scale bar shows 0.1 changes.
Fig. 2 – Occultibambusa jonesii (holotype, GZAAS 16-0162). a Appearance of ascostromata on dead bamboo culms. b Vertical section through ascostroma. c Section through peridium. d Pseudoparaphyses. e-h Asci with ascospores. i-m Ascospores. Scale bars: b = 100 µm, c = 30 µm, d-h = 20 µm, i-m = 10 µm
μm wide, thin at the base and becoming wider laterally, composed of several layers of dark brown cells, arranged in a **textura angularis**, and the outermost layer intermingled with host tissue. **Hamathecium** comprising dense, 2–3 μm wide, pseudoparaphyses, which anastomose above and between the asci, embedded in a gelatinous matrix. **Asci** (65–)75–89(–105) × 13.5–19 μm (x̄ = 85 × 16.5 μm, n = 20), 8-spored, bitunicate, fissitunicate, broadly cylindrical to clavate, short pedicellate, apically rounded to truncate, with an ocular chamber. **Ascospores** 27–33.5 × 5.5–6.5 μm (x̄ = 29.5 × 6 μm, n = 20), 1–3-seriate, 2-celled, constricted at the septum, and the upper cell swollen near the septum, inequilateral-fusiform, slightly curved, hyaline and guttulate when young and becoming brown to grayish when mature, wall smooth, without any mucilaginous sheath and appendages. Asexual morph – Undetermined.

Culture characters – Ascospores geminating on WA within 12 hours. Colonies reaching 35 mm diameter on PDA in three weeks at 25 °C, circular, dense, regular at the margin, raised at the center, gray from above and dark olive-green to black from below.

Material examined – CHINA, Guizhou Province, Maolan Town, on dead bamboo culms, 20 July 2016, J.F. Zhang, MLC’12, (GZAAS 16-0162, holotype); ex-type living culture, GZCC 16-0117; Ibid., 10 November 2016, J.F Zhang (HKAS96379); living culture KUMCC 17-0136.


- **Faces of fungi number:** IF 552744
- **Etymology** – Refers to the holotype was collected from Maolan Town.
- **Holotype** – GZAAS 16-0161
- **Saprobiec** on dead bamboo culms, forming dark, rounded spots on the host surface. Sexual morph – **Ascostromata** 544–600 μm diameter, solitary to gregarious, immersed under the epidermis, subglobose, coriaceous, slightly conical in vertical section, and flattened at the base, ostiolate, with a short, rounded, shiny, black papilla. **Peridium** up to 20–42 μm laterally composed of several layers of brown cells, becoming thin-walled and hyaline towards the centrum, arranged in a **textura angularis**, thick and darkly pigmented around ostiole, intermingled with host tissue. **Hamathecium** comprising dense, 1.5–2.4 μm wide, hypha-like pseudoparaphyses, branched and swollen towards the terminal cells, anastomosing above and between the asci, embedded in a gelatinous matrix. **Asci** (66–)77–85(–94) × 17–20(–24) μm (x̄ = 81 × 20 μm, n = 20), 8-spored, bitunicate, fissitunicate, broadly cylindrical to clavate, short pedicellate, apically rounded to truncated with a visible ocular chamber (2.5–3.5 μm wide). **Ascospores** 25–31 × 8–10 μm (x̄ = 28 × 9 μm, n = 30), 2–4-seriate, 2-celled, and moderately constricted at the septum, inequality-fusiform, apical cells 14–18 μm, basal cells 11–15 μm, slightly curved, hyaline and guttulate when young and become light brown when mature, wall smooth, without any mucilaginous sheath and appendages. Asexual morph – Undetermined.

Culture characters – Ascospores geminating on WA within 24 hours. Colonies reaching 30 mm diameter on PDA in three weeks at 25 °C, circular, dense, regular at the margin, gray from above and black from below.

Specimens examined – CHINA, Guizhou Province, Maolan Town, on dead bamboo culms, 8 July 2015, J.F. Zhang, MLC-29, (GZAAS 16-0161, holotype); ex-type living culture, GZCC 16-0116; Ibid., 10 November 2016, J.F Zhang (HKAS96380); living culture KUMCC 17-0137.

**Discussion**

The taxa that occur on bamboo are rather unique, often family specific grouping that appear to have a considerable diversity (Hyde et al. 2002, Liu et al. 2011, Jaklitsch et al. 2015, Dai et al. 2017). In this paper, we introduce two new species, **Occultibambusa jonesii** and **O. maolanensis** from bamboo, with molecular and morphological support. **Occultibambusa jonesii** is phylogenetically close to **O. aquatica**, but can be distinguished from it by its larger ascii (65–105 × 13.5–19 μm vs. 73–86 × 9–13 μm), longer ascospores (27–33.5 μm vs. 19–25 μm), and the new taxa also lacks a mucilage sheath surrounding the ascospores. **Occultibambusa maolanensis** clusters
Fig. 3 – *Occultibambusa maolanensis* (holotype, GZAAS 16-0161). a Appearance of ascostromata on dead bamboo culms. b Rounded, shiny black papilla. c Vertical section through ascostroma. d Section through peridium. e Pseudoparaphyses. f-h Asci with ascospores. i-l Ascospores. Scale bars: a = 500 µm, b = 200 µm, c = 100 µm, d = 10 µm, e-n = 20 µm

with *O. fusispora* in a well-supported clade in the phylogenetic analysis, but they can be distinguished readily by the difference in appearance of ascostromata, the wider asci (17–24 µm vs. 11–16 µm) and larger ascospores (25–31 × 8–10 µm vs. 20–26 × 5–6.5 µm). Both species have
pseudoparaphyses embedded in a gelatinous matrix and anastomose between and above the asci and are more like trabeculae in *Occultibambusa maolanensis*, but typical of cellular pseudoparaphyses in *O. jonesii*. Liew et al. (2000) show that the nature of pseudoparaphyses had little relevance above the family level, while in this study different types of pseudoparaphyses are found even in the same genus (Figs 2d, 3e).

The family of *Occultibambusaceae* includes four genera: *Neooccultibambusa*, *Occultibambusa*, *Seriascoma* and *Versicolorisporium*, however, the phylogenetic placement of *Versicolorisporium* is not well-resolved. Its placement is not stable and when used in analyses it affects the molecular placements of genera (results not shown). Therefore, we excluded the molecular data of *Versicolorisporium* in our phylogenetic analysis. The two new taxa are both morphologically and phylogenetically with described *Occultibambusa* species, and the lack of *Versicolorisporium* sequence data has no effect on phylogenetic relationships of species in *Occultibambusa*.

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