



Additions to Karst Fungi 5: *Sardiniella guizhouensis* sp. nov. (Botryosphaeriaceae) associated with woody hosts in Guizhou province, China

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Abstract

During an investigation of ascomycetous fungi in Karst formations of the Asian region, three interesting taxa were found on dead aerial stems of woody hosts in Guizhou province, China. Their morphology assigned them as typical botryosphaeriaceous species. Phylogenetic analyses based on a combined DNA dataset of large subunit (LSU), internal transcribed spacer (ITS) and part of the translation elongation factor 1- α (*tef1*) gene confirmed their placement within Botryosphaeriaceae. In the phylogenetic tree, the three isolates formed a well-supported monotypic clade as a distinct lineage within the genus *Sardiniella*. Therefore, a new species *Sardiniella guizhouensis* sp. nov. is introduced to accommodate these taxa and detailed, illustrated descriptions of the asexual and sexual morphs are provided. This study reveals the first sexual morph of *Sardiniella*, which is characterized by 2–4(–6)-spored asci with hyaline to brown, aseptate to 1-septate, ovate to subclavate ascospores.

Keywords: 1 new species, Dothideomycetes, multi-gene phylogeny, sexual morph, taxonomy

Introduction

The Botryosphaeriaceae are amongst the most widespread, common and important fungal pathogens of woody plants. Many are also known to exist as endophytes in healthy plant tissues and as saprobes in dead, woody materials. Botryosphaeriaceae has gone significant revisions over the last decade due to the introduction of several new genera and species while some taxa have been reduced to synonymy, mainly on the basis of combined morphological and multiple gene sequence data analyses (Crous *et al.* 2006, Liu *et al.* 2012, Phillips *et al.* 2013, 2019, Hyde *et al.* 2014, Dissanayake *et al.* 2016, Yang *et al.* 2017, Zhang *et al.* 2021).

Linaldeddu *et al.* (2016) introduced the genus *Sardiniella* Linald., A. Alves & A.J.L. Phillips in Botryosphaeriaceae, with *Sardiniella urbana* as the type. This taxon causes a decline of European hackberry (*Celtis australis* L.) in Sardinia, Italy. Though *Sardiniella* is morphologically similar to brown-conidiospored genera *Diplodia* and *Dothiorella*, it differs in colony appearance, conidial shape and DNA sequence data (LSU, ITS and *tef1*) from all known Botryosphaeriaceae species. Subsequently, Hyde *et al.* (2017) introduced a second species to the genus, *Sardiniella celtidis*, based on a phylogenetic analysis of combined ITS and *tef1* sequence data.

So far, *Sardiniella* species have been reported only from Italy, with *S. urbana* isolated from Sassari in Sardinia, while *S. celtidis* was reported from Forlì-Cesena Province (Linaldeddu *et al.* 2016, Hyde *et al.* 2017). Both species were isolated from the same host *Celtis australis* (Cannabaceae). As a part of our ongoing studies on ascomycetous fungi from Karst formations of the Asian region (Ariyawansa *et al.* 2016, Chen *et al.* 2017, 2020, Zhang *et al.* 2017a, b, 2018, 2019, 2020, Dissanayake *et al.* 2020a), three isolates were identified within Botryosphaeriaceae. Based on phylogenetic and morphological studies, they were identified as a new species of *Sardiniella*, namely *Sardiniella guizhouensis* sp. nov. This study also suggests that more investigations are still needed in the Karst region, and many novel microfungi remain to be discovered.

Material and Methods

Sample collection, isolation and specimen examination

Specimens from woody hosts were collected from three locations (Huaxi wetland park, Xiaochehe wetland park in Guiyang District and Maolan nature reserve in Libo District) in Guizhou province during April to July in 2017 and were taken to the laboratory. Morphological observations were made using a Motic SMZ 168 Series stereomicroscope and digital images were recorded with a Nikon E80i microscope-camera system. Measurements were made with the Tarosoft (R) Image Frame Work (Liu *et al.* 2010) and images used for figures were processed with Adobe Photoshop CS v. 5.

Isolations were made from single spores as described by Liu *et al.* (2010). Spore germination on 2% water agar (WA) was observed after 12 h and germinating spores were transferred to potato dextrose agar (PDA). Cultures were incubated at 25 °C in the dark and colony morphology and conidial characteristics were studied for a total of 3 isolates. Colony color was determined according to Rayner (1970) after 5 d to 10 d on PDA at 25 °C in the dark. More than 20 ascostromata or conidiomata, 25 asci, and 40 ascospores or conidia were measured to calculate the mean length and width. Spore shape, color and arrangement were also recorded.

The type specimen was deposited in the herbarium of Cryptogams, Kunming Institute of Botany Academia Sinica (KUN-HKAS) in Kunming, China and herbarium of Guizhou Academy of Agricultural Sciences (GZAAS) in Guiyang, China. The cultures isolated were deposited at the China General Microbiological Culture Collection Center (CGMCC) in Beijing, China and in Guizhou Culture Collection (GZCC) in Guiyang, China. Faces of fungi number and index fungorum number were obtained for the taxonomic novelty as outlined in Jayasiri *et al.* (2015) and Index Fungorum (2021) respectively.

DNA extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA for 5 days at 25 °C in the dark. Genomic DNA was extracted from fresh mycelium of 3–4 days old cultures with Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China). DNA was amplified performed by polymerase chain reaction (PCR) for three gene regions, namely large subunit (LSU), internal transcribed spacer (ITS) and translation elongation factor 1- α gene (*tefl*). The primers used were LR0R/LR5 (Vilgalys & Hester 1990) for LSU, ITS5/ITS4 (White *et al.* 1990) for ITS, EF1-728F/EF1-986R (Carbone & Kohn 1999) for *tefl*. The amplification procedure was performed as detailed in Doilom *et al.* (2014). PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham product code: 27-9602-01). Sequencing was done by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, P.R. China).

Sequence alignment and phylogenetic analyses

Resulting DNA sequences were checked with BioEdit v.5 (Hall 1999). A BLAST search of LSU, ITS and *tefl* sequence data was used to find the closest matching taxa in Botryosphaeriaceae. The relevant sequences were retrieved from GenBank. *Melanops tulasnei* (CBS 116805) was selected as the outgroup taxon and aligned with the sequences obtained in this study using MAFFT (<http://www.ebi.ac.uk/Tools/msa/mafft/>) (Katoh *et al.* 2013). LSU, ITS and *tefl* sequence datasets were manually edited, portions with missing bases at the start and end of the sequences were removed with BioEdit v.5 and then the individual datasets were concatenated into a combined dataset.

Phylogenetic analysis was performed by maximum parsimony (MP), maximum likelihood (ML) and bayesian inference (BI) as detailed in Dissanayake *et al.* (2020b). Maximum-parsimony analysis (MP) was performed with PAUP v. 4.0b10 (Swofford 2002). Ambiguous regions in the MP alignment were excluded, gaps were treated as missing data and robustness of the branches was determined with 1000 bootstrap replicates along with 1000 of max-trees. Branches of zero length were collapsed, and all multiple most parsimonious trees saved. Statistics including tree length (TL), consistency index (CI), retention index (RI), relative consistency index (RC) and homoplasy index (HI) were calculated. Differences between the trees inferred under different optimality criteria were evaluated using Kishino-Hasegawa tests (KHT). Maximum likelihood analysis was performed by RAxML GUIv.0.9b2 (Kishino & Hasegawa 1989, Silvestro & Michalak 2010). Bayesian analyses was performed with MrBayes v.3.1.2 (Huelsenbeck & Ronquist 2001) and posterior probabilities (PP) were determined by Markov Chain Monte Carlo sampling (MCMC).

MrModeltest v. 2.3 (Nylander 2004) was used to select of the best-fit model of nucleotide substitutions and was integrated into ML and BI analysis.

Fungal strains used in this study are listed in TABLE 1 with information of the ex-type cultures and GenBank accession numbers. Sequences generated in this study were deposited in GenBank (TABLE 1). Alignments and trees were deposited in TreeBASE (www.treebase.org, study ID: 28099).

TABLE 1. Taxa used in this study and their GenBank accession numbers.

Species name	Isolate number	LSU	ITS	<i>tefl</i>
<i>Barriopsis stevensiana</i>	CBS 174.26	KF766317	EU673330	EU673296
<i>Botryobambusa fusicoccum</i>	MFLUCC 11-0143	JX646809	JX646792	JX646857
<i>B. fusicoccum</i>	MFLUCC 11-0657	JX646810	JX646793	JX646858
<i>Botryosphaeria agaves</i>	MFLUCC 11-0125	JX646808	JX646791	JX646856
<i>B. dothidea</i>	CBS 115476	DQ377852	AY236949	AY236898
<i>Diplodia mutila</i>	CBS 112553	AY928049	AY259093	AY573219
<i>Di. rosulata</i>	CBS 116470	DQ377896	EU430265	EU430267
<i>Di. seriata</i>	CBS 112555	AY928050	AY259094	AY573220
<i>Dothiorella citricola</i>	ICMP 16828	EU673242	EU673323	EU673290
<i>Do. iberica</i>	CBS 115041	AY928053	AY573202	AY573222
<i>Do. mangiferae</i>	CBS 500.72	EU673237	EU673318	EU673285
<i>Do. plurivora</i>	CBS 117006	EU673236	AY905555	AY905562
<i>Do. prunicola</i>	CBS 124723	EU673232	EU673313	EU673280
<i>Do. sarmentorum</i>	IMI 63581b	AY928052	AY573212	AY573235
<i>Do. viticola</i>	CBS 117009	DQ377873	AY905554	AY905559
<i>Lasiodiplodia crassispora</i>	CBS 118741	DQ377901	DQ103550	EU673303
<i>L. rubropurpurea</i>	CBS 118740	DQ377903	DQ103553	DQ103571
<i>L. theobromae</i>	CBS 164.96	EU673253	AY640255	AY640258
<i>Melanops tulasnei</i>	CBS 116805	FJ824764	FJ824769	FJ824774
<i>Neofusicoccum arbuti</i>	CBS 116131	DQ377915	AY819720	KF531792
<i>N. luteum</i>	CBS 110299	AY928043	AY259091	AY573217
<i>N. mangiferae</i>	CBS 118531	DQ377920	AY615185	DQ093221
<i>N. parvum</i>	CMW 9081	AY928045	AY236943	AY236888
<i>Phaeobotryon mamane</i>	CPC 12264	DQ377898	EU673331	EU673297
<i>P. mamane</i>	CPC 12440	EU673248	EU673332	EU673298
<i>Sardiniella celtidis</i>	MFLUCC 17-0981	N/A	MF443249	MF443248
<i>S. guizhouensis</i>	CGMCC 3.19222	<i>MW886225</i>	<i>MW886228</i>	<i>MZ231048</i>
<i>S. guizhouensis</i>	<i>GZCC 19-0094</i>	<i>MW886226</i>	<i>MW886229</i>	<i>MZ231049</i>
<i>S. guizhouensis</i>	<i>GZCC 19-0216</i>	<i>MW886227</i>	<i>MW886230</i>	<i>MZ231050</i>
<i>S. urbana</i>	CBS 141580	KX379676	KX379674	KX379675
<i>S. urbana</i>	BL180	KX379679	KX379677	KX379678
<i>Sphaeropsis citrigena</i>	ICMP 16812	EU673246	EU673328	EU673294
<i>S. eucalypticola</i>	MFLUCC 11-0579	JX646819	JX646802	JX646867

Ex-type and reference strains are indicated in bold and newly generated sequences are indicated in italic. Abbreviations of isolates and culture collections: **BL**: B.T. Linaldeddu culture collection housed at Dipartimento di Agraria, Università di Sassari, Italy; **CBS**: Centraalbureau voor Schimmelcultures, The Netherlands; **CGMCC**: China General Microbiological Culture Collection Center; **CMW**: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, Pretoria South Africa; **CPC**: Collection of Pedro Crous housed at CBS; **GZCC**: Guizhou Culture Collection, Guiyang, China; **ICMP**: International Collection of Micro-organisms from Plants, Landcare Research, New Zealand; **IMI**: CABI Bioscience, Egham, UK; **MFLUCC**: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

Results

Phylogenetic analyses

The combined dataset of 33 taxa including *Melanops tulasnei* (Melanopsaceae) as the outgroup taxon comprised 1,834 characters (LSU: 1-839; ITS: 840-1,470; *tefl*: 1,471-1,834) including alignment gaps. Of these, 1,326 were constant, 170 were variable and parsimony-uninformative and 345 were parsimony informative. The equally weighted maximum parsimony tree resulted in a single tree of 1,241 steps, a consistency index (CI) of 0.608, a retention index (RI) of 0.774, a rescaled consistency index (RC) of 0.471 and homoplasy index (HI) of 0.392. In the ML analyses, the best scoring RAxML tree (FIG. 1) with a final likelihood value of -8863.905208 is presented. The matrix had 630 distinct alignment patterns, with 17.89% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.225568, C = 0.258004, G = 0.287112, T = 0.229317; substitution rates AC = 0.906523, AG = 2.023119, AT = 0.800723, CG = 1.057633, CT = 3.784672, GT = 1.000000; gamma distribution shape parameter alpha = 0.191768. The MP and BI phylogenetic analyses produced trees with similar topologies to ML. Phylogenetic analysis indicated eleven major clades in Botryosphaeriaceae (FIG. 1). Three isolates representing the new species *Sardiniella guizhouensis* clustered with the other two known *Sardiniella* species in a well-supported clade (ML/MP/BI = 96/99/1.0) and represented a distinct lineage (FIG. 1).

Taxonomy

Sardiniella guizhouensis Y.Y. Chen & Jian K. Liu., *sp. nov.* (FIGURES 2, 3)

Index Fungorum number: IF558352, Facesoffungi number: FoF09647.

Etymology:—Name refers the location where the fungus was collected, Guizhou province, China.

Saprobic on decaying aerial stem. **Sexual morph:** *Ascostromata* 175–250 µm high × 220–285 µm diam. (\bar{x} = 190 × 265 µm, n = 20), black, pseudothecial, solitary, initially immersed in host, erumpent at maturity, uniloculate. *Peridium* 50–80 µm thick, outer layer composed of heavily pigmented thick-walled cells of *textura angularis*, inner layer composed of hyaline thin-walled cells of *textura angularis*. *Pseudoparaphyses* up to 3–4 µm wide, hyphae-like, septate, slightly constricted at septum. *Asci* 78–92 × 23–27 µm (\bar{x} = 85 × 24 µm, n = 25), 2–4(–6)-spored, bitunicate, fissitunicate, clavate, pedicellate, with a well-developed ocular chamber, arising from base of the ascoma. *Ascospores* 26–31 × 14–16 µm (\bar{x} = 29 × 15 µm, n = 40), uniseriate or irregularly biseriate, initially hyaline and aseptate, becoming pigmented brown and 1-septate, ovate to subclavate, constricted at septum, thick-walled. **Asexual morph:** *Conidiomata* 95–151 µm high × 114–294 µm diam. (\bar{x} = 129 × 187 µm, n = 20), immersed, arranged singly or in small groups within the bark, globose to subglobose, dark brown to black, solitary or gregarious. *Ostiole* central. *Peridium* 22–27 µm thick, outer layer composed of pigmented thick-walled cells of *textura angularis*, inner layer composed of hyaline thin-walled cells of *textura angularis* (3–5-layered). *Conidiogenous cells* lining the inner surface of the conidioma, hyaline, short obpyriform to subcylindrical. *Conidia* 16–28 × 9–12 µm (\bar{x} = 26 × 10, n = 40), ellipsoid to obovoid, immature conidia hyaline, mature conidia becoming medium to dark brown.

Culture characteristics:—Ascospores germinating on WA within 18 h and producing germ tubes from the septum. Colonies growing on PDA, reaching a diam. of 4 cm after 3d at 25°C, effuse, velvety, with entire to slightly undulate edge. Colonies initially white and later turning green.

Materials examined:—China, Guizhou province, Guiyang District, Xiaochehe wetland park, on decaying aerial stem, 20 May 2017, Y.Y. Chen, 18-53 (HKAS 113023, holotype; GZAAS 19-1809, isotype); ex-type living culture CGMCC 3.19222 (GZCC 19-0090); *ibid*, Huaxi wetland park, 16 April 2017, 19-62 (GZAAS 19-1935, paratype), living culture GZCC 19-0216; *ibid*, Libo District, Maolan nature reserve, 19 July 2017, 18-60 (GZAAS 19-1813, paratype), living culture GZCC 19-0094.

Notes:—The phylogenetic results showed that six taxa clustered together and formed a well-supported clade (ML/MP/BI = 96/99/1.0) representing the genus *Sardiniella* (FIG. 1). *Sardiniella guizhouensis* can be distinguished from *S. celtidis* (9/631 in ITS and 24/364 in *tefl*), and from *S. urbana* (5/839 in LSU, 6/631 in ITS and 25/364 in *tefl*). In addition, *Sardiniella guizhouensis* differs from other two known *Sardiniella* species in having multiloculate conidiomata (FIG. 2 c,d). The sexual morph is known only in *Sardiniella guizhouensis* and thus no comparisons between species can be made.

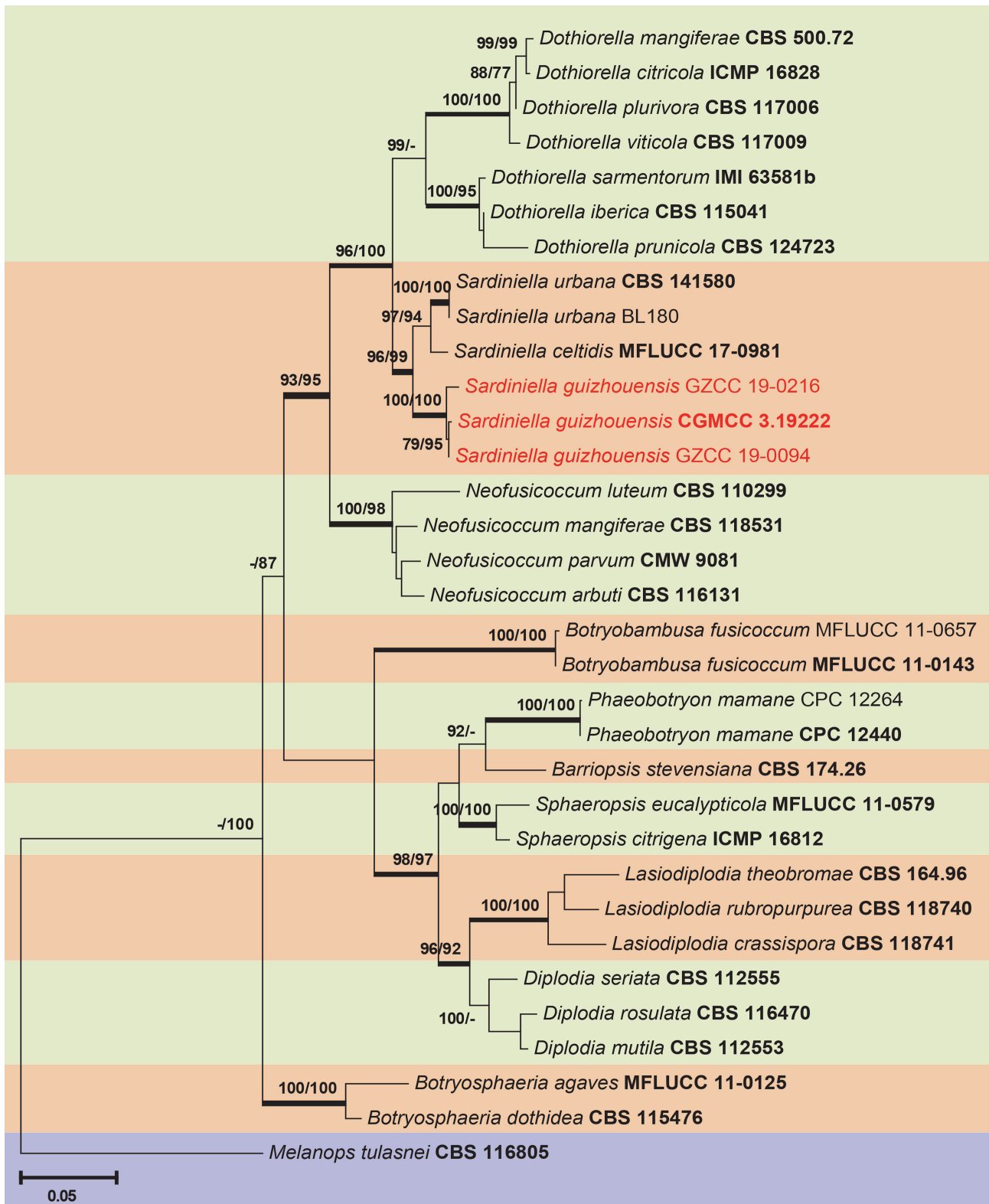


FIGURE 1. Maximum likelihood (ML) majority rule consensus tree for the analyzed Botryosphaeriaceae genera based on combined LSU, ITS and *tef1* sequence data. RAxML bootstrap support values (ML) and maximum parsimony (MP) are given at the nodes (ML/MP). Branches in bold indicate Bayesian posterior probabilities > 0.95. Isolate numbers of ex-types and reference strains are in bold. Species isolated in this study are in red. The tree was rooted to *Melanops tulasnei* (CBS 116805).

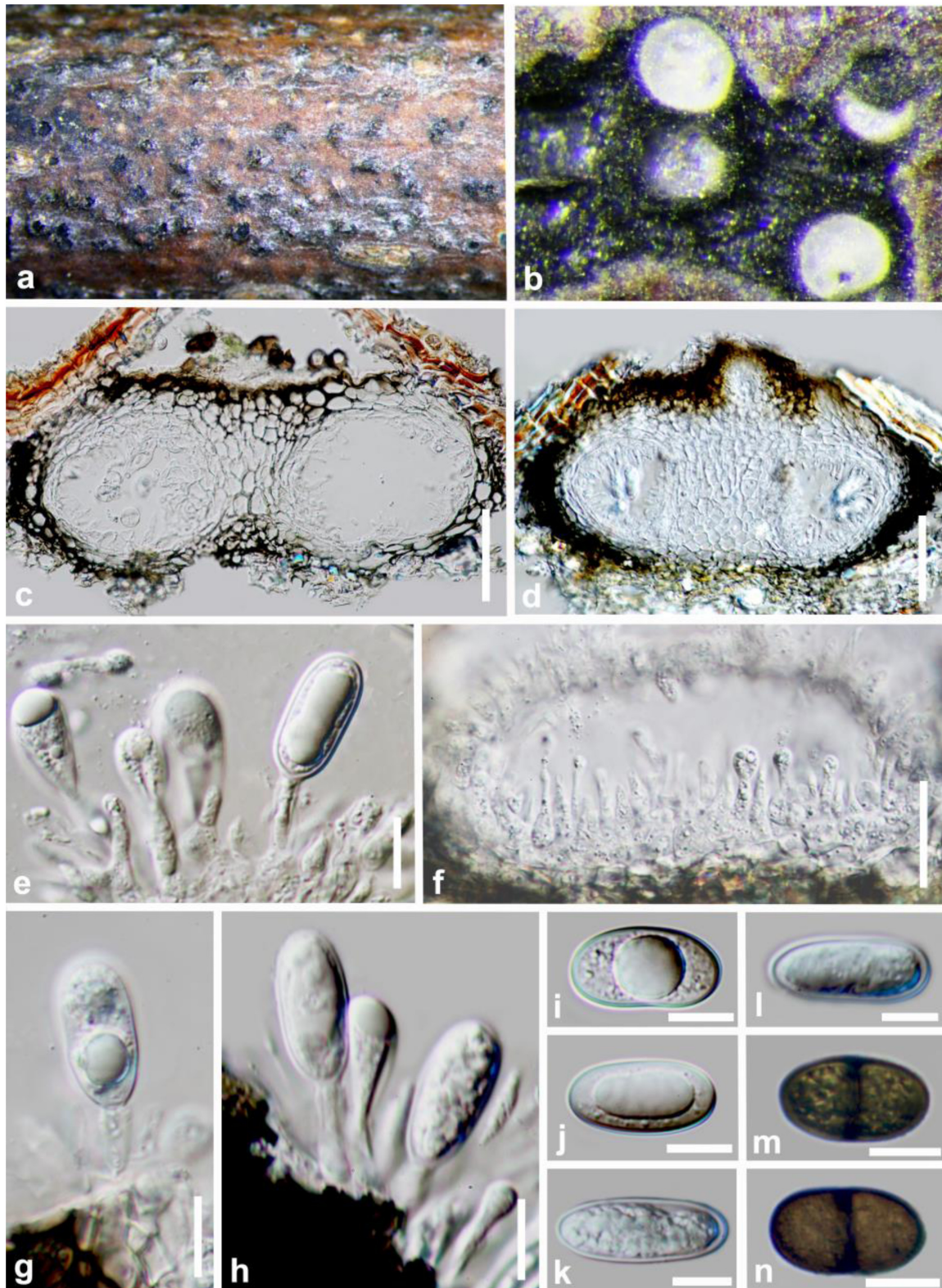


FIGURE 2. *Sardiniella guizhouensis* (HKAS 113023, holotype). a, b. Conidiomata on host surface. c, d. Vertical section of multiloculate conidiomata. e–h. Conidiogenous cells and developing conidia. i–l. Immature, hyaline conidia. m, n. Mature, brown 1-septate conidia. **Scale bars:** c=50 μ m, d=10 μ m, e=50 μ m, f=20 μ m, g–n=10 μ m.



FIGURE 3. *Sardiniella guizhouensis* (GZAAS 19-1935, sexual morph) a, b. Appearance of ascostromata on decaying aerial stem. c. Peridium. d. Vertical section of ascostromata. e. Mature and immature asci. f. Immature ascus. g–i. Mature asci. j–l. Mature brown 1-celled ascospores. m, n. Mature brown 1-septate ascospores. o. 5d old culture on PDA from above. p. 5d old culture on PDA from reverse. **Scale bars:** c=50µm, d=100µm, e=20µm, f–n=10µm.

Discussion

In this study, a third species, *Sardiniella guizhouensis* is described and assigned to the genus *Sardiniella* based on the specimens collected from woody hosts in Guizhou province, China. Phylogenetic analyses (FIG. 1) showed that *S. guizhouensis* is phylogenetically distinct from *S. urbana* and *S. celtidis*, which are reported on *Celtis australis* (Cannabaceae) from Sardinia and Forlì-Cesena Province in Italy respectively. This study reveals the sexual morph of *Sardiniella* for the first time, which is characterized by 2–4(–6)-spored asci with a well-developed ocular chamber and ascospores that are initially hyaline and aseptate, becoming pigmented brown and 1-septate. The asexual morph of *Sardiniella guizhouensis* is morphologically distinguished from *S. urbana* and *S. celtidis* by the presence of its multi-loculate conidiomata.

Sardiniella is morphologically similar to *Diplodia* and *Dothiorella* in the family Botryosphaeriaceae. Based on phylogenetic evidence, Linaldeddu *et al.* (2016) demonstrated that *Sardiniella* is distinct from *Diplodia* and *Dothiorella* based on different conidial morphology, ecology and sequence data. Although in *Dothiorella* and some species of *Diplodia* the conidia become pigmented while still attached to the conidiogenous cells, this character is absent in *Sardiniella*. In *Diplodia*, the conidial wall is thicker than in *Sardiniella* and these two genera are clearly phylogenetically separated. Nevertheless, *Sardiniella* and *Dothiorella* are phylogenetically closely related to *Neofusicoccum*, but the oblong conidia that become pigmented and 1-septate differentiate these two genera from *Neofusicoccum*. The molecular analysis in this study provided an additional support for the phylogenetic placement of the genus *Sardiniella* within Botryosphaeriaceae.

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