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Additions to Karst Fungi 4: *Botryosphaeria* spp. associated with woody hosts in Guizhou province, China including *B. guttulata sp. nov.*

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Abstract

Members of *Botryosphaeria* encompass important plant pathogens, saprobes and endophytes on a wide range of woody hosts worldwide. *Botryosphaeria* species are difficult to differentiate due to the overlapping morphological characteristics and the molecular data analyses are necessary recently when species identification is carried out. In this study, 28 *Botryosphaeria* isolates were obtained from decaying woody hosts in six nature reserves in Guizhou province, China. Based on both morphological characteristics and molecular analysis of combined ITS and *tef1-α* sequence data, four known species (*Botryosphaeria dothidea*, *B. minutispermatia*, *B. sinensia* and *B. wangensis*) are identified and one new species *B. guttulata* is introduced. *Botryosphaeria sinensia* (32% of the isolates obtained from various hosts) is the abundant species, followed by *B. dothidea* (28.5% of the isolates), *B. guttulata* (28.5% of the isolates), *B. minutispermatia* (7% of the isolates) and *B. wangensis* (4% of the isolates). These results represent the first study of *Botryosphaeria* species associated with woody hosts from nature reserves in Guizhou province, China. Our findings indicate that there is a potential of *Botryosphaeria* species remain to be discovered in this unique landform (Karst formations) in Guizhou province, China.

Keywords: 1 new taxon, Botryosphaeriaceae, phylogeny, taxonomy, wood-inhabiting fungi

Introduction

Botryosphaeria Ces. & De Not. (Botryosphaeriaceae, Botryosphaeriales) includes saprobic, endophytic and pathogenic species that cause canker and dieback disease of woody plants and mostly found on terrestrial habitats (Crous *et al*. 2006, Liu *et al*. 2012, Phillips *et al*. 2013, 2019, Dissanayake *et al*. 2016). Some of those species cause significant harvest decline, ecological damage and severe commercial losses (Dissanayake *et al*. 2016). Cesati & de Notaris (1863) introduced the genus and revised by Saccardo (1877) based on the type genus *Botryosphaeria* and typified with *B. dothidea* (Moug.: Fr.) Ces. & de Not. Over the years, this genus has been undergone various revisions and updated at times encompassing a diverse range of morphologies (Liu *et al*. 2012, Phillips *et al*. 2013, 2019). *Botryosphaeria dothidea* is one of the most commonly reported species as a significant pathogen of woody plants (Dissanayake *et al*. 2016). According to USDA fungal database (Farr & Rossman 2016, accessed in April 2020), this species has been reported in 184 host genera belonging to 79 plant families. However, the validity of many of these records is questionable before Slippers *et al*. (2004), who established a modern and reliable concept for this species, made the re-evaluation of *B. dothidea*. No ex-type cultures were available for *B. dothidea* so that Slippers *et al*. (2004) designated a neotype based on a specimen of *Sphaeria dothidea* and designated it as an epitype to stabilize the type species with molecular data of combined ITS, *tef1-α* and TUB2 sequences. This data was used to elucidate the taxonomic complications of *B. dothidea* and set a stable basis for the resolution of the species (Slippers *et al*. 2004).

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There are more than 280 species records of *Botryosphaeria* listed in Index Fungorum (2020) (http://www.indexfungorum.org/, accessed in April 2020), but most of them lack specimens/living cultures and DNA sequence data. Species identification criteria of *Botryosphaeria* were previously based on host affiliations and morphology. However, these approaches were unreliable due to the uninformative illustrations and descriptions, weak host specificity and overlapping morphological characteristics (Phillips *et al*. 2013). Recent studies have been able to use multiphase approaches to solve and address the taxonomy of *Botryosphaeria* (Crous *et al.* 2006, Liu *et al*. 2012, Phillips *et al*. 2013, 2019, Dissanayake *et al*. 2016). Dissanayake *et al*. (2016) summarized ten species of *Botryosphaeria* (*Botryosphaeria sensu stricto*): *B. agaves*, *B. auasmontanum*, *B. corticis*, *B. dothidea*, *B. fabicerciana*, *B. fusispora*, *B. minutispermatia*, *B. ramosa*, *B. scharifii* and *B. sinensia* using three gene matrix (ITS, *tef1-α* and LSU). Subsequently, Xu *et al*. (2015) designated an epitype of *B. kuwatsukai*, while Zhou *et al*. (2017) described and illustrated *B. rosaceae*. Li *et al*. (2018) introduced three novel species, *B. pseudoramosa*, *B. qingyuanensis* and *B. wangensis* from Eucalyptus trees in China.

Guizhou province (China) is called as a "natural park" with picturesque natural scenery, ethnic cultures, waterfalls, valleys, karst caves and landscapes. The nature reserves in Guizhou province consist of high plant and animal diversity, which is considered as a biodiversity hotspot in this region (Wang & Wen 2011). The Fanjing Mountain National Nature Reserve was established in 1978 and designated as a UNESCO Biosphere Reserve in 1986 which became a UNESCO World Heritage Site in 2018 (Wang & Wen 2011). The Huaxi National Wetland Park is approved as the first national wetland park in southwest China. The nature reserves provide a safe place as a habitat for animals and create favorable conditions for carrying out scientific researches. In these nature reserves, more than 1000 different vascular plants and few kinds of precious animals are protected by the state in Guizhou province (Xu 2003, Yao 2011). These nature reserves have attracted a large number of visitors and have helped to integrate resources such as mountains, forests, water and fresh air in Guizhou province (Xu 2003, Lan *et al*. 2009). As most fungi are often linked to particular host plants as saprobes, endophytes and pathogens, therefore future studies will explore a high fungal diversity in nature reserves in Guizhou province.

During a biodiversity survey of ascomycetous fungi on decaying wood in several nature reserves in Guizhou Province, we have collected numerous *Botryosphaeria* species and the taxonomy and phylogeny studies of these taxa were carried out. In this study, we report four known *Botryosphaeria* species and introduce one novel species with descriptions, illustrations, and phylogenetic analysis.

Material & methods

Sample collection and isolation

Decaying wood samples with conidiomata/ascomata on host materials were randomly collected from six nature reserves in Guizhou province, China (Fanjing Mountain, Guiyang Huaxi Wetland Park, Guiyang Xiaochehe Wetland Park, Maolan Natural water Reserve, Suiyang broad water nature reserve and Xingyi Wanfenglin) (TABLE 1). Collected samples were taken to the laboratory for isolation and photographed, documented and then kept at 4 °C for further study. A total of 28 isolates was obtained following the single spore isolation (Chomnunti *et al*. 2014). Single germinating spores were transferred onto fresh PDA plates and incubated at 25-30 °C. Cultures were grown for 7d and morphological characters such as colour, colony shape, and texture were recorded. Herbarium specimens were deposited at the Herbarium of Cryptogams, Kunming Institute of Botany Academia Sinica (KUN-HKAS), Kunming, China and herbaria of Guizhou Academy of Agricultural Sciences (GZAAS), Guiyang, China. The living cultures were deposited in the Guizhou Culture Collection (GZCC) in Guiyang, China and China General Microbiological Culture Collection Center (CGMCC) in Beijing, China (TABLE 1).

Morphological analysis

Species identification was primarily based on morphological observation of the conidiomata or ascosmata from host materials and micromorphology supplemented by culture characteristics. Morphological observations were made using a Motic SMZ 168 Series stereomicroscope and photographed by a Nikon E80i microscope-camera system. Measurements were made with the Tarosoft (R) Image FrameWork (Liu *et al.* 2010). Adobe Photoshop CS v. 5 was used for manual editing. More than 10 conidiomata/ascomata, 10 asci, and 30 conidia/ascospores were measured to

calculate the mean size/length and respective standard deviations (SD). Measurements of asexual morph structures (Conidiomata, Conidiophore, Conidiogenous cell and Conidia) of all type species of *Botryosphaeria* were compared (TABLE 2). Colony diameters were measured and the colony features were described using the colour charts of Rayner (1970).

TABLE 1. Strains and GenBank accession numbers used in the phylogenetic analyses.

Taxa	Strain numbers	GenBank Accession numbers	
		ITS	*tef1-α*
Botryosphaeria agaves	MFLUCC 11-0125[T]	JX646791	JX646856
B. agaves	MFLUCC 10-0051	JX646790	JX646855
B. auasmontanum	CMW 25413[T]	KF766167	EU101348
B. corticis	CBS 119047[T]	DQ299245	EU017539
B. corticis	ATCC 22927	DQ299247	EU673291
B. dothidea	CMW 8000[T]	AY236949	AY236898
B. dothidea	CBS 110302	AY259092	AY573218
B. dothidea	GZCC 19-0144	**MT327825**	**MT331608**
B. dothidea	GZCC 19-0148	**MT327826**	**MT331609**
B. dothidea	GZCC 19-0149	**MT327827**	**MT331610**
B. dothidea	GZCC 19-0158	**MT327828**	**MT331611**
B. dothidea	GZCC 19-0164	**MT327829**	**MT331612**
B. dothidea	GZCC 19-0180	**MT327830**	**MT331613**
B. dothidea	GZCC 19-0182	**MT327831**	**MT331614**
B. dothidea	GZCC 19-0190	**MT327835**	**MT331615**
B. fabicerciana	CBS 127193[T]	HQ332197	HQ332213
B. fabicerciana	CMW 27108	HQ332200	HQ332216
B. fusispora	MFLUCC 10-0098[T]	JX646789	JX646854
B. fusispora	MFLUCC 11-0507	JX646788	JX646853
B. guttulata	CGMCC3.20094[T]	**MT327839**	**MT331606**
B. guttulata	GZCC 19-0186	**MT327832**	**MT331600**
B. guttulata	GZCC 19-0188	**MT327833**	**MT331601**
B. guttulata	GZCC 19-0189	**MT327834**	**MT331602**
B. guttulata	GZCC 19-0194	**MT327836**	**MT331603**
B. guttulata	GZCC 19-0196	**MT327837**	**MT331604**
B. guttulata	GZCC 19-0201	**MT327838**	**MT331605**
B. guttulata	GZCC 19-0205	**MT327840**	**MT331607**
B. kuwatsukai	CBS 135219[T]	KJ433388	KJ433410
B. kuwatsukai	LSP5	KJ433395	KJ433417
B. minutispermatia	GZCC 16-0013[T]	KX447675	KX447678
B. minutispermatia	GZCC 16-0014	KX447676	KX447679
B. minutispermatia	GZCC 19-0087	**MT327817**	**MT331616**
B. minutispermatia	GZCC 19-0091	**MT327818**	**MT331617**
B. qingyuanensis	CGMCC3.18742[T]	KX278000	KX278105
B. qingyuanensis	CGMCC3.18743	KX278001	KX278106
B. ramosa	CBS 122069[T]	EU144055	EU144070
B. rosaceae	CGMCC3.18007[T]	KX197074	KX197094
B. rosaceae	CGMCC3.18008	KX197075	KX197095
B. scharifii	CBS 124703[T]	JQ772020	JQ772057
B. scharifii	CBS 124702	JQ772019	JQ772056

.....continued on the next page

TABLE 1 (Continued**)**

Taxa	Strain numbers	GenBank Accession numbers	
		ITS	*tef1-α*
B. pseudoramosa	CGMCC3.18740	KX277992	KX278097
B. sinensia	CGMCC 3.17722[T]	KT343254	KU221233
B. sinensia	CGMCC 3.17724	KT343256	KU221234
B. sinensia	GZCC 19-0073	**MT327815**	**MT331618**
B. sinensia	GZCC 19-0083	**MT327816**	**MT331619**
B. sinensia	GZCC 19-0093	**MT327819**	**MT331620**
B. sinensia	GZCC 19-0099	**MT327820**	**MT331621**
B. sinensia	GZCC 19-0100	**MT327821**	**MT331622**
B. sinensia	GZCC 19-0109	**MT327822**	**MT331623**
B. sinensia	GZCC 19-0116	**MT327823**	**MT331624**
B. sinensia	GZCC 19-0126	**MT327824**	**MT331625**
B. sinensia	GZCC 19-0341	**MT327842**	**MT331626**
B. wangensis	CGMCC3.18744[T]	KX278002	KX278107
B. wangensis	CGMCC3.18745	KX278003	KX278108
B. wangensis	GZCC 19-0340	**MT327841**	**MT331627**

Abbreviations: **ATCC**: American Type Culture Collection; **CBS**: Westerdijk Fungal Biodiversity Institute (CBS-KNAW Fungal Biodiversity Centre), Utrecht, The Netherlands; **CGMCC**: China General Microbiological Culture Collection, **CMW**: Culture collection of Michael Wingfield, university of Pretoria, South africa; **GZCC**: Guizhou Culture Collection, **MFLUCC**: Mae Fah Luang University Culture Collection, Thailand; NA: not applicable. The newly generated sequences in this study are in bold and ex-type/ex-epitype isolates are indicated with (T).

DNA extraction, PCR amplification and sequencing

Fungal mycelium of 7d pure cultures were scraped for the extraction of genomic DNA using Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China). The ITS region was amplified using primers ITS1 and ITS4 (White *et al*. 1990). The target region of the *tef1-α* gene was amplified using primer pairs EF-728F and EF-986R (Carbone & Kohn 1999). The PCR reactions were accomplished in a Bio Rad C1000 thermal cycler. The PCR mixture was composed of 0.3 µl of TaKaRa *Ex-Taq* DNA polymerase, 2.5 µl of 10 × *Ex-Taq* DNA polymerase buffer, 3.0 µl of dNTPs, 5–20 ng of genomic DNA, 1 µl of each primer and ddH_2O up to 25 µl. Following the PCR amplification, products were visualized on 1% agarose gel under UV light using a Gel Doc™ XR. PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham product code: 27-9602-01). Sequence analysis was carried out by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, P.R. China).

Phylogenetic analyses

Resulted sequence chromatograms were checked using BioEdit v.5 (Hall 2006) to assure the sequence quality. Phylogenetic analysis based on the combined ITS and *tef1-α* was performed to identify the taxa isolated in this study together with the reference sequences from GenBank. Reference sequences were obtained based on ex-type or ex-epitype sequences available from recent publications (Xu *et al*. 2015, Dissanayake *et al*. 2016, Zhou *et al*. 2017, Li *et al*. 2018, Jayawardena *et al*. 2019) (TABLE 1). *Macrophomina phaseolina* (CBS 227.33) was selected as the outgroup taxon. Subsequent alignments for each gene were generated using MAFFT v.7 (Katoh & Standley 2013). The alignments were checked visually and improved manually where necessary. Phylogenetic analyses were performed by using PAUP v.4.0b10 for maximum parsimony (MP) method (Swofford 2003), RAxML for maximum likelihood (ML) method (Stamatakis 2006) and MrBayes v.3.1.2 for Bayesian Inference (BI) method (Ronquist & Huelsenbeck 2003).

Maximum Parsimony analysis (MP) was performed to test the discrepancy among the ITS and *tef1-α* sequence datasets in a heuristic search option of 1,000 random-addition sequences with a tree bisection and reconnection (TBR)

branch swapping algorithm (Swofford 2003). The branches of zero length were collapsed and all equally parsimonious trees were saved. Clade stability was assessed with a bootstrap analysis of 1,000 replicates (Hillis & Bull 1993). Other parsimony scores such as tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency (RC) were calculated (Swofford 2003). Maximum Likelihood analysis (ML) was performed with GTR+G+I model of site substitution. The branch support was evaluated with a bootstrapping method of 1000 replicates (Hillis & Bull 1993). Bayesian Inference analysis (BI) was performed using a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities (Rannala & Yang 1996). A nucleotide substitution model was estimated by MrModeltest v.2.3 (Posada & Crandall 1998), and a weighted Bayesian analysis was considered. Two MCMC chains were run from random trees for 1,000,000 generations and trees were sampled each 100th generation. The first 25% of trees were discarded as the burn-in phase of each analysis, and the posterior probabilities (BPP) were calculated to assess the remaining trees (Rannala & Yang 1996). The branch support from MP and ML analysis was evaluated with a bootstrapping (BPP) method of 1,000 replicates (Hillis & Bull 1993). Phylograms were plotted in treeview and edited in Adobe Illustrator CS6 v.16.0.0 (https://www.adobe.com/cn/products/illustrator.html). The sequences generated in this study were deposited in GenBank (TABLE 1) and the alignment used for phylogenetic analyses was submitted in TreeBASE (www.treebase.org, study ID S26092). Taxonomic novelties were submitted to the Faces of Fungi database (Jayasiri *et al*. 2015) and Index Fungorum (Index Fungorum 2020).

Results

Fungal isolation

In this study, 28 *Botryosphaeria* isolates were obtained from decaying woody hosts from six nature reserves in Guizhou province, China. Ten isolates from Xingyi Wanfenglin, seven isolates from Maolan Natural Reserve, two isolates from Fanjing mountain and each three isolates respectively from Guiyang Huaxi Wetland Park, Xiaochehe Wetland Park and Suiyang broad water nature reserve were isolated (TABLE 1).

Phylogenetic analyses

The combined alignment matrix of ITS and *tef1-α* included 57 accessions (28 from this study and 29 retrieved from GenBank) and counted 940 characters including gaps (580 characters for ITS and 360 for *tef1-α*), of which 778 characters were constant, 89 variable characters were parsimony-uninformative and 78 characters were variable and parsimony-informative. The MP analysis generated 100 parsimonious trees of which the first tree is presented in FIG. 1 (TL = 216, CI = 0.875, RI = 0.899, RC = 0.787, HI = 0.125). Tree topologies of ML and BI analyses were similar to the MP tree. Based on the multi-locus phylogeny and morphology, 28 strains can be recognized as five species with four known species, namely as *Botryosphaeria dothidea* (FIG. 3), *B. minutispermatia* (FIG. 4), *B. sinensia* (FIG. 5), *B. wangensis* (FIG. 6) and one new species *B. guttulata* (FIG. 2). Eight taxa formed a phylogenetically distinct lineage (FIG. 1) with well-support (MP/ML/BPP = 93/90/0.9), therefore we introduce *B. guttulata* as a new species in this study.

Species residing in the genus

The isolates obtained in this study were clustered into five phylogenetic groups (FIG. 1). Eight isolates (GZCC 19-0202/CGMCC3.20094, GZCC 19-0186, GZCC 19-0188, GZCC 19-0189, GZCC 19-0194, GZCC 19-0196, GZCC 19-0201 and GZCC 19-0205) were found to be consistently distinct from other known phylogenetically related species of *Botryosphaeria* by congruent distinction in the molecular sequence data, and representing as the novel species *B. guttulata*. The phylogenetic analyses based on ITS and *tef1-α* sequences showed that nine isolates (GZCC 19-0073, GZCC 19-0083, GZCC 19-0093, GZCC 19-0099, GZCC 19-0100, GZCC 19-0109, GZCC 19-0116, GZCC 19-0126 and GZCC 19-0341) were identical to *B. sinensia*, eight isolates (GZCC 19-0144, GZCC 19-0148, GZCC 19-0149, GZCC 19-0158, GZCC 19-0164, GZCC 19-0180, GZCC 19-0182 and GZCC 19-0190) phylogenetically clustered with *B. dothidea*, two isolates (GZCC 19-0087 and GZCC 19-0091) were identified as *B. minutispermatia* and one isolate (GZCC 19-0340) was phylogenetically closely related to *B. wangensis*.

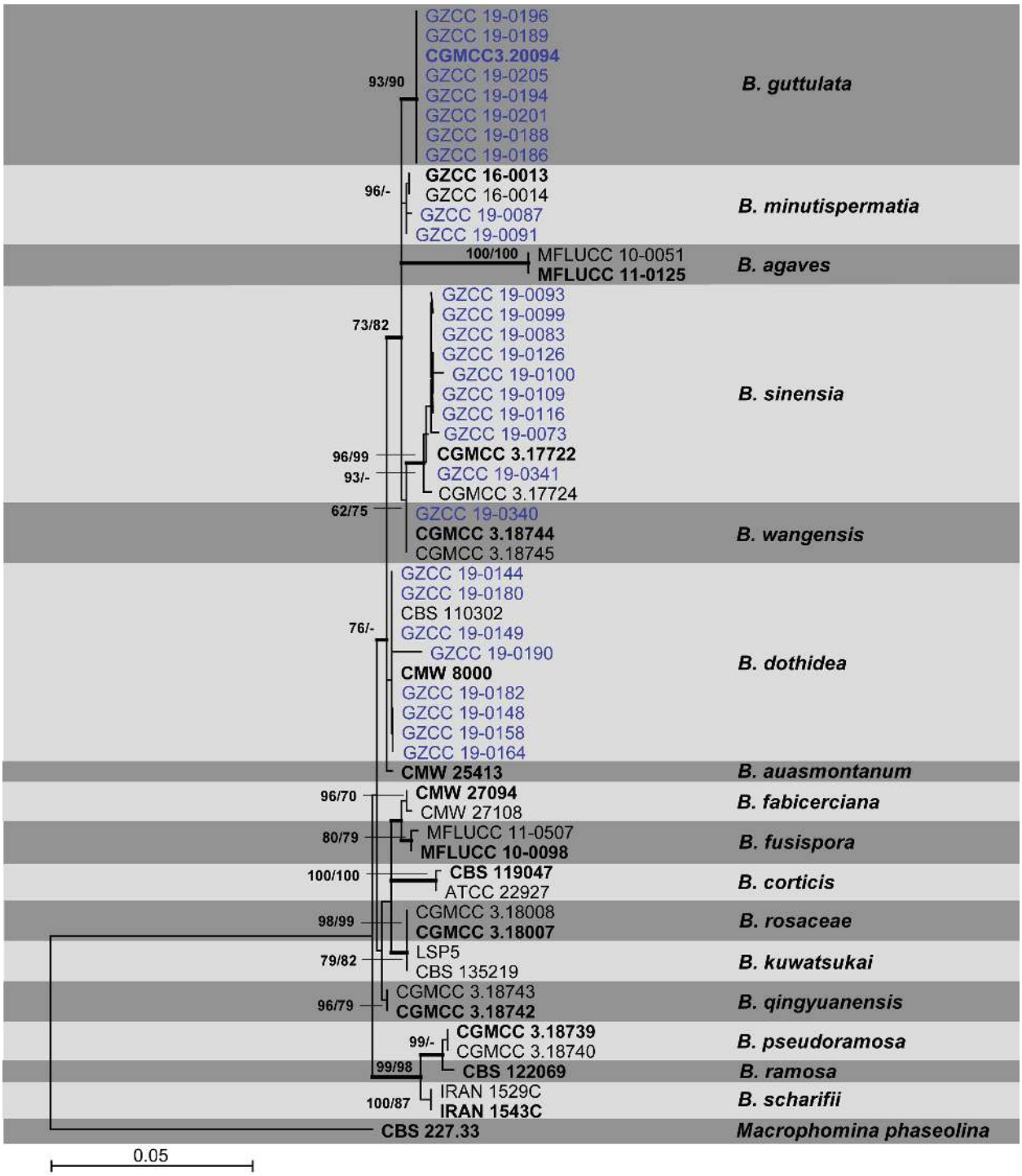

FIGURE 1. Phylogenetic tree based on maximum parsimony (MP) analysis of a combined ITS and *tef1-α* dataset. Bootstrap support values for MP and RAxML greater than 70% are indicated above the nodes. Bayesian posterior probabilities greater than 0.90 (BPP) are indicated in thickened branches. The tree is rooted with *Macrophomina phaseolina* (CBS 227.33). The type species are in bold and new isolates obtained in this study are in blue.

Taxonomy

Botryosphaeria guttulata Y.Y. Chen, A.J. Dissanayake & Jian K. Liu *sp. nov*. (FIG. 2)

Index Fungorum number: IF557253, Facesoffungi number: FoF 07604.

Etymology:—Named according to the account of the guttulation of conidia.

Saprobic on dead wood. **Sexual morph:** unknown. **Asexual morph:** *Conidiomata* up to 402 μm in diam and 213 μm in height, sometimes with a neck up to 435 μm long, arising from the substrate embedded in the host, becoming partially erumpent at maturity, solitary or aggregate, globose, unilocular with a central ostiole, brown to black. *Peridium* 5 to 18 μm in width ($\overline{x}$ = 12 μm, n=10), consisting of 2 regions of hyaline and brown cells lining the locule. *Conidiophores* 16–21 × 4–5 μm, cylindrical, hyaline, smooth, thin-walled, septate, lining the entire inner surface of the conidiomata. *Conidiogenous cells* 6–8 × 3–4 discrete, hyaline, cylindrical to lageniform, phialidic with periclinal thickening, holoblastic producing a single conidium at the tip. *Conidia* (17.1–)18.5–19.3(–20.3) × (4.1–)4.4–4.9(–5.2) μm ($\overline{x}$ = 18.9 × 4.7 μm, n= 30, TABLE 2), guttulate, irregularly fusiform, hyaline, aseptate, smooth-walled, with granular contents.

Culture characteristics:—Colonies on PDA growing rapidly, reaching 30–40 mm diam after 7 d at 25 ℃, aerial mycelium at first white becoming dark grey to black.

TABLE 2. Measurements of asexual morph structures (Conidiomata, conidiophore, conidiogenous cell and conidia) of *Botryosphaeria* species. The measurements of newly introduced species is in bold. All measurements are in μm.

Species	Conidiomata	Conidiophore	Conidiogenous cell	Range of conidial size	Mean	L/W	Reference
B. agaves	Asexual morph not reported						
B. auasmontanum	up to 410 diam	N/A	5.5–8.5 × 2–2.5	(8.1–)8.8–11.3(–13) × (2.5–)2.9–3.9(–5)	10.1 × 3.4	3.0	Slippers *et al*. (2014)
B. corticis	up to 450 diam	7.5–14 × 3.5–4.5	12.5–17.5 × 2.5–4.5	(20.5–)23.5–32.5(–34.5) × (5.0–)5.5–7(–7.5)	28.9 × 6.4	4.5	Phillips *et al*. (2006)
B. dothidea	up to 500 diam	23–35 × 4–5	6–20 × 2–5	(20–)23–27(–30) × 4–5(–6)	26.2 × 5.4	4.9	Slippers *et al*. (2004)
B. fabicerciana	up to 470 diam	Absent	10.5–13.5 × 2.5–3.5	(16.5–)19.5–24.5(–26) × (4.5–)5–6.5(–7.5)	22.0 × 5.8	3.8	Chen *et al*. (2011)
B. fusispora	up to 210 diam	18–21 × 2–4.5	N/A	16–22 × 4–5.5	20.0 × 5.0	4.0	Liu *et al*. (2012)
B. guttulata	**up to 402 diam**	**16–21 × 4–5**	**6–8 × 3–4**	**(17.1–)18.5–19.3(–20.3) × (4.1–)4.4–4.9(–5.2)**	**18.9 × 4.7**	**4.0**	This study
B. kuwatsukai	N/A	N/A	7–18×2–4	(18.5–)20–24.5(–26) × 5–7(–8)	22.3 × 6.2	3.6	Xu *et al*. (2015)
B. minutispermatia	N/A	N/A	5–9×1–3	8–14×3–4	13.0 × 3.5	3.7	Ariyawansa *et al*. (2016)
B. pseudoramosa	up to 698 diam	N/A	11–16 × 2–3.5	(8–)10–13(–16) × (4–)4.5–5(–6)	11.5 × 4.6	2.5	Li *et al*. (2018)
B. qingyuanensis	up to 317 diam	N/A	7.5–12 × 2.5–3.5	(15–)19.5–24.5(–28.5) × (5–)6–6.5(–7.5)	22.0 × 6.2	3.5	Li *et al*. (2018)
B. ramosa	up to 510 diam	N/A	7.5–10 × 2–3	(11–)12–15(–16) × (4.7–)5–6(–7)	13.5 × 5.5	2.3	Pavlic *et al*. (2008)
B. rosaceae	up to 290 diam	20–55 × 5–6	6–15 × 2.5–5	20–31 × 6–8	26.2 × 6.7	3.9	Zhou *et al*. (2017)
B. scharifii	up to 760 diam	7.5–33.5 × 2–4.5	7–15 × 1.5–3.5	(11.5–)13–17(–19) × 4–6.5	15.4 × 5.2	2.7	Abdollahzadeh *et al*. (2013)
B. sinensia	N/A	N/A	8–15 × 2–3	(15–)19–29 × 5–7	24.3 × 5.9	4.1	Zhou *et al*. (2016)
B. wangensis	up to 698 diam	N/A	8.5–13.5 × 2–3	(20.5–)22–26(–29) × (4.5–)5.5–6.5(–7.5)	23.8 × 6.0	3.9	Li *et al*. (2018)

Material examined:—CHINA, Guizhou Province, Xingyi District, Wanfenglin, Saprobic on decaying branch, June 2019, Y.Y. Chen (HKAS 107541, holotype; GZAAS 19-1921, isotype), ex-type living culture CGMCC 3.20094 = GZCC 19-0202.

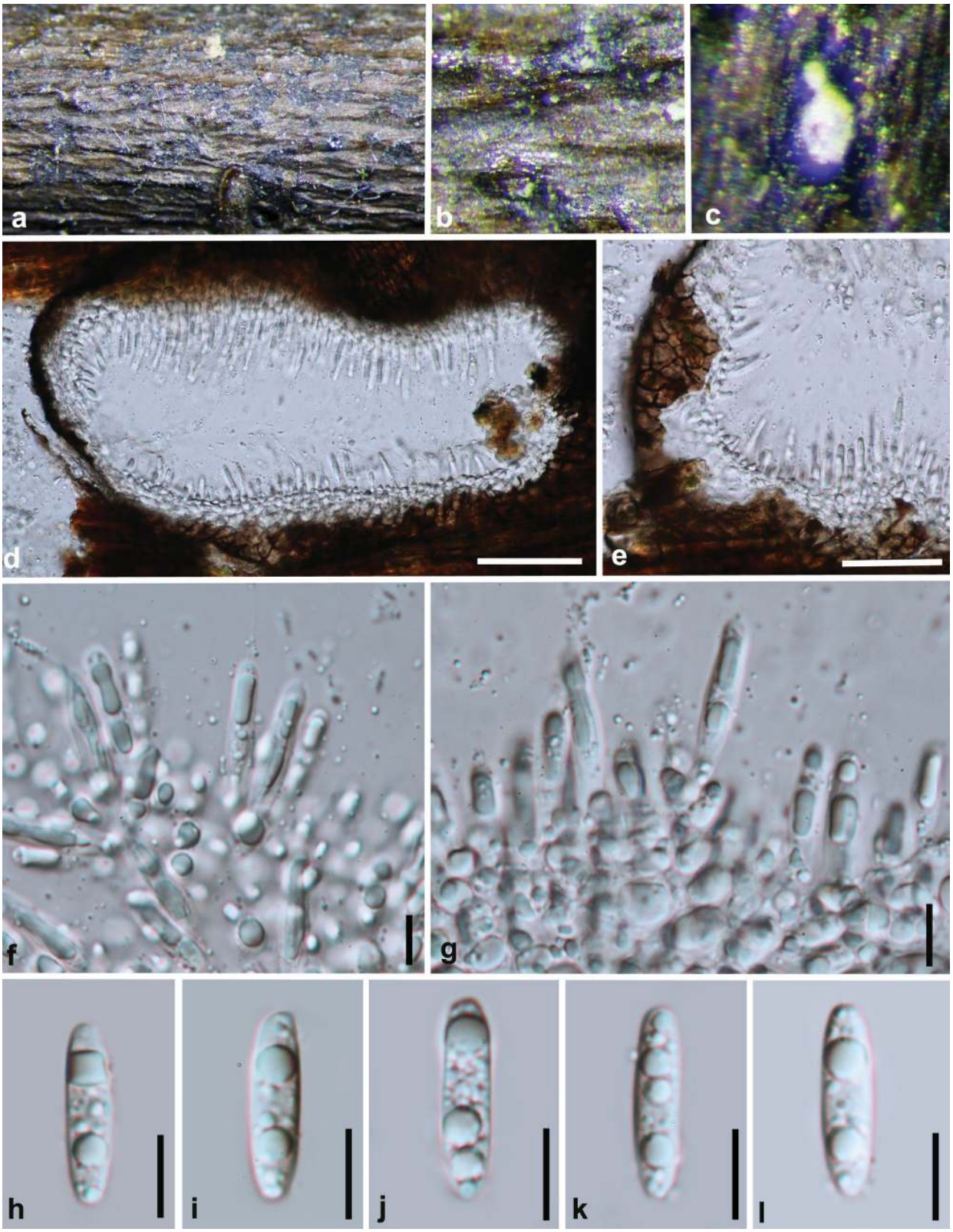

FIGURE 2. *Botryosphaeria guttulata* (HKAS 107541, holotype). a–c. Colonies on host surface. d. Cross section of conidiomata. e. Peridium. f, g. conidiogenous cells and developing conidia. h–l. hyaline aseptate conidia. **Scale bars:** d,e = 100 μm; f,g = 20 μm; h–l = 10 μm.

Additional materials examined:—CHINA. Guizhou Province, Tongren District, Fanjing mountain, Saprobic on decaying branch, May 2019. Y.Y. Chen (GZAAS 19-1905, paratype), living culture GZCC 19-0186; *ibid*., Xingyi District, Wanfenglin, Saprobic on decaying branch, June 2019. Y.Y. Chen, (GZAAS 19-1907, living culture GZCC 19-0188; GZAAS 19-1908, living culture GZCC 19-0189; GZAAS 19-1913, living culture GZCC 19-0194).

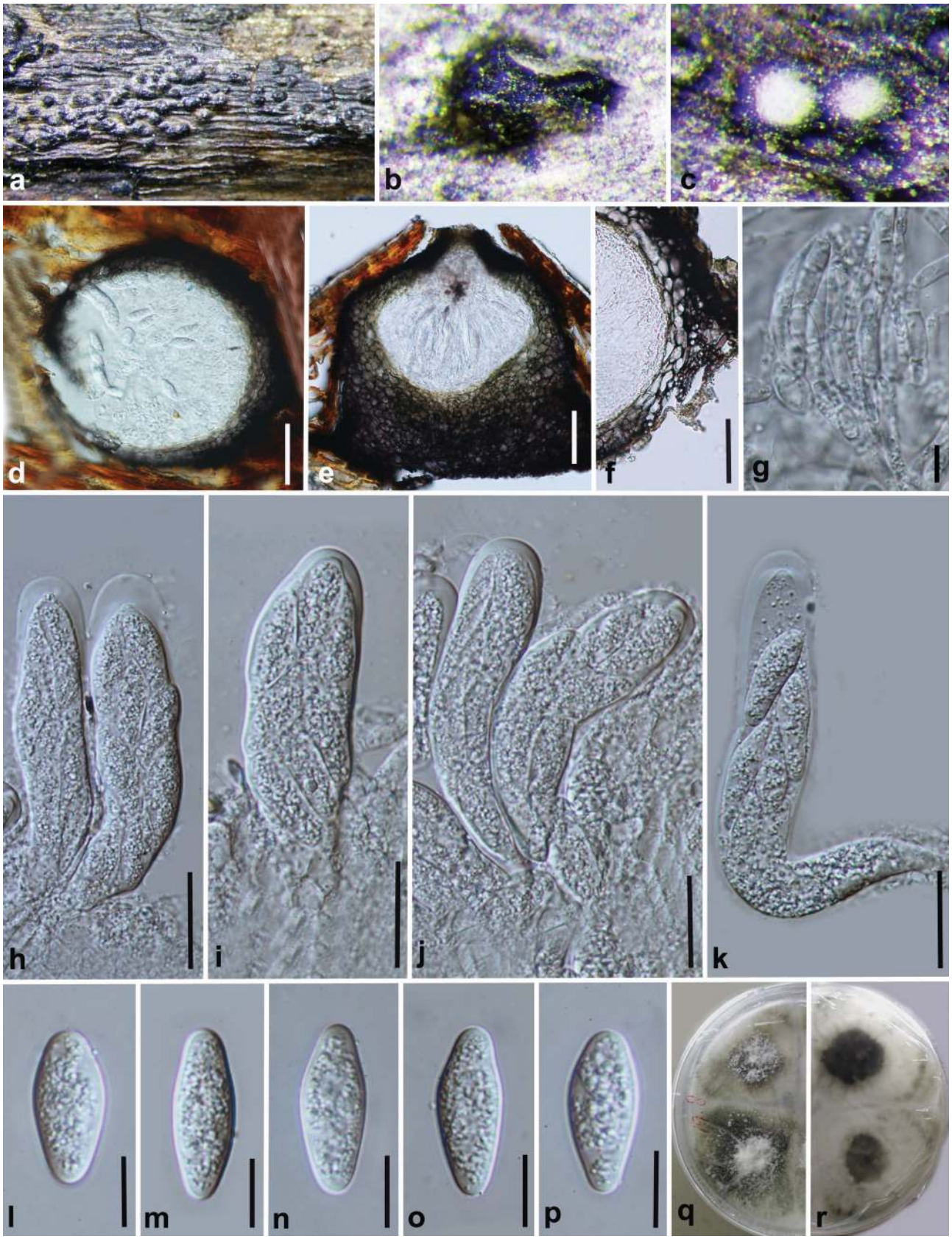

FIGURE 3. *Botryosphaeria dothidea* (GZAAS 19-1863). a, b. Ascomatal necks emerging through the host bark. c. Vertical section of ascoma. d, e. Section through an ascoma. f. Section through the ascomal wall. g. Pseudoparaphyses. h–k. Asci with ascospores. l–p. Ascospores. q,r. Culture characteristics on PDA, q from above, r from reverse. **Scale bars:** f=50μm, g=10μm, h–k=20μm, l–p=10μm.

Notes:—*Botryosphaeria guttulata* fits in the generic concept of *Botryosphaeria* by having irregularly fusiform, hyaline, aseptate, smooth-walled conidia. Eight isolates (GZCC 19-0186, GZCC 19-0188, GZCC 19-0189, GZCC

19-0194, GZCC 19-0196, GZCC 19-0201, GZCC 19-0202/CGMCC3.20094 and GZCC 19-0205) representing *B. guttulata* cluster together as a distinct lineage and appears mostly related to *B. agaves* and *B. minutispermatia* (Liu *et al*. 2012, Ariyawansa *et al*. 2016) (FIG. 1). The asexual morph of *B. agaves* has not been reported, and *Botryosphaeria guttulata* can be morphologically distinguished from *B. minutispermatia* by having larger conidia ($\overline{x}$ = 18.9 × 4.7; L/W = 4.0 vs. 13.0 × 3.5, L/W = 3.7) (Ariyawansa *et al*. 2016) (TABLE 2). In addition, *Botryosphaeria guttulata* differs from other *Botryosphaeria* species by its prominent guttules in conidia. To further justify its classification, pairwise dissimilarities of DNA sequence data was compared following the phylogenetic result (FIG. 1). There are 5 bp (base pair) and 8 bp differences in ITS and *tef1-α* respectively between *B. guttulata* and *B. minutispermatia*, while 9 bp and 18 bp differences (ITS and *tef1-α* respectively) between *B. guttulata* and *B. agaves*. Based on morphology and molecular evidences, we hereby describe *B. guttulata* as a new species.

Botryosphaeria dothidea (Moug.) Ces. & De Not., Comm. Soc. crittog. Ital: 212 (1863) (FIG. 3)

Index Fungorum number: IF183247, Facesoffungi number: FoF03512.

Pathogenic or saprobic on dead wood. **Sexual morph**: *Ascomata* 210−400 µm diam erumpent, pseudothecial, forming a botryose aggregate, sometimes solitary, globose with a central ostiole, emergent or embedded, papillate, black, wall comprising 4–13 layers of *textura angularis*, outer region of dark brown cells, inner region of 2–3 layers of hyaline cells lining the locule. *Asci* 60−120 × 18–25 µm, bitunicate, clavate, forming between pseudoparaphyses. *Pseudoparaphyses* filiform, septate, constricted at the septa, rarely branched, 2–4 µm wide. *Ascospores* (15–)19–27(–33) × (6–)7–8(–10) µm ($\overline{x}$ = 21.5 × 7.5 µm, n=30), fusoid to ovoid, tapered ends appearing spindle-shaped, biseriate in the ascus. **Asexual morph**: *Conidiomata* stromatic. *Conidiophores* hyaline, cylindrical, smooth. *Conidiogenous cells* 11.5–14×4–.5 µm ($\overline{x}$ = 13×6 µm, n=20) hyaline, sub-cylindrical. *Conidia* 15–10×7–5 µm ($\overline{x}$ = 12.5×6 µm n=30), hyaline, unicellular, narrowly fusiform, with a sub-truncate to bluntly rounded base, forming a septum before germination, smooth-walled with a granular content.

Culture characteristics:—Colonies on PDA reaching 50 mm diam., after 4 d at 28 ℃. Initially white becoming grey, moderately dense, margin smooth, olivaceous.

Material examined:—CHINA, Guizhou Province, Libo District, Maolan Natural Reserve, saprobic on dead wood, July 2017, Y.Y. Chen, GZAAS 19-1863, living culture GZCC 19-0144; *ibid*., Zunyi District Suiyang broad water nature reserve, saprobic on dead wood, April 2018, Y.Y. Chen, (GZAAS 19-1867, living culture GZCC 19-0148; GZAAS 19-1868, living culture GZCC 19-0149; GZAAS 19-1877, living culture GZCC 19-0158); *ibid*., Xingyi District, Wanfenglin, saprobic on dead wood, July 2018, Y.Y. Chen, (GZAAS 19-1883, living culture GZCC 19-0164; GZAAS 19-1899, living culture GZCC 19-0180; GZAAS 19-1901, living culture GZCC 19-0182; GZAAS 19-1909, living culture GZCC 19-0190).

Notes:—Slippers *et al.* (2004) designated a specimen as the neotype of *Botryosphaeria dothidea* and also designated an epitype (PREM 57372) on *Prunus* sp. collected from Crocifisso, Switzerland, with an ex-epitype culture (CBS 115476 = CMW 8000). The colony morphology of the taxa isolated from decaying woody hosts in this study is similar to *B. dothidea* (Slippers *et al.* 2004, Phillips *et al*. 2013). In the multi-gene phylogenetic analysis, these eight isolates clustered together (76% ML, 0.9 BPP) with the ex-epitype of *B. dothidea* and could be identified as *B. dothidea*. *Botryosphaeria dothidea* has a wide range of hosts and has a worldwide distribution (Phillips *et al*. 2013, Dissanayake *et al*. 2016).

Botryosphaeria minutispermatia Ariyawansa, K.D. Hyde & Z.Y. Liu, Phytotaxa 275: 40 (2016) (FIG. 4)

Index Fungorum number: IF552252, Facesoffungi number: FoF 02393.

Saprobic on decaying wood. **Sexual morph**: *Ascomata* 220−310 µm erumpent or embedded in the host, mostly solitary, globose with a central ostiole, papillate, brown to black. *Peridium* 35–72 µm ($\overline{x}$ = 52 µm, n=10) wide, consisting of 5–10 layers with 2–4 layers of hyaline cells lining the locule. *Hamathecium* 2–4 µm wide, filiform, septate, rarely branched pseudoparaphyses, constricted at the septa. *Asci* 85–110×12–20 µm ($\overline{x}$ = 91×17 µm, n=30), 8-spored, bitunicate, fissitunicate, broadly clavate, with short, broad pedicel with well-developed ocular chamber, forming between pseudoparaphyses. *Ascospores* 24–28×7–12 µm ($\overline{x}$ = 26×9 µm, n=30), biseriate, fusoid to ovoid, hyaline, thick-walled, aseptate with tapered ends. **Asexual morph**: Not observed.

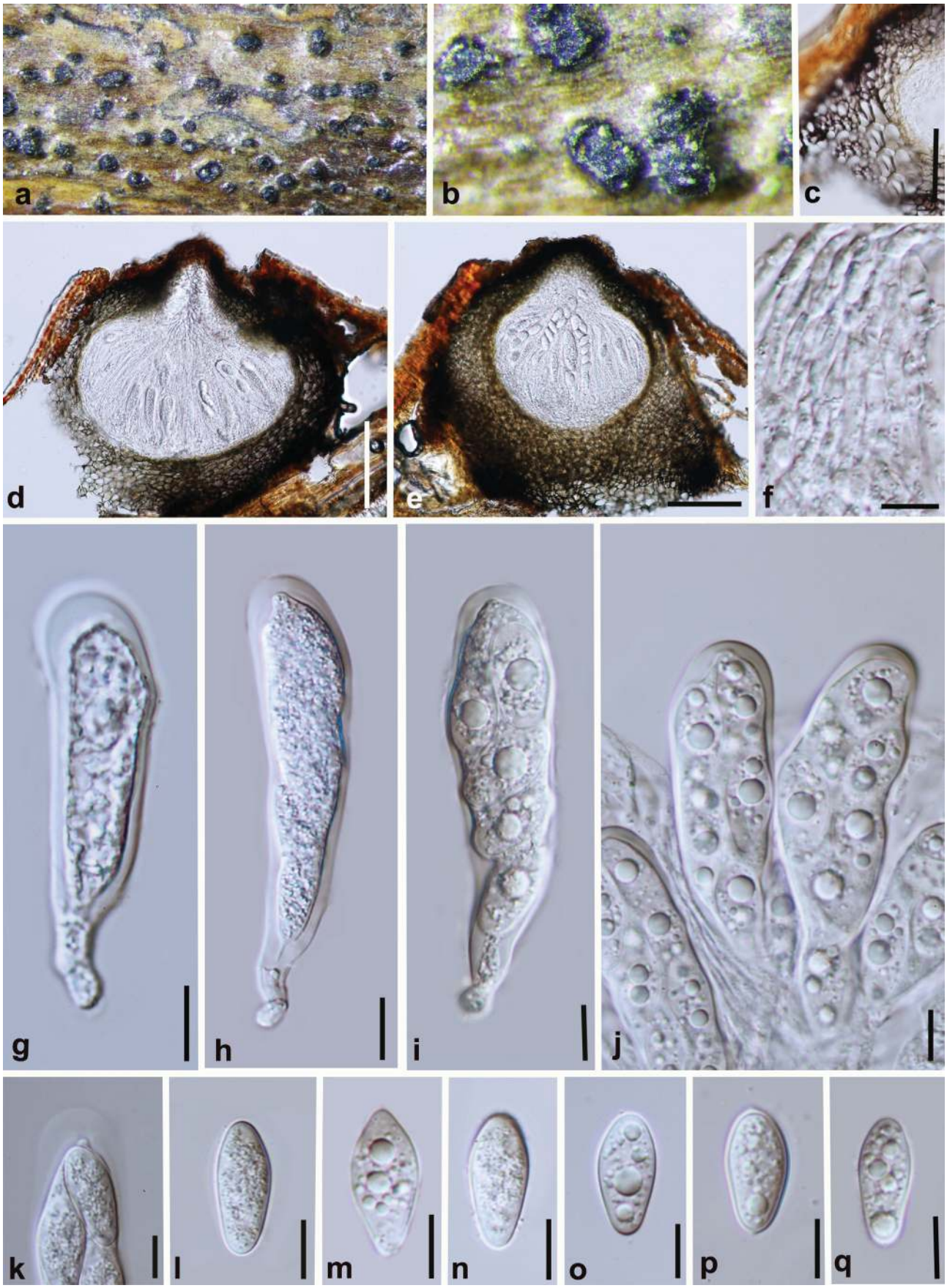

FIGURE 4. *Botryosphaeria minutispermatia* (GZAAS 19-1806). a, b. Ascomata on host surface. c. Section through the ascomal wall. d, e. Section through an ascoma. f. Pseudoparaphyses. g, h. Immature asci. i, j. Asci with ascospores. k–q. Ascospores. **Scale bars:** c=50μm, d, e=100μm, f–q=10μm.

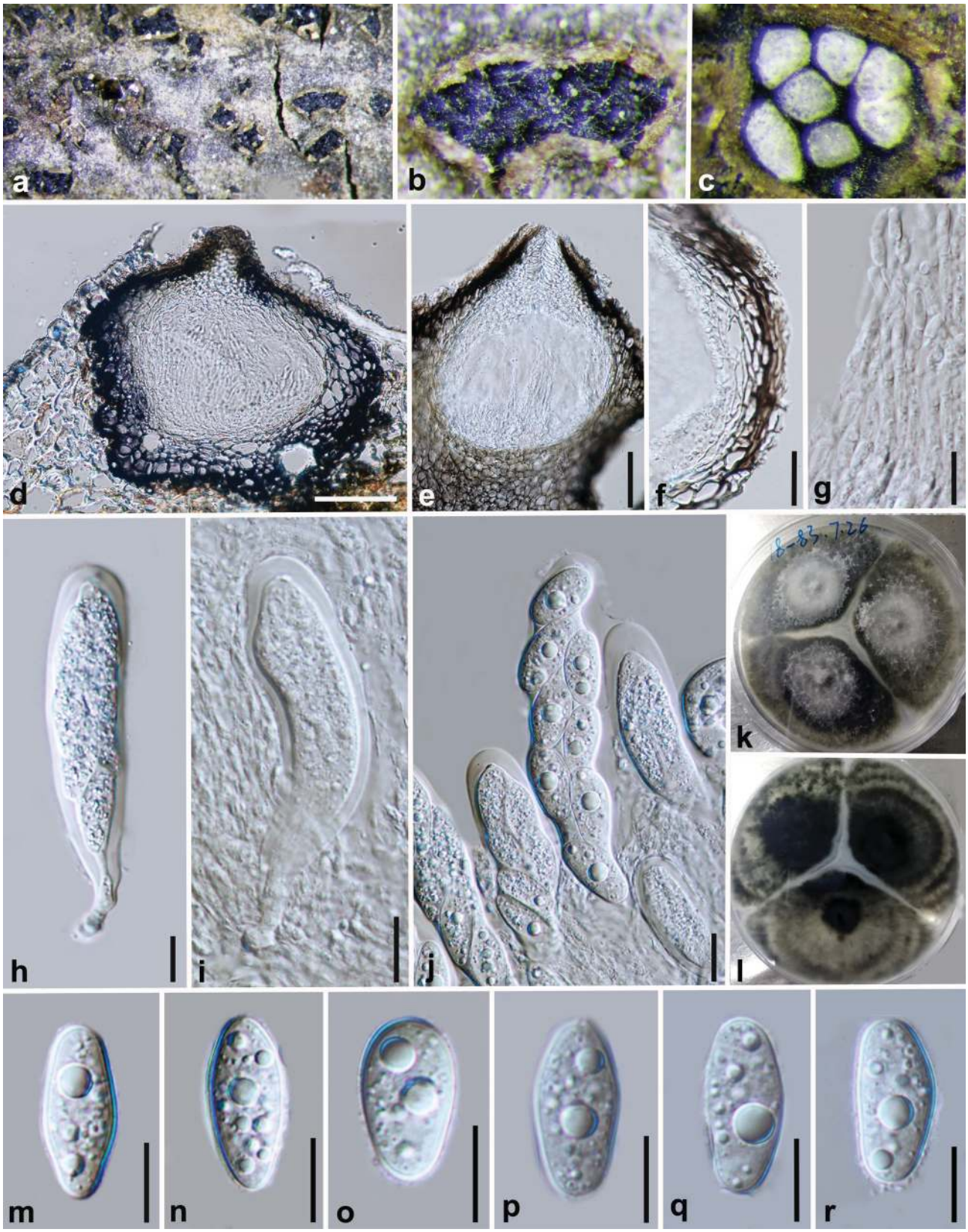

FIGURE 5. *Botryosphaeria sinensia* (GZAAS 19-1792) a, b. Ascomata on host surface. c. Vertical section of ascoma. d, e. Section through an ascoma. f. Section through the ascomal wall. g. pseudoparaphyses. h–j. asci. k,l. Culture on PDA, k from above, l from reverse, m–r. Ascospores. **Scale bars:** d = 100 μm, e=50μm, f 20μm, g–j=10μm, m–r=10μm

Material examined:—CHINA, Guizhou Province, Guiyang Xiaochehe Wetland Park, saprobic on dead wood, May 2017, Y.Y. Chen, GZAAS 19-1806, living culture GZCC 19-0087; GZAAS 19-1810, living culture GZCC 19-0091.

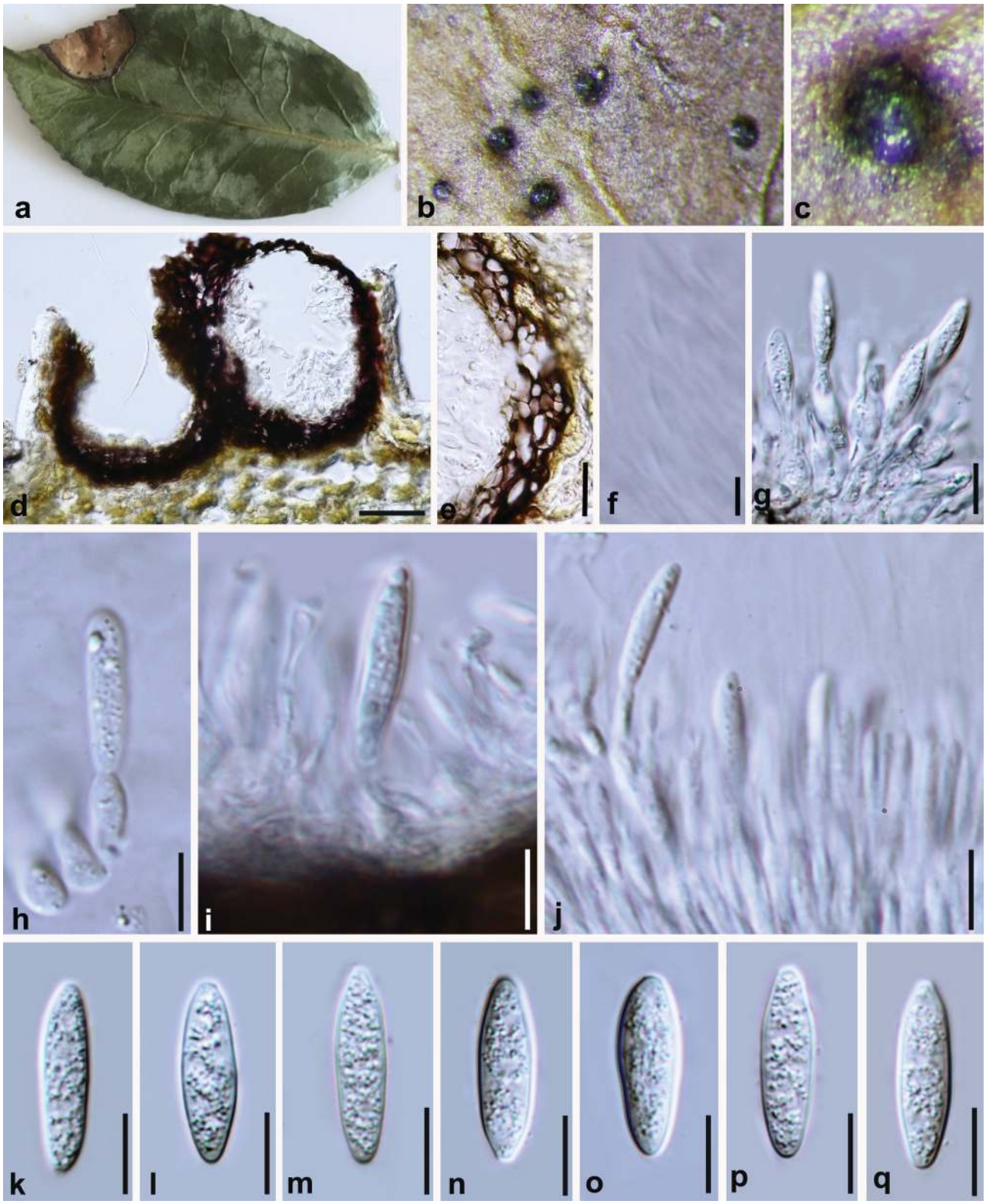

FIGURE 6. *Botryosphaeria wangensis* (GZAAS 19-2059). a–c. Conidiomata on host surface. d. Cross section of conidiomata. e. Peridium. f. Pseudoparaphyses. g–j. conidiogenous cells and developing conidia. k–q. hyaline aseptate conidia. **Scale bars:** d = 50 μm, e = 20 μm, f–q =10 μm.

Notes:—Our two collections are morphologically identical to *Botryosphaeria minutispermatia* (Ariyawansa *et al*. 2016). The multi-gene phylogenetic analysis showed that the newly obtained two isolates (GZCC 19-0087 and GZCC 19-0091) clustered together with ex-type of *B. minutispermatia* (GZCC 16-0013) and this species is only reported from Guizhou province, China (Ariyawansa *et al*. 2016).

Botryosphaeria sinensia Y.P. Zhou & Y. Zhang ter, Phytotaxa 245: 45 (2016) (FIG. 5)

Index Fungorum number: IF819599, Facesoffungi number: FoF07975.

Saprobic on decaying wood. **Sexual morph**: *Ascomata* 175−280 µm diam., mostly erumpent, pseudothecial, clustered, forming botryose clusters aggregate of up to 40, spherical to globose with a central ostiole, papillate, brown to black, wall comprising 5–15 layers of *textura angularis*, outer region of dark brown cells, inner region of 2–4 layers of hyaline cells lining the locule. *Pseudoparaphyses* 2–5 µm wide, filiform, septate, constricted at the septa, rarely branched. *Asci* 85−135 × 18–28 µm, bitunicate, broadly clavate, short pedicellate with a well-developed apical chamber, forming between pseudoparaphyses. *Ascospores* 19–30 × 7–11 µm ($\overline{x}$ = 24.6 × 8.7 µm, n=50), fusoid to ellipsoid, widest in the upper third or middle, obtuse apex, sometimes with tapered end, partially overlapping, hyaline, aseptate, biseriate in the ascus. **Asexual morph**: Not observed.

Culture characteristics:—Colonies on PDA reaching 65 mm diam., after 4 d at 28 ℃. Initially white becoming grey, moderately dense, margin smooth, olivaceous.

Material examined:—CHINA, Guizhou Province, Guiyang District, Huaxi Wetland Park, saprobic on dead wood, April 2017, Y.Y. Chen, (GZAAS 19-1792, living culture GZCC 19-0073; GZAAS 19-2060, living culture GZCC 19-0341); *ibid*., Xiaochehe Wetland Park, saprobic on dead wood, May 2017, Y.Y. Chen, GZAAS 19-1802, living culture GZCC 19-0083; *ibid*., Libo Distract, Maolan Natural Reserve, saprobic on dead wood, July 2017, Y.Y. Chen, (GZAAS 19-1812, living culture GZCC 19-0093; GZAAS 19-1818, living culture GZCC 19-0099; GZAAS 19-1819, living culture GZCC 19-0100; GZAAS 19-1828, living culture GZCC 19-0109; GZAAS 19-1835, living culture GZCC 19-0116; GZAAS 19-1845, living culture GZCC 19-0126).

Notes:—*Botryosphaeria sinensia* was introduced by Zhou *et al*. (2016) and it has a wide host range including *Juglans regia* (Jualandaceae), *Morus alba* (Moraceae) and *Populus* sp. (Salicaceae). Nine of our collections are morphologically similar to the original description of *B. sinensia* (Zhou *et al*. 2016), and all the sequences generated in this study are identical to the previous data (99–100%). Furthermore, this is the first time *B. sinensia* is reported from Guizhou province and it extends the species distribution in China, as well as the landform habitats (In Karst region).

Botryosphaeria wangensis G.Q. Li & S.F. Chen, Persoonia 40: 84 (2017) (FIG. 6)

Index Fungorum number: IF822325, Facesoffungi number: FoF07976.

Saprobic on decaying wood. **Sexual morph**: Not observed. **Asexual morph:** *Conidiomata* stromatic, Conidiophores hyaline, cylindrical, smooth. *Conidiogenous cells* 11.5–14×4–6.5 µm ($\overline{x}$ = 13×6 µm, n=20) hyaline, sub-cylindrical. *Conidia* 15–10×7–5 µm ($\overline{x}$ =12.5×6 µm n=20), hyaline, unicellular, narrowly fusiform, with a sub-truncate to bluntly rounded base, forming a septum before germination, smooth-walled with a granular content.

Culture characteristics:—Colonies on PDA reaching 65 mm diam., after 4 d at 28 ℃. Initially white becoming grey, moderately dense, margin smooth, olivaceous.

Material examined:—CHINA, Guizhou Province, Guiyang Huaxi Wetland Park, saprobic on dead wood, April 2017, Y.Y. Chen, GZAAS 19-2059, living culture GZCC 19-0340.

Notes:—Based on the morphology and phylogeny, we identify this species as *Botryosphaeria wangensis* (Li *et al*. 2018). This species is only reported from China and it was isolated from *Cedrus deodara* (Pinaceae) when it was described (Li *et al*. 2018).

Discussion

In this study, decaying woody samples were collected from six nature reserves in Guizhou province, China. This study aims to understand the diversity of *Botryosphaeria* species associated with woody hosts in nature reserves in Guizhou province and a polyphasic approach of combined morphological features and molecular phylogeny has been employed to justify these isolated fungi. Four commonly encountered species, namely *Botryosphaeria dothidea*, *B. minutispermatia*, *B. sinensia* and *B. wangensis* were identified. Among the 28 obtained isolates, eight taxa formed a unique lineage which was identified by incorporating reference type and non-type isolates in multi-gene phylogenetic analyses. Therefore, it is introduced as a new species *Botryosphaeria guttulata*.

Up to date, there are fifteen *Botryosphaeria* species reported in worldwide, and most of them have been justified with molecular data (Xu *et al*. 2015, Dissanayake *et al*. 2016, Zhou *et al*. 2017, Li *et al*. 2018, Jayawardena *et al*. 2019). Among them, ten species are reported from China (66% of reported species). Many of them have been isolated from a large assemblage of agriculturally (e.g. grapevine, *Camellia sinensis*) and ecologically (e.g. *Eucalyptus* sp.) important perennial hosts in China (Yan *et al.* 2013; Dissanayake *et al.* 2016). *Botryosphaeria dothidea* has been reported over 35 plant species in China, causing cankers and branch dieback of many orchard trees such as Apple, Chestnut, English walnut and Kiwi fruit (Xu *et al*. 2015, Zhou *et al*. 2015, Li *et al*. 2018); ornamental trees and shrubs (e.g. *Rosa chinensis*) and forest trees including *Acer platanoides*, *Cornus alba*, *Eucalyptus grandis*, *Helwingia chinensis*, *Morus alba* and *Populus* sp. (Pan *et al*. 2019). *Botryosphaeria fabicerciana* and *B. fusispora* have been isolated from *Eucalyptus* sp. (Chen *et al*. 2011, Li *et al*. 2018); *B. kuwatsukai* from *Malus domestica* and *Pyrus* sp. (Xu *et al*. 2015); *B. minutispermatia* from an unknown host (Ariyawansa *et al*. 2016) while *B. rosaceae* from *Malus* sp. and *Amygdalus* sp. (Zhou *et al*. 2017). *Botryosphaeria sinensia* has been isolated from dieback and cankers of *Juglans regia*, *Malus pumila*, *Morus sinensia* sp. and *Populus* sp. in China (Zhou *et al*. 2016, 2017, Li *et al*. 2018). In this study, *Botryosphaeria sinensia* was isolated more frequently (32%) than any other *Botryosphaeria* species from deadwood in nature reserves from Guizhou province. This result suggests that *B. sinensia* may be quite well adapted to deadwood communities in the nature reserves. Meanwhile *Botryosphaeria pseudoramosa*, *B. qingyuanensis* and *B. wangensis* have been reported only from *Eucalyptus* sp. in China (Li *et al*. 2018). The newly identified species *Botryosphaeria guttulata* has been shown as the second highest frequency (28.5%) indicating it's prevalence in nature reserves. Therefore, it was not surprising to isolate *Botryosphaeria* species from decaying woody hosts in nature reserves from Guizhou province, China.

The genus *Botryosphaeria* causes severe decline of habitat productivity and sustainability. This work adds to knowledge on the genus by introducing and recognizing the new and existing *Botryosphaeria* species occurring on decaying wood in nature reserves from Guizhou Province. Hence, it is necessary for continuous exploration and identification of these fungi in nature reserves in Karst formations in Guizhou province for protection of its biodiversity. We suggest our findings to be undertaken by relevant authorities to take preventive measures as the saprobic *Botryosphaeria* could alter to be pathogenic fungi with the changes in abiotic factors.

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