



***Botryosphaeria rosaceae* sp. nov. and *B. ramosa*, new botryosphaeriaceous taxa from China**

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Abstract

A new species of *Botryosphaeria* (*B. rosaceae*) is described and illustrated from Rosaceae in China. Phylogenetic analysis based on combined ITS, LSU, *tef1-α* and TUB loci supported its separation from another species of *Botryosphaeria*. Morphologically, *B. rosaceae* was comparable with *B. sinensia*, but the smaller and slenderer ascospores and conidia as well as the broader host spectrum of *B. sinensia* differentiate it from *B. rosaceae*. In addition, *Botryosphaeria ramosa* was found associated with an *Acacia* species in this study, which is a new host and first report in the Northern Hemisphere.

Keywords – Asia – Multilocus phylogeny – Rosaceae – Taxonomy

Introduction

Botryosphaeria Ces. & De Not. was established in 1863 by Cesati & De Notaris and earlier species were described mostly based on their sexual morph and host occurrences (Cesati & De Notaris 1863, De Notaris 1863, Grossenbacher & Duggar 1911, Putterill 1919, Saccardo 1877, 1882, Trotter 1928). The polyphyletic status of *Botryosphaeria sensu lato* has been well documented, and the species encompassed several distinct genera of Botryosphaeriaceae (Crous et al. 2006, Phillips et al. 2008, 2013, Slippers et al. 2004, Dissanayake et al. 2016). Based on the phylogenetic analysis of five loci (SSU, LSU, ITS, TUB and *tef1-α*), Phillips et al. (2013) recognized seven species in *Botryosphaeria* s. str., namely *B. agaves* (Henn.) E.J. Butler, Slippers & G.J. Marais, *B. cortices* (Demaree & Wilcox) Arx & E. Müll., *B. dothidea* (Moug.) Ces. & De Not., *B. fabicerciana* (Pavlic, M.J. Wingf. & X.D. Zhou, S.F. Chen bis.) A.J.L. Phillips & A. Alves, *B. fusispora* Boonmee, J.K. Liu & K.D. Hyde, *B. ramosa* (Pavlic, T.I. Burgess & M.J. Wingf.) A.J.L. Phillips & A. Alves and *B. scharifii* Abdoll., Zare & A.J.L. Phillips. Subsequently, another three species of *Botryosphaeria*, *B. auasmontanum* F.J.J. Van der Walt, *B. minutispermata* Ariyawansa, K.D. Hyde & Z.Y. Liu and *B. sinensia* Y.P. Zhou, Y. Zhang ter., were described (Ariyawansa et al. 2016, Slippers et al. 2014, Zhou et al. 2016). In total, ten species have been confirmed within *Botryosphaeria*, three of which have been reported from China, viz. *B. fabicerciana*, *B. minutispermata* and *B. sinensia* (Ariyawansa et al. 2016, Phillips et al. 2013, Dissanayake et al. 2016, Zhou et al. 2016).

In the course of an ongoing survey of biodiversity of botryosphaeriaceous ascomycetes in China, a new taxon with general characteristics of *Botryosphaeria* was collected. Phylogenetic analysis based on combined ITS, LSU, TUB and *tef1-a* nrDNA sequences verified its status as a new species within *Botryosphaeria*. Based on a combination of morphological and molecular phylogenetic differences, a new species, *B. rosaceae*, is introduced. In addition, *B. ramosa* is first reported in China

Materials & Methods

Isolates and morphology

Specimens were collected in temperate region of China during 2014 and 2015 (Table 1). Twigs were air-dried at room temperature and examined with an Olympus SZ 61 dissecting microscope without prior incubation in a moist chamber. Photomicrographs were taken with a Nikon Coolpix 995 digital camera fitted with an eyepiece adapter to the dissecting microscope. Microscopic observations of ascostromatal contents were made from material mounted in water. Thin vertical free-hand sections were cut with a razor blade under the dissecting microscope and mounted in water. Photomicrographs were taken with a Nikon Coolpix 995 digital camera connected to a trinocular Leitz Orthoplan microscope and processed with Adobe Photoshop Elements 10 software. Measurements of ascospores, asci and hamathecial elements were made from water mounts.

Isolations were made from single ascospores or conidia of ascomata or conidiomata on dead or dying twigs of different hosts and grown on 2 % water agar (WA) (Biolab, S.A.), and subsequently transferred to synthetic nutrient-poor agar (SNA) with sterilized pine needles. Isolates were grown on malt extract agar (MEA, Biolab, S.A.) at ambient temperatures (about 26–28 °C) in the dark to establish colony characteristics. Isolates and herbarium specimens were deposited at Beijing Forestry University (BJFU) with duplicates in the China General Microbiological Culture Collection Center (CGMCC) and the Mycological Herbarium of the Institute of Microbiology, Chinese Academy of Sciences (HMAS).

DNA extraction, PCR amplification

DNA was extracted from mycelium grown on MEA plates with CTAB plant genome DNA fast extraction kit (Aidlab Biotechnologies Co., Ltd, Beijing, China). The internal transcribed spacer of rDNA (ITS) was amplified and sequenced with primers ITS-4 and ITS-5 (White et al. 1990). The 28S large subunit nrDNA (LSU) was amplified and sequenced with primers LR0R and LR5 (Vilgalys & Hester 1990). The translation elongation factor-1 α (*tef1-a*) was amplified and sequenced with primers EF1-688F and EF1-1251R (Alves et al. 2008). The β -tubulin gene (TUB) was amplified and sequenced with primers Bt2a and Bt2b (Glass & Donaldson 1995). PCR amplification and sequencing was done following the protocol of Zhang et al. (2009).

Sequence alignment and phylogenetic analysis

Sequence data of combined ITS, LSU, *tef1-a* and TUB loci were used to infer the phylogenetic relationships among all confirmed *Botryosphaeria* species for which sequence data were available from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>), as well as the sequences generated in this study. Concatenated sequences were aligned with MEGA v. 6 (Tamura et al. 2013) and phylogenetic analyses performed in PAUP v. 4.0b10 (Swofford 2002) and MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003). Prior to phylogenetic analysis, ambiguous sequences at the start and the end of sequences were deleted and gaps manually adjusted to optimize the alignments. Maximum Parsimony (MP) was used to conduct heuristic searches as implemented in PAUP with the default options method (Zhang et al. 2008). Analyses with gaps treated as missing data were done under different parameters of maximum parsimony criteria as outlined in Zhang et al. (2008). Clade stability was assessed in a bootstrap analysis with 1 000 replicates, random sequence additions with maxtrees set to 1 000 and other default parameters as implemented in PAUP. For the

Species	Culture/Specimen No.	Host	Location	GenBank accession numbers			
				ITS	LSU	tef1- α	TUB
<i>Botryosphaeria agaves</i>	CBS 133992 ex-neotype	<i>Agave</i> sp.	Thailand	JX646791	JX646808	JX646856	JX646841
<i>B. agaves</i>	MFLUCC 10-0051	<i>Agave</i> sp.	Thailand	JX646790	JX646807	JX646855	JX646840
<i>B. auasmontanum</i>	CMW 25413 ex-type	<i>Pinus</i> sp.	Namibia	KF766167	KF766332	N/A	N/A
<i>B. corticis</i>	CBS 119047 ex-epitype	<i>Vaccinium corymbosum</i>	New Jersey, USA	DQ299245	EU673244	EU017539	EU673107
<i>B. corticis</i>	ATCC 22927	<i>Vaccinium</i> sp.	North carolina, USA	DQ299247	EU673245	EU673291	EU673108
<i>B. dothidea</i>	CBS 115476 ex-epitype	<i>Prunus</i> sp.	Crocifisso, Switzerland	AY236949	AY928047	AY236898	AY236927
<i>B. dothidea</i>	CBS 110302	<i>Vitis vinifera</i>	Portugal	AY259092	EU673243	AY573218	EU673106
<i>B. fabicerciana</i>	CBS 127193 ex-type	<i>Eucalyptus</i> sp.	Fujian, China	HQ332197	N/A	HQ332213	N/A
<i>B. fabicerciana</i>	CMW 27108	<i>Eucalyptus</i> sp.	Fujian, China	HQ332200	N/A	HQ332216	N/A
<i>B. fuispora</i>	MFLUCC 10-0098 ex-type	<i>Entada</i> sp.	Thailand	JX646789	JX646806	JX646854	JX646839
<i>B. minutispermata</i>	GZCC 16-0013 ex-type	dead wood	China	KX447675	N/A	KX447678	N/A
<i>B. minutispermata</i>	GZCC 16-0014	dead wood	China	KX447676	N/A	KX447679	N/A
<i>B. ramosa</i>	CBS 122069 ex-type	<i>E. camaldulensis</i>	Bell Gorge, Australia	EU144055	N/A	EU144070	N/A
<i>B. ramosa</i>	CGMCC3.18004	<i>Acacia</i> sp.	Hainan, China	KX197073	KX197082	KX197093	KX197100
<i>B. ramosa</i>	CGMCC3.18005	<i>Corymbia citriodora</i>	Guangdong, China	KX197071	KX197080	N/A	KX197098
<i>B. ramosa</i>	CGMCC3.18006	Myrtaceae	Guangdong, China	KX197072	KX197081	KX197092	KX197099
<i>B. rosaceae</i>	CGMCC3.18007	<i>Malus</i> sp.	Shandong, China	KX197074	KX197083	KX197094	KX197101
<i>B. rosaceae</i>	CGMCC3.18008	<i>Amygdalus</i> sp.	Shandong, China	KX197075	KX197084	KX197095	KX197102
<i>B. rosaceae</i>	CGMCC3.18009	<i>Malus</i> sp.	Henan, China	KX197076	KX197085	KX197096	KX197103
<i>B. rosaceae</i>	CGMCC3.18010	<i>Pyrus</i> sp.	Henan, China	KX197077	KX197086	N/A	KX197104
<i>B. rosaceae</i>	CFCC 82350	<i>Malus</i> sp.	Beijing, China	KX197079	KX197088	KX197097	KX197106

Table 1 Species and sequences database accession numbers used in this study (newly generated sequences are indicated in bold).

Table 1 continued.

Species	Culture/Specimen No.	Host	Location	GenBank accession numbers			
				ITS	LSU	tef1- α	TUB
<i>B. rosaceae</i>	CGMCC3.18011	<i>Pyrus</i> sp.	Henan, China	KX197078	KX197087	N/A	KX197105
<i>B. rosaceae</i>	CFCC 82350	<i>Malus</i> sp.	Beijing, China	KX197079	KX197088	KX197097	KX197106
<i>B. scharifii</i>	CBS 124703 ex-type	<i>Mangifera indica</i>	Iran, Tehran	JQ772020	N/A	JQ772057	N/A
<i>B. scharifii</i>	CBS 124702	<i>M. indica</i>	Iran, Hormozgan	JQ772019	N/A	JQ772056	N/A
<i>B. sinensia</i>	CGMCC3.17723	<i>Morus</i> sp.	Henan, China	KT343254	KX197090	KU221233	KX197107
<i>B. sinensia</i>	CGMCC3.17724	<i>Juglans regia</i>	Henan, China	KT343256	N/A	KU221234	KX197108
<i>B. sinensia</i>	CFCC 82346	<i>Juglans regia</i>	Beijing, China	KT343257	KX197091	KU221235	KX197109
<i>Cophinforma atrovirens</i>	MFLUCC 11-0425 ex-type	<i>Eucalyptus</i> sp.	Thailand	JX646800	JX646817	JX646865	JX646848
<i>C. atrovirens</i>	MFLUCC 11-0655	<i>Eucalyptus</i> sp.	Thailand	JX646801	JX646818	JX646866	JX646849
<i>Macrophomina phaseolina</i>	CBS 227.33	<i>Zea mays</i>	Unknown	KF531825	DQ377906	KF531804	KF531806
<i>M. phaseolina</i>	CBS 162.25	<i>Eucalyptus</i> sp.	Uganda	KF531826	DQ377905	KF531803	KF531805
<i>Neofusicoccum luteum</i>	CBS 110299 ex-type	<i>Vitis vinifera</i>	Portugal	AY259091	AY928043	AY573217	DQ458848
<i>N. parvum</i>	ATCC 58191 ex-type	<i>Populus nigra</i>	New Zealand	AY236943	AY928045	AY236888	AY236917
<i>Neoscytalidium dimidiatum</i>	CBS 499.66	<i>Mangifera indica</i>	Unknown	KF531820	DQ377925	KF531798	KF531800
<i>N. dimidiatum</i>	CBS 251.49	<i>Juglans regia</i>	USA	KF531819	DQ377923	KF531797	KF531799
<i>N. dimidiatum</i>	CBS 145.78 ex-isotype	<i>Homo sapiens</i>	United Kingdom	KF531816	DQ377922	KF531795	KF531796

* N/A: no sequence available in GenBank.

Results

Phylogenetic analyses

After alignment, the combined four-locus dataset consisted of 2265 characters (including alignment gaps) for 34 ingroup taxa and two outgroup taxa. Of the 2280 characters, 1964 were constant and 39 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 277 parsimony-informative characters resulted in 33 equally most parsimonious trees (CI = 0.769, RI = 0.879, RC = 0.676, HI = 0.231), one of which is shown in Fig. 1. The phylogenetic tree resulting from the Bayesian analysis using the general time reversible model of DNA evolution (Rodríguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+ Γ +G), had a topology identical to the MP tree presented. In both analyses (MP and Bayesian) the clade of *Botryosphaeria* received a high level of support (100 % for MP and 100 % for MrBayes). Within this clade ten subclades corresponding to eleven species were resolved, and *B. dothidea* and *B. auasmontanum* clustered together (Fig. 1). Most clades received moderate to high bootstrap support (≥ 60 % for MP or ≥ 80 % MrBayes), while some other clades received no bootstrap support. The subclade of *Botryosphaeria rosaceae* formed a sister group to other species of *Botryosphaeria*, and closely related to *B. auasmontanum*, *B. dothidea*, *B. fabicerciana*, *B. fusispora*, *B. minutispermata* and *B. sinensia* (Fig. 1).

Taxonomy

Botryosphaeria rosaceae Y.P. Zhou, Y. Zhang ter., *sp. nov.*

Fig. 2

Mycobank 817187; Facesoffungi number: FoF 02920

Etymology – The epithet *rosaceae* refers to the hosts within the family Rosaceae from which this species was isolated.

Ascostroma erumpent. Ascromata brown to black, 170–290 μm diam, pseudothecial, forming a botryose aggregate of up to 15, sometimes solitary, globose with a central ostiole, $\frac{1}{4}$ to $\frac{1}{2}$ emergent, rarely embedded, with or without papilla. Wall pseudothecial, composed of 8–14 layers of textura angularis, outer region of dark brown cells, inner region of 2–4 layers of hyaline cells lining the locule. Pseudoparaphyses filiform, septate, constricted at the septa, rarely branched, 2–4 μm wide. Asci bitunicate, clavate, 87–125 \times 22–26 μm . Ascospores ellipsoidal to fusiform, hyaline, thin-walled, sometimes with tapered ends and appearing spindle-shaped, biseriate in the ascus, 22–31(–35) \times 8–13(–15) μm (\bar{x} = 26.1 \times 10.8 μm , n = 30), L/W = 2.4. Conidiomata stromatic, morphologically indistinguishable from the ascromata. Paraphyses, hyaline, septate, up to 55 μm long, 5–6 μm wide at the base tapering to acutely rounded apices, 2–2.5 μm wide at the tip. Conidiogenous cells cylindrical to lageniform, hyaline, smooth, thin-walled, producing a single conidium at the tip, rarely proliferating at the same level giving rise to periclinal thickenings, 6–15 \times 2.5–5 μm . Conidia hyaline, narrowly fusiform, or irregularly fusiform, base subtruncate to bluntly rounded, 20–31 \times 6–8 μm (\bar{x} = 26.2 \times 6.7 μm , n = 30), L/W = 3.9, aged conidia not observed. Spermatogenous cells hyaline, thin-walled, smooth, 8–11 \times 2–4 μm , producing conidia at their tips, proliferating internally to form periclinal thickenings. Spermatia rod-shaped with obtuse ends, hyaline, thin-walled, smooth, 5–8 \times 1.5–3 μm .

Culture characteristics – Colonies initially white turning grey olivaceous from the middle of colonies after 4 d, becoming smoke grey to olivaceous grey on the surface and olivaceous green to black underneath within 7–10 d in the dark at 28°C.

Material examined – CHINA, Shandong Province, Yantai, Laishan, on twigs of *Malus* sp. (Rosaceae), 19 May 2015, Y Zhang & JQ Zhang (holotype HMAS 246964), ex-type culture CGMCC3.18007; Qixia, on twigs of *Amygdalus* sp. (Rosaceae), 19 May 2015, leg. Y Zhang, JQ Zhang & ZP Dou (paratype, HMAS 246965), isolate CGMCC3.18008; Henan Province, Puyang, Qingfeng County, on twigs of *Pyrus* sp. (Rosaceae), 10 November 2014, leg. ZP Dou (paratype, HMAS 246966), isolate CGMCC3.18009; on twigs of *Pyrus* sp. (Rosaceae), 10 November 2014, leg. ZP Dou (paratype, HMAS 246967), isolate CGMCC3.18010.

Notes – Morphologically, *B. rosaceae* was most comparable to *B. sinensia*, but the smaller and more slender ascospores and conidia of *B. sinensia* differentiate this species from *B. rosaceae*.

Phylogenetic analyses based on combined ITS, LSU, TUB and *tef1-a* sequences indicated that *B. rosaceae* and *B. sinensia* are separate species. In this study, all collections of *B. rosaceae* are from plants of Rosaceae, such as *Amygdalus* sp., *Malus* sp. and *Pyrus* sp., while *B. sinensia* has been reported from *Populus* sp. (Salicaceae), *Morus* sp. (Moraceae) and *Juglans regia* (Juglandaceae).

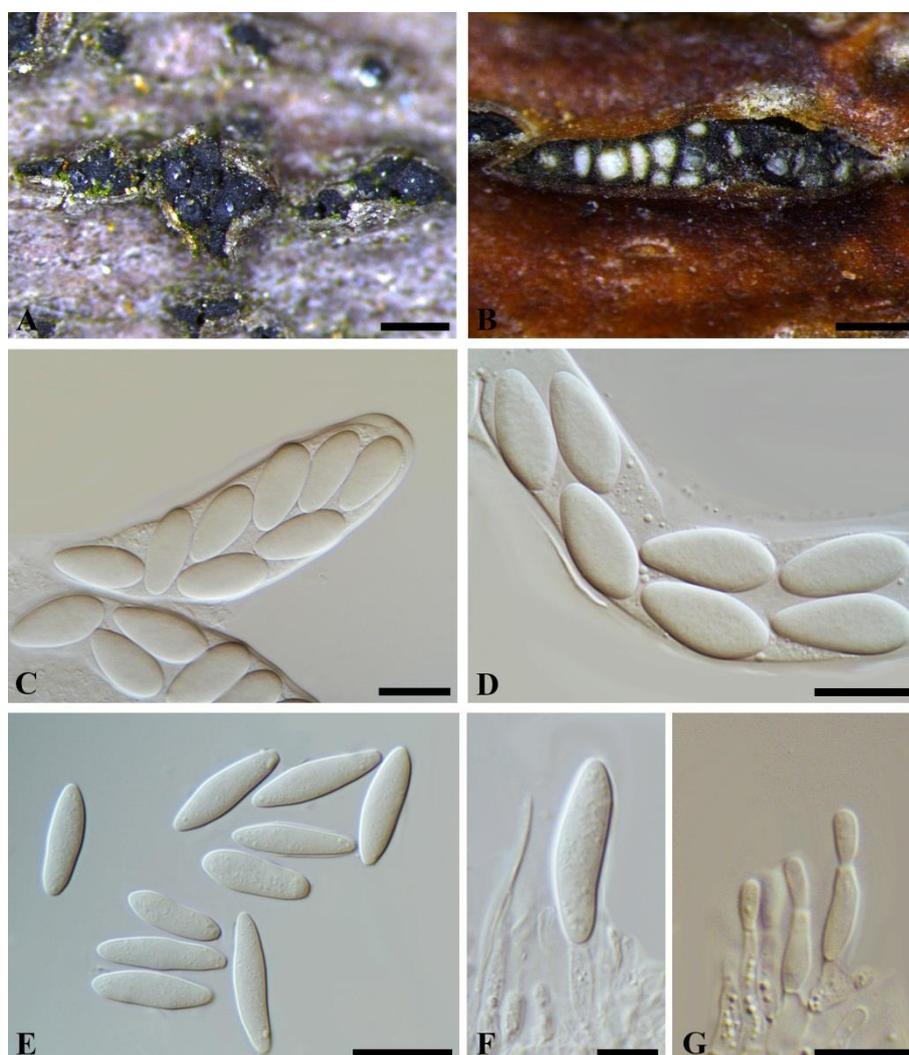


Figure 2 – *Botryosphaeria rosaceae*. A Botryose clusters of ascomata erumpent through the bark of an *Amygdalus* sp. twig (HMAS 246965, paratype). B Transverse section through ascomata revealing brilliant white contents. C Asci with ascospores. D Ascospores. E Conidia. F Conidia developing on conidiogenous cells. G Branched spermatogenous cells and developing spermatia. – Bars: A, B = 0.5 mm, C–E= 20 μ m, F, G= 10 μ m.

Botryosphaeria ramosa (Pavlic, T.I. Burgess & M.J. Wingf.) A.J.L. Phillips & A. Alves, Stud. Mycol. 76: 77 (2013) \equiv *Fusicoccum ramosum* Pavlic, T.I. Burgess & M.J. Wingf., Mycologia 100(6): 861 (2008)

Material examined – CHINA, Hainan Province, Wuzhi mountain, on twigs of *Acacia* sp. (Mimosaceae), 5 Nov., 2015, ZP Dou and W He (culture CGMCC 3.18004); Guangdong Province, Zhanjiang, on twigs of *Corymbia citriodora* (Myrtaceae), 1 Nov., 2015, ZP Dou and W He (culture CGMCC 3.18005); on twigs of Myrtaceae, 1 Nov., 2015, ZP Dou and W He (culture CGMCC 3.18006).

Discussion

The erumpent, botryose clustered ascostromata, ostiolate ascomata, bitunicate, 8-spored and clavate asci, filiform, rarely branched pseudoparaphyses, hyaline, 1-celled, fusiform ascospores, as

well as the *Fusicoccum* asexual morph of *B. rosaceae* point to *Botryosphaeria* (Slippers et al. 2004, Crous et al. 2006, Phillips et al. 2008, 2013). Polyphasic taxonomy has been used in classification of Botryosphaeriaceae, such as morphological, cultural and multi-allelic DNA sequence datasets as well as host spectrum (Slippers et al. 2004, 2013, Crous et al. 2006, Phillips et al. 2008, 2013). The sexual and asexual morphological characteristics have been used for species identification, such as the dimension of stroma, asci, ascospores or conidia and pigmentation and septation of ascospores or conidia (Slippers et al. 2004, Phillips et al. 2013). The morphological characteristics of *B. rosaceae* and *B. sinensia* are comparable, but the smaller and more slender ascospores ($24.6 \times 8.7 \mu\text{m}$, l/w 2.8) and conidia ($24.3 \times 5.9 \mu\text{m}$, l/w 4.1) of *B. sinensia* differ from those of *B. rosaceae* (Zhou et al. 2016). In particular, the molecular phylogeny based on combined ITS, LSU, *tef1- α* and TUB loci indicate that they are separate species. Aged conidia of *B. rosaceae* were not observed in this study and thus cannot be compared with the septate but hyaline aged conidia of *B. sinensia*. All the specimens of *B. rosaceae* obtained were from Rosaceae in the northern part of China, which also differs from the broader host spectrum of *B. sinensia* (Zhou et al. 2016). We therefore introduce a new species for this taxon herein.

Botryosphaeria ramosa was first reported on *Eucalyptus camaldulensis* (Myrtaceae) from Western Australia (Pavlic et al. 2008, Phillips et al. 2013). In this study *Botryosphaeria ramosa* was found associated with an *Acacia* species (Mimosaceae), *Corymbia citriodora* (Myrtaceae) and another unidentified myrtaceous host in Hainan and Guangdong Province. This is a new host and first report of this species in the Northern Hemisphere.

Compared with African, American and European regions, botryosphaeriaceous fungi are more poorly studied in Asian area. For example, no more than 5 % members of Botryosphaeriaceae have been reported in China so far (data unpublished). During the last few years more studies have been conducted in this region. For example, new botryosphaeriaceous species or records have been reported from China (Phillips et al. 2013, Tennakoon et al. 2016, Dissanayake et al. 2016, Zhang et al. 2016, Zhou et al. 2016, Dou et al. 2017) and Thailand (Trakunyingcharoen et al. 2015, Dissanayake et al. 2016, Konta et al. 2016a, b). By exploring the biodiversity of Botryosphaeriaceae in these poorly studied area, researchers can shed light on a global vision of them, and also help to understand their global transmission, pathogenicity or to estimate their potential damages.

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