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Multigene phylogeny and morphology reveal *Phaeobotryon rhois* sp. nov. (Botryosphaeriales, Ascomycota)

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Abstract

The family *Botryosphaeriaceae* encompasses important plant-associated pathogens, endophytes and saprobes with a wide geographical and host distribution. Two dark-spored botryosphaeriaceous taxa associated with *Rhus typhina* dieback and canker disease were collected from Ningxia Province, in northwestern China. Morphology and multigene analysis (ITS, LSU and EF-1α) clearly distinguished this clade as a distinct species in the genus. *Phaeobotryon rhois* is introduced and illustrated as a new species in this paper. The species is characterized by its globose, unilocular fruiting bodies and small, brown, 1-septate conidia. It can be distinguished from the similar species *P. cercidis*, *P. cupressi*, *P. mamane* and *P. quercicola* based on host association and conidial size and colour.

Key words: biodiversity, Botryosphaeriaceae, molecular phylogeny, new species, taxonomy

Introduction

Phaeobotryon (*Botryosphaeriaceae*) was established by Theissen & Sydow (1915) to accommodate *Dothidea cercidis* Cooke and subsequently entered a long period of confusion with a broad concept of the *Botryosphaeria* species. Phillips *et al.* (2008) redefined *Phaeobotryon* using the characteristic of 2-septate, brown ascospores, with bipolar conical apiculi. *Phaeobotryon* is a monophyletic genus with a single name for both sexual and asexual morphs (Phillips *et al.* 2008, Liu *et al.* 2012). Seven *Phaeobotryon* epithets are listed in Index Fungorum (2015) while Kirk *et al.* (2008) estimated there are four species. Sequence data or living cultures, are however, available for only a very few species and many taxa need verification. Recent studies suggest that this genus comprises four species (*P. cercidis* Cooke, *P. cupressi* Abdollahzadeh *et al.*, *P. mamane* Crous & Phillips and *P. quercicola* (Phillips) Crous & Phillips), of which only two species (*P. cupressi* and *P. mamane*) have been verified and studied based on available living cultures (Phillips *et al*. 2005, 2008, 2013; Abdollahzadeh *et al*. 2009). Until now, *Phaeobotryon* has been recorded from *Cercis canadensis* L., *Cupressus sempervirens* L., *Juniperus scopulorum* Sargent, *Quercus* sp. and *Sophora chrysophylla* (Salisb.) Seem. only in Germany, Iran and the USA (Abdollahzadeh *et al*. 2009, Phillips *et al*. 2013). Although *Phaeobotryon* species have been isolated from diseased plants, their pathogenicity has not been tested and their role as causal agents of disease is unresolved.

During an investigation of forest pathogens that cause canker or dieback disease in China, two *Phaeobotryon* specimens were collected from *Rhus typhina* L. in Yinchuan City, Ningxia Province, China. This species is characterized by globose, unilocular, fruiting bodies and brown, 22 × 11 µm, ellipsoid to oblong, 1-septate conidia. Phylogenetic analysis inferred from combined ITS, LSU and EF-1α sequence data provided strong support that this is a new species. We introduce *Phaeobotryon rhois* sp. nov. in this paper with a description and illustrations and compare it with other species in the genus.

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Material and methods

Isolation

Two isolates were obtained by single spore isolation following the methods of Chomnunti *et al*. (2014). After incubation at 25 °C for 24 h, single germinating conidia were transferred to fresh plates of PDA. The representative isolates are maintained in the China Forestry Culture Collection Center (CFCC), and duplicate isolates of the new species cultures are deposited in the China Center for Type Culture Collection (CCTCC). Specimens are deposited in the Museum of Beijing Forestry University (BJFC).

Morphology

The conidiomata and conidia characters, including size, shape, colour, septation, wall thickness and texture were recorded from specimens on infected plant tissues. Vertical and horizontal sections were cut through the conidiomata by hand using a razor blade. Over 20 conidiomata were sectioned, and 50 conidia were randomly selected for measurement using a Leica light microscope (LM, DM 2500). Isolates cultured on PDA were incubated in the dark at 25 °C and culture characteristics (i.e., colony colour, texture and arrangement of the conidiomata) were recorded at 3, 7 and 30 days.

DNA extraction, PCR amplification and sequencing

Genomic DNA was obtained from colonies grown on PDA with cellophane using a modified CTAB method (Doyle and Doyle 1990). DNA concentrations were estimated by electrophoresis in 1.2 % agarose gels, qualities were measured with a NanoDrop™ 2000 (Thermo, USA) according to the user's manual (Desjardins *et al*. 2009), and DNA was stored at -80°C. The PCR amplifications were performed in a DNA Engine (PTC-200) Peltier Thermal Cycler (Biorad Laboratories, CA, USA). ITS, LSU and EF-1α sequence data were generated in this study. The ITS region was amplified using ITS1 and ITS4 primers (White *et al*. 1990). The LSU region was amplified using NL1 and NL4 primers (O'Donnell 1993). The EF-1α region was amplified using EF1-688F and EF1-1251R primers (Alves *et al*. 2008). The PCR amplification products were visually estimated by electrophoresis in 2 % agarose gels. DNA sequencing was performed using an ABI PRISM® 3730XL DNA Analyzer with the BigDye® Terminater Kit v.3.1 (Invitrogen), and all primers were synthesized by Invitrogen Biotech Co. Limited (Beijing, China).

DNA sequence analysis

The DNA sequences generated by the forward and reverse primers were used to obtain consensus sequences using Seqman v.7.1.0 (DNASTAR Inc, Madison, USA). Subsequently, the sequences that included reference sequences from GenBank were aligned using MAFFT v.6 (Katoh & Toh 2010), and the alignments were then edited manually using MEGA v.6 (Tamura *et al*. 2013). A partition homogeneity test (PHT) test with heuristic search and 1,000 homogeneity was performed using PAUP v.4.0b10 to test the discrepancy among the ITS-LSU and EF-1α in reconstructing phylogenetic trees. Phylogenetic analysis was performed with PAUP v.4.0b10 for maximum parsimony (MP) analysis (Swofford *et al*. 2003), MrBayes v.3.1.2 for Bayesian inference (BI) analysis (Ronquist & Huelsenbeck 2003) and PhyML v.3.1 for maximum likelihood (ML) analysis (Guindon *et al*. 2010). The analysis was performed on the multilocus alignments (ITS, LSU, and EF-1α). *Melanops* spp. (*Melanopsaceae*) and *Saccharata* sp. (*Saccharataceae*) belonging to the order Botryosphaeriales were selected as outgroups (Phillips *et al*. 2013). The illustrated trees were drawn with Figtree v.1.3.1 (Rambaut & Drummond 2010).

MP analysis was run using the heuristic search option of 1000 random-addition sequences and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All detected insertion-deletion events (Indels) were coded using the simple code method in the GapCoder software (Young & Healy 2003). The branches of zero length were collapsed, and all parsimonious trees were saved. Clade stability was assessed using a bootstrap analysis of 1000 replicates (Hillis & Bull 1993). Other parsimony scores that were calculated included the length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). The ML analysis was also performed using PhyML v.3.1 using a GTR model of site substitution that included estimation of the gamma-distributed rate heterogeneity and a proportion of invariant sites (Guindon *et al*. 2010). The branch support was evaluated with a bootstrapping method of

100 replicates (Hillis & Bull 1993). The Bayesian analysis was performed with a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities (Rannala & Yang 1996). The model of nucleotide substitutions was estimated with MrModeltest v.2.3 (Posada and Crandall 1998), and a weighted Bayesian analysis was considered. Two MCMC chains were run from random trees for 10^6 generations, and the trees were sampled every 100^{th} generation, which resulted in 10^4 total trees. The first 25 % of the trees were discarded as the burn-in phase of each analysis, and the posterior probabilities were calculated using the remaining 7500 trees.

All sequence data from this study have been deposited in GenBank, the multi-gene sequence alignment files have been deposited in TreeBASE (www.treebase.org) under accession number S16768, and the taxonomic novelty has been deposited in MycoBank (Crous *et al.* 2004) and Faces of Fungi (www.facesoffungi.org).

Results

The combined ITS, LSU and EF-1α region dataset from 63 ingroup taxa representing 47 clades residing in primary 17 genera of *Botryosphaeriaceae* is listed in Table 1. A partition homogeneity test (PHT) was not significant (P = 0.012 > 0.01) indicating that the individual datasets were congruent and produced trees with the same topologies. Following alignment, the combined dataset had a length of 1502 characters, including the coded alignment gaps. Of these characters, 939 were constant, and 102 were variable and parsimony-uninformative. MP analysis of the remaining 461 parsimony-informative characters resulted in six equally parsimonious trees of 1884 lengths (CI = 0.503, RI = 0.807, RC = 0.406); the generated phylogram is shown in Fig. 1. The ML analysis was run using the general time reversible (GTR) model of DNA evolution, which applies a discrete gamma distribution with six rate categories (GTR+Γ+G), and resulted in an MP tree with the same topology as the presented tree. The BI analysis resulted in the same topology with an average standard deviation of split frequencies = 0.009984. The MP bootstrap support (BT) and ML were equal to or above 70 % and 75 %. The branches with significant Bayesian posterior probabilities (BPP) equal to or above 0.95 are thickened in the phylogram.

The two isolates of *Phaeobotryon rhois* from *Rhus typhina* clustered in the subclade of *Phaeobotryon* and are distinct from *P. mamane* and *P. cupressi*. The two strains clustered in an individual clade representing a novel species with high support values (100 for BT and 1.00 for BPP); this is also supported by morphology.

TABLE 1. Isolates used in phylogenetic analysis in this study.

Species	Isolate No.	GenBank Accession No.		
		ITS	LSU	EF-1α
Barriopsis fusca	**CBS 174.26***	EU673330	DQ377857	EU673296
Barriopsis iraniana	IRAN 1448	FJ919663	N/A	FJ919652
Botryobambusa fusicoccum	**CBS 134113***	JX646792	JX646809	JX646857
Botryobambusa fusicoccum	MFLUCC 110657	JX646793	JX646810	JX646858
Botryosphaeria corticis	**CBS 119047**	DQ299245	EU673244	EU017539
Botryosphaeria dothidea	**CBS 115476***	AY236949	AY928047	AY236898
Botryosphaeria fabicercianum	CMW 27106	HQ332199	N/A	HQ332215
Cophinforma atrovirens	**MFLUCC 110425***	JX646800	JX646817	JX646865
Cophinforma atrovirens	MFLUCC 110655	JX646801	JX646818	JX646866
Cophinforma eucalypti	CBS 124935	FJ888476	N/A	FJ888457
Cophinforma mamane	CBS 117444	KF531822	DQ377855	KF531801
Diplodia africana	**CBS 120835**	EF445343	N/A	EF445382
Diplodia mutila	**CBS 112553***	AY259093	AY928049	AY573219
Diplodia stevensii	CBS 230.30	DQ458886	EU673265	DQ458869
Dothiorella iberica	**CBS 115041**	AY573202	AY928053	AY573222
Dothiorella sarmentorum	**IMI 63581b**	AY573212	AY928052	AY573235
Endomelanconiopsis endophytica	**CBS 120397***	EU683656	EU683629	EU683637
Endomelanconiopsis microspora	**CBS 353.97**	EU683655	EU683628	EU683636
Lasiodiplodia crassispora	CBS 110492	EF622086	EU673251	EF622066

...Continue on the next page

TABLE 1. (Continued)

Species	Isolate No.	GenBank Accession No.		
		ITS	LSU	EF-1α
Lasiodiplodia crassispora	WAC 12533	DQ103550	N/A	DQ103557
Lasiodiplodia gonubiensis	**CBS 115812**	AY639595	DQ377902	DQ103566
Lasiodiplodia theobromae	**CBS 164.96***	AY640255	EU673253	AY640258
Macrophomina phaseolina	**CBS 162.25***	KF531826	DQ377905	KF531803
Macrophomina phaseolina	CBS 227.33	KF531825	DQ377906	KF531804
Melanops tulasnei	CBS 116805	FJ824769	FJ824764	KF766423
Melanops sp.	CBS 118.39	FJ824771	DQ377856	FJ824776
Neodeightonia palmicola	**MFLUCC 100822**	HQ199221	HQ199222	N/A
Neodeightonia palmicola	MFLUCC 100823	HQ199224	HQ199225	N/A
Neodeightonia phoenicum	**CBS 122528**	EU673340	EU673261	EU673309
Neodeightonia phoenicum	CBS 169.34	EU673338	EU673259	EU673307
Neodeightonia subglobosa	**CBS 448.91***	EU673337	DQ377866	EU673306
Neofusicoccum australe	CMW 6837	AY339262	N/A	AY339270
Neofusicoccum eucalypticola	**CBS 115679**	AY615141	N/A	AY615133
Neofusicoccum grevilleae	CPC 16999	JF951137	JF951157	N/A
Neofusicoccum luteum	CMW 10309	KF766369	KF766202	KF766424
Neofusicoccum mangiferae	CMW 7024	AY615185	DQ377920	DQ093221
Neofusicoccum parvum	**ATCC 58191***	AY236943	AY928045	AY236888
Neoscytalidium dimidiatum	CBS 499.66	KF531820	DQ377925	KF531798
Neoscytalidium hyalinum	**CBS 145.78***	KF531816	DQ377922	KF531795
Neoscytalidium novaehollandiae	**CBS 122071**	EF585540	N/A	EF585580
Phaeobotryon cupressi	IRAN 1445	KF766208	N/A	KF766428
Phaeobotryon cupressi	IRAN 1454	FJ919673	N/A	FJ919662
P. cupressi	**IRAN 1455**	FJ919672	N/A	FJ919661
P. cupressi	IRAN 1456	FJ919670	N/A	FJ919659
P. cupressi	IRAN 1458	FJ919671	N/A	FJ919660
P. mamane	CPC 12440	EU673332	EU673248	EU673298
P. mamane	CPC 12442	EU673185	EU673333	DQ377899
P. mamane	CPC 12443	EU673334	EU673249	EU673300
P rhois	**CFCC 89662[N]**	KM030584	KM030591	KM030598
P. rhois	CFCC 89663[N]	KM030585	KM030592	KM030599
Pseudofusicoccum adansoniae	CMW 26147	EF585523	N/A	EF585571
P. adansoniae	WAC 12689	EF585534	EF585554	EF585567
P. ardesiacum	**CBS 122062**	EU144060	N/A	EU144075
P. kimberleyense	**CBS 122058**	EU144057	N/A	EU144072
P. stromaticum	**CBS 117448***	AY693974	DQ377931	AY693975
Saccharata proteae	CBS 115206	KF766226	DQ377882	KF766438
Spencermartinsia viticola	**CBS 117009***	AY905554	DQ377873	AY905559
S. viticola	UCP 105	JF271748	N/A	JF271784
Spencermartinsia sp.	ICMP 16827	EU673322	EU673241	EU673289
Sphaeropsis citrigena	**ICMP 16812**	EU673328	EU673246	EU673294
S. citrigena	ICMP 16818	EU673329	EU673247	EU673295
S. eucalypti	MFLUCC 110579	JX646802	JX646819	JX646867
S. porosa	**CBS 110496**	AY343379	DQ377894	AY343340
S. visci	**CBS 122526***	EU673324	N/A	EU673292
Tiarosporella tritici	**CBS 118719**	KF531830	DQ377941	KF531809
T. urbis-rosarum	**CMW 36477**	JQ239407	JQ239420	JQ239394

The new species the current study are marked with N. Ex-type taxa are in bold. Type species representing the genus are marked with an *.

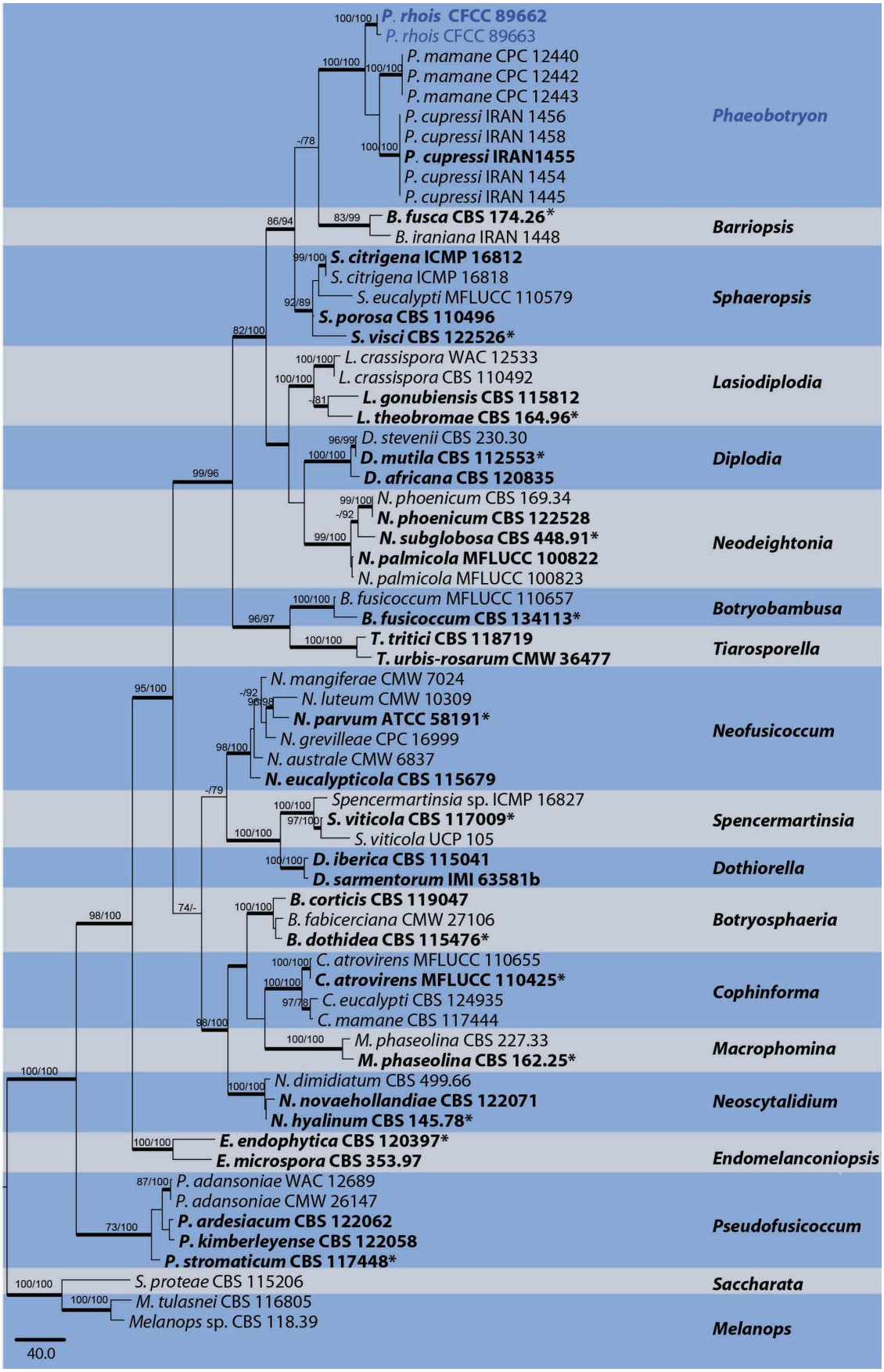

FIGURE 1. Phylogram of the combined genes of ITS, LSU and EF-1α based on MP, ML and BI analysis. The values above the branches indicate bootstrap values. The thickened branches indicate PP ≥ 0.95 from the Bayesian inferences. *Bars*: 40 nucleotide substitutions. The taxa resulting from the current study are shown in blue. Ex-type taxa are in bold. Type species representing the genus are marked with an *.

Taxonomy

Phaeobotryon rhois C.M. Tian, X.L. Fan & K.D. Hyde, ***sp. nov.***
MycoBank MB 811599; Facesoffungi number: 00596, **Fig. 2**

Differs from *Phaeobotryon* spp. by its globose, unilocular fruiting body, size of conidia (22 × 11 µm in average) and host.

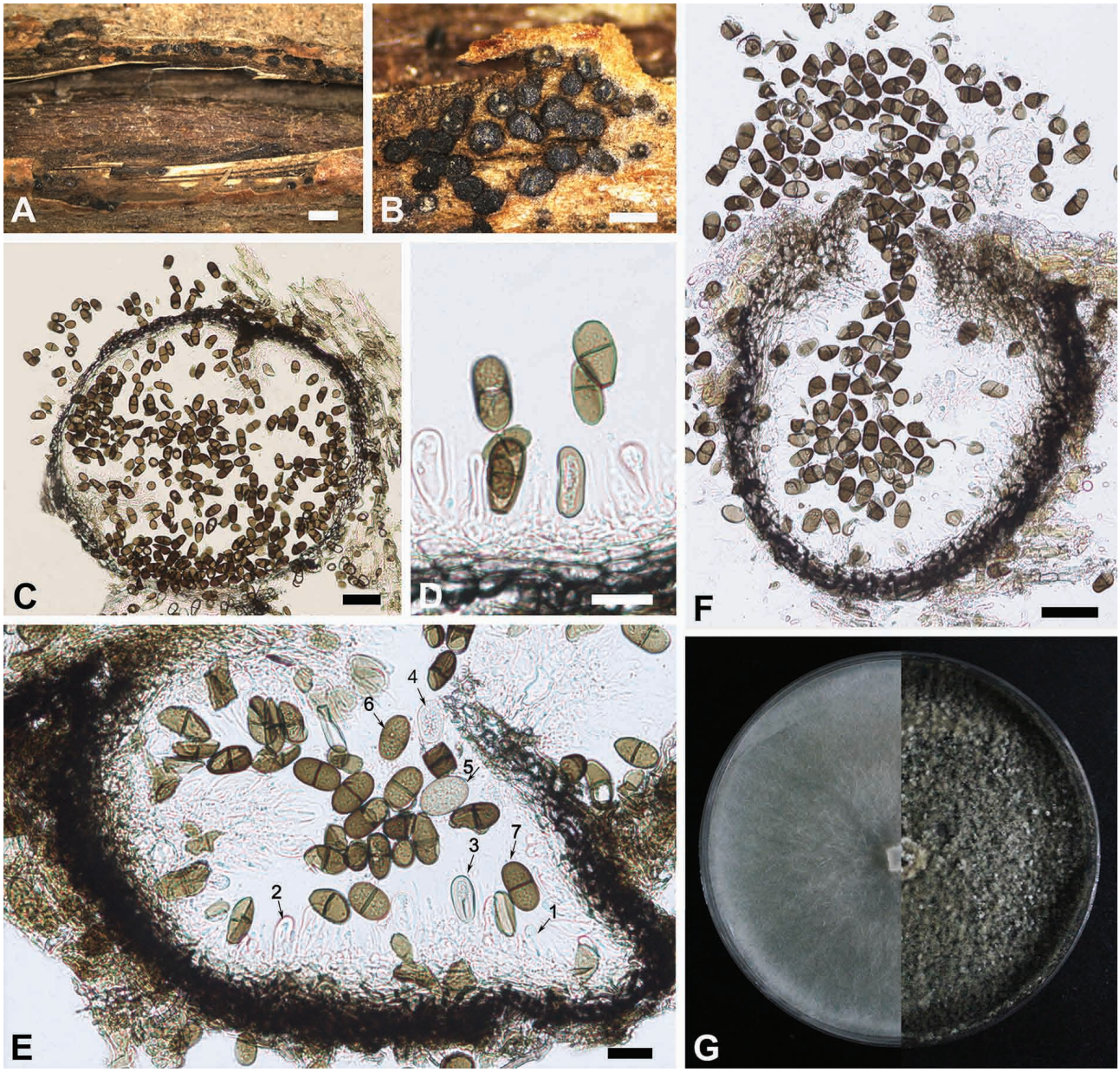

FIGURE 2. *Phaeobotryon rhois* (BJFC-S1007, holotype). A, B. Habit of conidiomata on a twig. C. Transverse sections through conidioma. F. Longitudinal section through conidioma. D, E. Conidiogenous cells, immature and mature conidia (arrows pointing to process of conidia maturity). G. Colonies on PDA at 3 days (left) and 30 days (right). Scale bars: A = 1 mm; B = 0.5 mm; C, F = 40 µm; D, E = 20 µm.

Etymology:—*rhois*, referring to the host *Rhus typhina* L.

Holotype:—BJFC-S1007.

Original description:—pathogen on twigs and branches of *Rhus typhina*. Sexual morph: Undetermined. Asexual morph: *Conidiomatal stromata* immersed in bark, erumpent slightly from surface of the bark, separate or aggregated, globose, dark-brown to black, unilocular, up to 380 µm diam, wall consisting of 4–6 layers of dark-brown cells *textura angularis*. *Ostiole* in the center of the disc, inconspicuous, at the same level as the disc surface, surrounded below disc by lighter entostroma, (17.2–)18.9–21.3(–22.8) µm ($\overline{x}$ = 19.6 µm, n = 20) in diam. *Locule* single, globose, (180–)240–360(–380) µm ($\overline{x}$ = 330 µm, n = 20) in diam. *Conidiogenous cells* formed from the cells lining the inner

walls of the locules, hyaline, smooth, inconspicuous. *Conidia* ellipsoid to oblong or subcylindrical or obovoid, smooth to verruculose, moderately thick-walled, guttulate, ends rounded, initial hyaline, aseptate, becoming brown, 1-septate when mature, (19–)20–25 × 10–12 µm ($\overline{x}$ = 22 × 11 µm, n = 50).

Culture characteristics:—colonies were originally white, and produced dark green to black pigments after 7–10 days. Texture felty with appressed mycelial mat and fluffy aerial mycelium in the centre, edges smooth. Colonies covering the 90 mm diameter Petri-dish after 3 day in the dark at 25 ºC.

Habitat/Distribution:—known from twigs and branches of *Rhus typhina* in China.

Material examined:—CHINA. Ningxia Province: Yinchuan City, Yongning County, Wanghong Town, Nongsheng Village, 38°22'97.02" N, 106°21'44.89" E, alt. 1101 m, on twigs and branches of *Rhus typhina* (*Anacardiaceae*), 26 July 2013, *Xin-lei Fan & Hong Fan* (BJFC-S1007!, **holotype**), living culture CFCC 89662 = CCTCC AF2014017; Yinchuan City, Yongning County, Wanghong Town, Nongsheng Village, 38°22'98.13" N, 106°21'43.77" E, alt. 1107 m, on twigs and branches of *Rhus typhina* (*Anacardiaceae*), 26 July 2013, *Xin-lei Fan & Hong Fan* (BJFC-S1008, paratype), living culture CFCC 89663 = CCTCC AF2014016.

Table 2. Comparison of species in *Phaeobotryon* (new species in bold).

Species	Strain	Conidial size	Conidial colour	Host	Location	Reference
P. cercidis	No strain	No record	No record	*Cercis canadensis*	USA	Phillips *et al*. 2008
P. cupressi	IRAN 1455C = CBS 124700	24.1–25 × 12.2 12.5	Initially hyaline and becoming brown	*Cupressus sempervirens* *Juniperus scopulorum*	Iran USA	Abdollahzadeh *et al*. 2009 Alves *et al*. 2013 Phillips *et al*. 2013
P. mamane	CPC 12440 = CBS 122980	35–38 × 14–15	brown	*Sophora chrysophylla*	USA	Phillips *et al*. 2008 Phillips *et al*. 2013
P. quercicola	No strain	28.8–30.8 × 15.9–17.1	hyaline	*Quercus* sp.	Germany	Phillips *et al*. 2005 Phillips *et al*. 2008
P. rhois	CFCC 89662 = CCTCC AF2014017	20.4–24.7 × 10.1–12.1	Initially hyaline and becoming brown	*Rhus typhina*	China	This study

Discussion

Species of *Botryosphaeriaceae* (Ascomycota) are cosmopolitan in their distribution and occur on a wide range of host plants. The family has been unsettled until Liu *et al*. (2012) listed all of the various primary treatments and redefined 29 genera using multilocus phylogenetic analysis, as well as examinations of the types of genera. However, the interrelations of several genera remained disordered, and Liu *et al*. (2012) noted that it is likely that Botryosphaeriales comprised more families (Crous *et al*. 2006). Subsequently, Slippers *et al*. (2013) introduced three families namely *Aplosporellaceae, Melanopsaceae* and *Saccharataceae* for Botryosphaeriales and this is followed in the outlines of Dothideomycetes in Hyde *et al*. (2013) and Wijayawardene *et al*. (2014). Phillips *et al*. (2013) established a starting point for the resolving genera; these authors considered the accepted genera and species with taxonomic evidence and living cultures, rather than the older taxa, which lacked definitions, unless they had been epitypified. The results cleared up much confusion and resulted in a total of 17 genera that were phylogenetically recognized within *Botryosphaeriaceae*; this classification focused on species with cultures.

Abdollahzadeh *et al*. (2009) accepted *P. cercidis* (only sexual morph known), *P. cupressi* (only asexual morph known), *P. mamane* and *P. quercicola* in the genus *Phaeobotryon*. Phillips *et al*. (2013) illustrated that *P. cupressi* and *P. mamane* known in culture. Molecular data from this study are used in the phylogram in Fig. 1. The current study introduces a novel species, *P. rhois*, which causes torched tree cankers and is distinguished from other species based on host association, and fruiting body and spore characteristics (Table 2). *Phaeobotryon rhois* is similar to *P. cupressi,* however, the conidia of *P. rhois* are initially hyaline, aseptate and become brown and 1-septate when mature; the sporulation is shown in Fig. 2E. Comparatively, *P. cupressi* conidia rarely become brown and 1-septate and clearly produce microconidia and microconidiomata (Abdollahzadeh *et al*. 2009, Phillips *et al*. 2013). *Phaeobotryon rhois* was isolated from diseased twigs and branches of *Rhus typhina* with obvious canker symptoms (Fig. 2A). However,

it has not been determined if this species is pathogenic, as is true of other *Phaeobotryon* species. The taxonomy of species requires a robust sampling from a wide distribution range and consequent pathogenicity testing as in other well-studied botryosphaeriaceous fungi. Future studies should clarify the species diversity in this genus and improve the understanding of its disease importance.

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