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Phyllosticta species from banana (*Musa* sp.) in Chongqing and Guizhou Provinces, China

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Abstract

Six *Phyllosticta* strains were isolated from diseased leaves of *Musa* species in Chongqing and Guizhou provinces, China. Morphological and molecular analysis of LSU and combined ITS, ACT, TEF-1, and GPDH gene sequences, identified these strains as *P. capitalensis* (3 strains), *P. musarum* (1 strain) and two isolates were distinct from known *Phyllosticta* species. The latter is herein introduced as *Phyllosticta musaechinensis* sp. nov. A description and illustrations are provided, and the new species is compared with other species from *Musa* in this paper.

Key words: *Musa*, Phylogeny, New species, Taxonomy

Introduction

The genus *Phyllosticta* is an important causal agent of banana (*Musa* sp.) leaf and post-harvest diseases (Meredith 1968, Jones & Alcorn 1982, Wulandari *et al*. 2010). Seven species of *Phyllosticta* have been recorded from banana although their identification is confusing (Meredith 1968, Chuang 1981, Brown *et al*. 1998, Photita *et al.* 2001, van der Aa & Vanev 2002, Pu *et al.* 2008, Wong *et al.* 2013, Wulandari *et al.* 2010). Wulandari *et al.* (2010) investigated the *Guignardia*/*Phyllosticta* species associated with freckle disease on banana leaves, re-examined the holotype of each epithet, and reported that the agents of banana freckle are *Guignardia musae* Racib. and *G. musicola* Wulandari, L. Cai & K.D. Hyde. Wong *et al.* (2012) used the name *Phyllosticta* rather than *Guignardia,* found five species on *Musa* in Australia, designated the epitypes for *Phyllosticta maculata* M.H. Wong & Crous and *P. musarum* (Cooke) Aa, and described *P. cavendishii* M.H. Wong & Crous as a new species. The history of *Phyllosticta* on banana was also discussed by Wulandari *et al.* (2010) and Wong *et al.* (2012). *Phyllosticta capitalensis* Henn., *P. cocoicola* (Bat.) Sivan., *Phyllosticta musae* (as *Guignardia musae*), *Phyllosticta musicola* and *Guignardia sydowiana* Trotter have been also recorded as endophytes on banana (Brown *et al*. 1998, Photita *et al*. 2001, 2002).

Wikee *et al*. (2013) provided a multilocus backbone tree for *Phyllosticta* species based on combined ITS, TEF-1, ACT, LSU and GPDH region genes, however, they did not include pathogens from banana (except the ubiquitous endophyte, *P. capitaliensis*). In the present study six, *Phyllosticta* strains were isolated from diseased leaves of *Musa* spp. from Chongqing and Guizhou provinces in China. Among them, one taxon differed from known *Phyllosticta* species from banana and other hosts. The aim of this paper is to describe the new species based on morphological and molecular data and investigate the relationship of *P. musaechinensis* with other species.

Materials and methods

Isolates

Symptomatic banana leaves with small to expanding lesions were selected for isolation. The leaves were cut into pieces

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approximately 3 × 5 cm, surface sterilized in 70% ethanol for 1 min, and then air-dried. Isolations were performed using two different laboratory methods. Firstly, a fruiting body was transferred into sterile water, allowed to soak overnight, and then single spore isolates were prepared using the method described in Chomnunti *et al.* (2014). Secondly, single fruiting bodies, observed under a stereo microscope were removed with a scalpel and plated onto potato dextrose agar (PDA) containing streptomycin sulphate to inhibit bacterial growth (Wong *et al*. 2012). The ex-type strain has been deposited in Guizhou Academy of Agricultural Sciences Collection (GZAAS) and an ex-paratype strain has been sent to International Collection of Microorganisms from Plants (ICMP) and Mae Fah Luang University Culture Collection (MFLUCC), respectively.

DNA isolation, amplification and phylogeny

DNA was extracted from isolates growing on PDA at 28°C for 30 d following the protocol of Cubero *et al.* (1999). The primers used were LROR (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990) for LSU region, ITS1 and ITS4 (White *et al.* 1990) for ITS region, EF1-728F and EF1-986R (Carbone & Kohn 1999) for translation elongation factor 1-α gene (TEF-1),ACT-512F and ACT-783R (Carbone & Kohn 1999) for the actin gene (ACT) and GDF1 (Guerber *et al.* 2003) and Gpd2-LM (Myllys *et al.* 2002) or GDR1 (Guerber *et al.* 2003) for the glyceraldehyde 3-phosphate dehydrogenase gene (GPDH). Amplification conditions followed Arzanlou *et al.* (2008). DNA sequencing was performed at the SinoGenoMax Company (Beijing, China) using corresponding primer pairs in sequencing. Novel sequences have been deposited in the GenBank (Tab. 1)

Sequences of our isolates together with reference sequences obtained from GenBank (Table 1) were aligned with MAFFT (Katoh *et al.* 2005). The alignments were checked visually and improved manually if necessary in BioEdit (v. 7.1.3.0). The index of substitution saturation was assessed using DAMBE v5.3.70 (Xia *et al.* 2003). The selection of conserved blocks was conducted using Gblocks v0.91b. The phylogenetic analysis of the aligned sequences was conducted using PAUP v.4.0b10 (Swofford 2003) for maximum-parsimonious analysis.

TABLE1 Sources of isolates and GenBank accession number used in this study

Species	Strain no.[a]	**Host**	**Locality**	**GenBank Accession number[b]**			
				ITS	**ACT**	**TEF-1**	**GPDH**
Botryosphaeria obtusa	CMW8232	*Conifers*	South Africa	AY972105	AY972111	DQ280419	
Guignardia alliacea	MUCC0014*	*Allium fistulosum*	Japan	AB454263			
G. mangiferae	IMI260.576*	*Manifera indica*	India	JF261459	JF343641	JF261501	JF343748
G. philoprina	CBS447.68*	*Taxus baccata*	Netherlands	AF312014			
G. rhodorae	CBS 901.69	*Rhododendron sp.*	Netherlands	KF206174	KF289256	KF289230	KF289166
Phyllosticta abieticola	CBS112067*	*Abies concolor*	Canada	KF170306	KF289238		
P. aloeicola	CPC21020*	*Aloe ferox*	South Africa	KF154280	KF289311	KF289193	KF289124
P. ampelicida	ATCC200578*	*Vitis riparia*	USA	KC193586	KC193581		KC193584
P. ardisiicola	NBRC102261*	*Ardisia crenata*	Japan	AB454274	AB704216		
P. aspidistricola	NBRC102244*	*Aspidistra elatior*	Japan	AB454260			
P. beaumarisii	IMI 298910 *	*Muehlenbekia adpressa*	Australia	AY042927	KF306232	KF289170	KF289074
P. bifrenariae	CBS128855*	*Bifrenaria harrisoniae*	Brazil	JF343565	JF343649	JF343586	JF343744
P. brazilianiae	CBS126270*	*Mangifera indica*	Brazil	JF343572	JF343656	JF343593	JF343758
P. capitalensis	CBS128856*	*Stanhopea sp.*	Brazil	JF261465	JF343647	JF261507	JF343776
P. capitalensis	CBS117118	*Musa acuminata*	Indonesia	FJ538339	FJ538455	FJ538397	KF289090
P. cavendishii	BRIP554196*	*Musa cv. Formosana*	Taiwan	JQ743562	KF014080	KF009743	
P. cavendishii	BRIP58008	*Musa* sp.	Australia	KC988365	KF014071	KF009742	
P. citriasiana	CBS 120486*	*Citrus maxima*	Thailand	FJ538360	FJ538476	FJ538418	JF343686
P. citribraziliensis	CBS100098*	*Citrus limon*	Brazil	FJ538352	FJ538468	FJ538410	JF343691
P. citricarpa	CBS127454*	*Citrus limon*	Australia	JF343583	JF343667	JF343604	JF343771

.....Continued on the next page

TABLE1 (Continued)

Species	Strain no.[a]	Host	Locality	GenBank Accession number[b]			
				ITS	ACT	TEF-1	GPDH
P. cordylinophila	CPC20261*	*Cordyline fruticosa*	Thailand	KF170287	KF289295	KF289172	KF289076
P. cornicola	CBS111639	*Cornus florida*	USA	KF170307	KF289234		
P. cussoniae	CBS136060*	*Cussonia sp.*	South Africa	JF343578	JF343662	JF343599	JF343764
P. elongata	CBS 126.22*	*Oxycoccus macrocarpos*	USA	FJ538353	FJ538469	FJ538411	KF289164
P. ericarum	CBS132534*	*Erica gracilis*	South Africa	KF206170	KF289291	KF289227	KF289162
P. eugeniae	CBS 445.82	*Eugenia aromatica*	Indonesia	AY042926	KF289246	KF289208	KF289139
P. fallopiae	MUCC0113*	*Fallopia japonica*	Japan	AB454307			
P. foliorum	CBS 447.68*	*Taxus baccata*	Netherlands	KF170309	KF289247	KF289201	KF289132
P. gaultheriae	CBS 447.70*	*Gaultheria humifusa*	USA	JN692543	KF289248	JN692531	JN692508
P. hamamelidis	MUCC149	*Hamamelis japonica*	Japan	KF170289	KF289309		
P. hostae	CGMCC3.14355*	*Hosta plantaginea*	China	JN692535	JN692511	JN692523	JN692503
P. hubeiensis	CGMCC3.14986*	*Viburnum odoratissimim*	China	JX025037	JX025032	JX025042	JX025027
P. hymenocallidicola	CBS 131309*	*Hymenocallis littoralis*	Australia	JQ044423	KF289242	KF289211	KF289142
P. hypoglossi	CBS 434.92*	*Ruscus aculeatus*	Italy	FJ538367	FJ538483	FJ538425	JF343695
P. ilicis-aquifolii	CGMCC3.14358*	*Ilex aquifolium*	China	JN692538	JN692514	JN692526	
P. kerriae	MAFF240047*	*Kerria japonica*	Japan	AB454266			
P. leucothoicola	MUCC0553*	*Leucothoe catesbaei*	Japan	AB454370	KF289310		
P. ligustricola	MUCC0024*	*Ligustrum obtusifolium*	Japan	AB454269	AB704212		
P. maculata	CPC18347*	*Musa cv.Goly-goly pot-pot*	Australia	JQ743570	KF014016	KF009700	
P. maculata	BRIP46622	*Musa* sp.	Australia: Northern Territory	JQ743567	KF014013	KF009692	
P. mangifera-indica	CPC20264*	*Mangifera indica*	Thailand	KF170305	KF289296	KF289190	KF289121
P. minima	CBS 585.84*	*Acer rubrum*	USA	KF206176	KF289249	KF289204	KF289135
P. musarum	BRIP55434*	*Hill banana*	India	JQ743584			
P. musarum	BRIP57803	*Musa* sp.	Australia	JX997138	KF014055	KF009737	
P. musarum	BRIP58028	*Musa* sp.	Australia	KC988377	KF014054	KF009738	
P. musicola	CBS123405*	*Musa acuminata*	Thailand	FJ538334	FJ538450	FJ538392	
P. neopyrolae	MUCC0125*	*Pyrola asarifolia*	Japan	AB454318	AB704233		
P. owaniana	CBS776.97*	*Brabejum stellatifolium*	South Africa	FJ538368	KF289254	FJ538426	JF343767
P. pachysandricola	MUCC0124*	*Pachysandra terminalis*	Japan	AB454317	AB704232		
P. parthenocissi	CBS111645*	*Parthenocissus quinquefolia*	USA	EU683672	JN692518	JN692530	
P. paxistimae	CBS112527*	*Paxistima mysinites*	USA	KF206172	KF289239	KF289209	KF289140
P. philoprina	CBS616.72	*Ilex aquifolium*	Germany	KF154279	KF289251	KF289205	KF289136
P. podocarpi	CBS111647	*Podocarpus lanceolata*	South Africa	KF154276	KF289235	KF289232	KF289168
P. podocarpicola	CBS728.79*	*Podocarpus maki*	USA	KF206173	KF289252	KF289203	KF289134

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TABLE1 (Continued)

Species	Strain no.[a]	Host	Locality	GenBank Accession number[b]			
				ITS	ACT	TEF-1	GPDH
P. spinarum	CBS292.90	*Chamaecyparis pisifera*	France	JF343585	JF343669	JF343606	JF343773
P. styracicola	CGMCC3.14985*	*Styrax grandiflorus*	China	JX025040	JX025035	JX025045	JX025030
P. telopeae	CBS777.97*	*Telopea speciosissima*	Tasmania	KF206205	KF289255	KF289210	KF289141
P. vaccinii	ATCC46255*	*Vaccinium macrocarpon*	USA	KC193585	KC193580	KC193582	KC193583
P. vacciniicola	CPC18590*	*Vaccinium macrocarpum*	USA	KF170312	KF289287	KF289229	KF289165
P. yuccae	CBS117136	*Yucca elephantipes*	New Zealand	JN692541	JN692517	JN692529	JN692507
P. capitalensis	GZAAS6.1201	*Musa* sp.	China: Guizhou	KF955290	KM816623	KM816635	KM816629
P. capitalensis	GZAAS6.1202	*Musa* sp.	China: Guizhou	KF955291	KM816624	KM816636	KM816630
P. capitalensis	GZAAS6.1242	*Musa* sp.	China: Guizhou	KF955292	KM816625	KM816637	KM816631
P. musarum	GZAAS6.1228	*Musa* sp.	Thailand: Chiang Rai	KF955293	KM816626	KM816638	KM816632
P. musaechinensis	GZAAS6.1247	*Musa* sp.	China: Chongqing	KF955294	KM816627	KM816639	KM816633
P. musaechinensis	GZAAS6.1384	*Musa* sp.	China: Chongqing	KF955295	KM816628	KM816640	KM816634

Note:

a ATCC: American Type Culture Collection, Virginia,USA; **BRIP**: Plant PathologyHerbarium,BiosecurityQueensland,DuttonPark,Que ensland,Australia;**CBS**: CBS KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; **CGMCC**: China GeneralMicrobialCultureC ollection;**CPC**:CulturecollectionofP.W.Crous,housedatCBS;**IMI**InternationalMycologicalInstitute,Bioscience,Egham,BakehamLane,U. K.;**MAFF**:theMicrobiologicalGenebank,NationalInstituteofAgrobiologicalSciences,Japan;**MUCC**: Culture Collection, Laboratory of Plant Pathology, Mie University, Tsu, Mieprefecture,Japan;**NBRC**:BiologicalResourceCenter,theNationalInstituteof502Technology and Evaluation, Japan;**ZJUCC**:Zhejiang University Culture Collection, China.

* indicates the ex–type cultures

b **ITS**: Internal transcribed spacers 1 and 2 togetherwith 5.8S nrDNA;**TEF-1**: partial translationelongationfactor1-alphagene;**ACT**: actingene;**GAPDH**:glyceraldehyde–3–phosphate dehydrogenase gene.

The models of evolution were estimated with MrModeltest v2.3 (Nylander 2004). Bayesian analyses with the selected evolutionary model were performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Six simultaneous Markov chains were run for 1×10^6 generations and trees were sampled every 100th generation. The first 2000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 8000 trees used for calculating posterior probabilities (PP) in the majority rule consensus tree (Liu *et al*. 2012).

The models of evolution were estimated with MrModeltest v2.3 (Nylander 2004). The alignments were converted by ALTER on http://sing.ei.uvigo.es/ALTER (Glez-Peña *et al.* 2010). A maximum likelihood analysis was performed (Silvestro *et al.* 2012). One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution. The resulting replicates were plotted on to the best scoring tree obtained previously. The representative sequences and phylogenetic analyses were performed according to Wong *et al*. (2012).

Morphological characters

Specimens were observed by a Nikon eclipse 80i compound microscope with DS-5Mc camera and an Olympus SZX2 stereomicroscope. Hand sections were made for microscopic examination. Measurements were made in water. The morphological characters of colony were assessed after 60 d growth on PDA, MEA (malt extract agar) and OA (oat agar).

Results

Phylogenetic analysis

The six isolates from banana obtained in this study were sequenced using five (LSU, ITS, ACT, TEF-1 and GPDH) genes.

The LSU alignment contained 22 sequences (including the outgroup *Botryosphaeria dothidea*) and consisted of 780 (including alignment gaps) total characters, of which 60 characters (7.7%) are parsimony informative. A heuristic search with random addition of taxa (1000 replicates) and treating gaps as missing characters generated 1 parsimonious tree(TL = 127, CI = 0.764, RI= 0.880, RC= 0.672, HI= 0.236) shown in Fig. 1. Bootstrap support values of MP (BS) (equal to or above 50%) and Bayesian posterior probabilities (PP) (equal to or above 0.90 based on 1,000,000 generations) are shown on the upper branches. The phylogenetic tree of the LSU region indicated that all *Phyllosticta* species and the six new isolates form a monophyletic lineage sister to *Botryosphaeria dothidea* (BS = 100). It confirms that the six isolates, GZAAS6.1201, GZAAS6.1202, GZAAS6.1228, GZAAS6.1242, GZAAS6.1247 and GZAAS6.1384 were members of *Phyllosticta sensu stricto.*

The combined dataset of ITS, ACT, TEF-1 and GPDH contained 75 combined 250 sequences from 64 taxa and comprised 1644 total characters including gaps, of which 887 characters were constant; 535 characters(32.5%) were parsimony informative; 211 variable characters are parsimony-uninformative. A heuristic search with random addition of taxa (1000 replicates) and treating gaps as missing characters generated 2496 equally parsimonious trees. All trees were similar in topology, first of which equally most parsimonious tree (TL = 2760, CI = 0.435, RI = 0.707, RC = 0.307, HI = 0.565) shown in Fig. 2. Bootstrap support values of most parsimonious (MPBS) and maximum likelihood (MLBS) (equal to or above 50 %) are shown on the upper branches. The phylogenetic tree based on four gene loci analysis indicated that three isolates, GZAAS6.1201, GZAAS6.1202 and GZAAS6.1242, clustered with *P. capitalensis* and formed a branch with high support (MPBS = 83, MLBS = 87). GZAAS6.1228 was shown to be species of *P. musarum* (MPBS = 77, MLBS = 94). GZAAS6.1247 and GZAAS6.1384 formed a strong single lineage (MPBS = 100, MLBS = 100) relative to *P. musarum*, *P. maculata* and *P. cavendishii*.

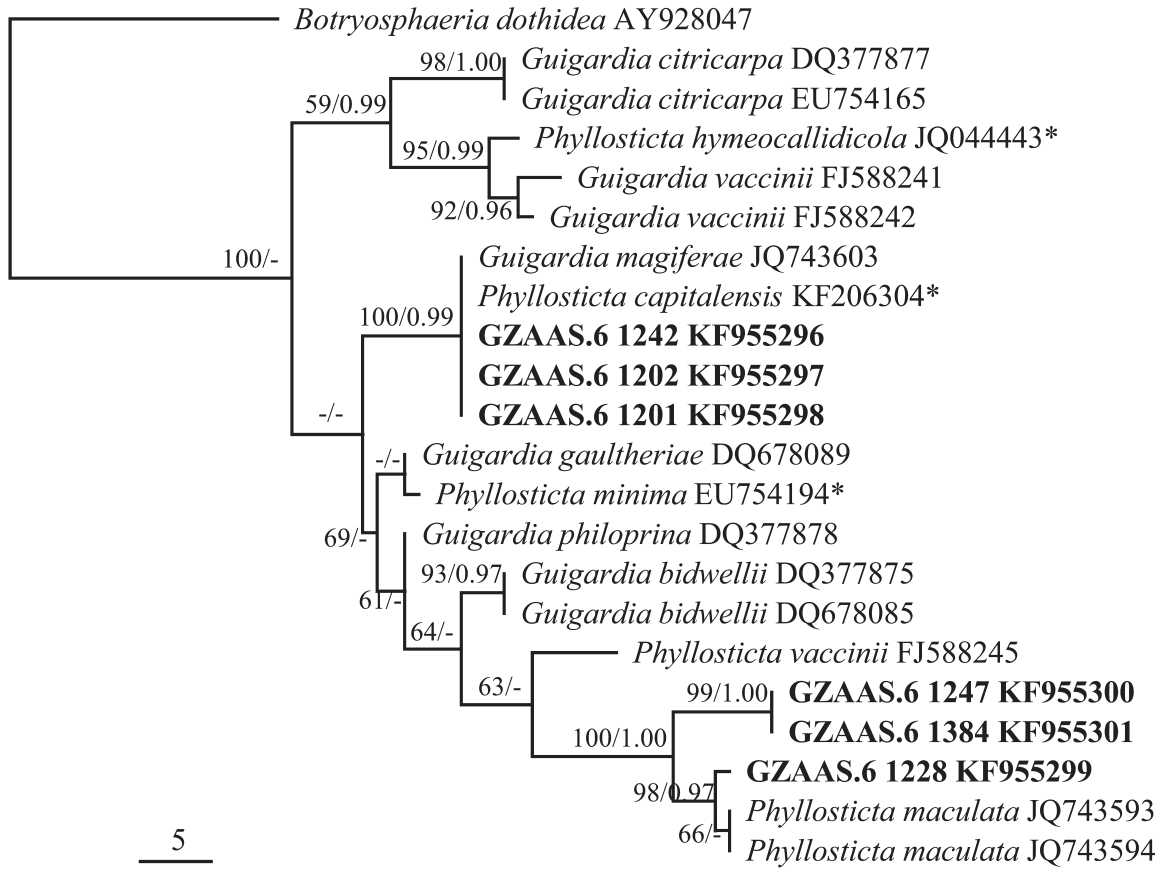

FIGURE 1. The most parsimonious trees obtained from a heuristic search with 1000 random taxon additions of the LSU sequences using PAUP v. 4.0b10. The scale bar shows 5 changes. Bootstrap support values for maximum parsimony (MP) and Bayesian posterior probabilities above 0.90are shown. A hyphens (–) indicates the value lower than 50% (BS) or 0.90 (PP). The tree is rooted to *Botryosphaeria dothidea*. Ex-type/ex-epitype isolates are marked by an asterisk *. Novel sequences are in boldface.

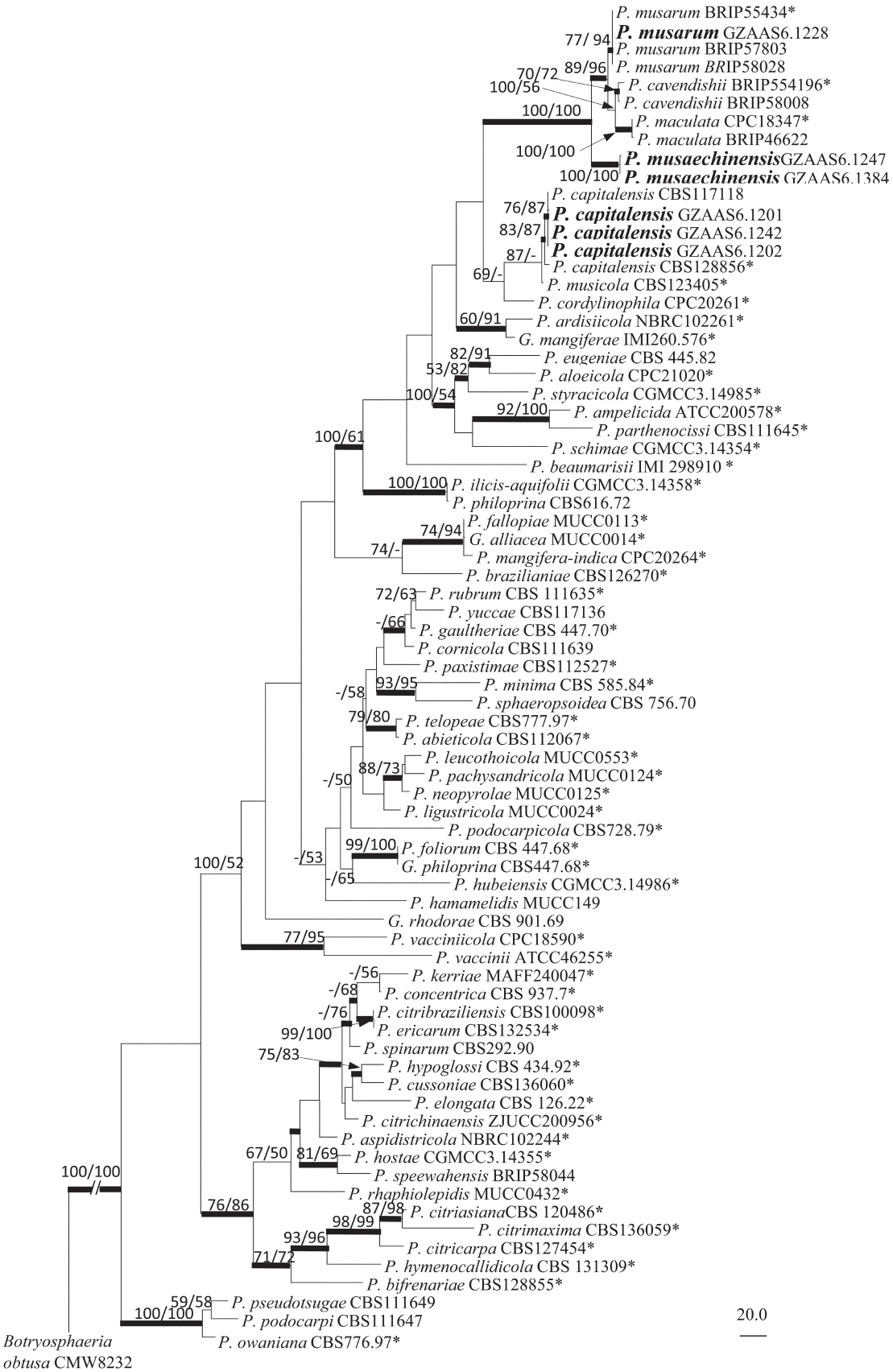

FIGURE 2. The first of 2496 equally most parsimonious trees obtained from a heuristic search with 1000 random taxon additions of the ITS, ACT, TEF-1 and GPDH sequences using PAUP v. 4.0b10. The scale bar shows 20 changes. Bootstrap support values for maximum parsimony (MPBS) and maximum likelihood (MLBS)(equal to or above 50 %)were shown. Thickened branches represent significant Bayesian posterior probability (≥90%). A hyphen (–) indicates the value lower than 50%. The tree is rooted *Botryosphaeria dothidea.*An asterisk (*) indicates the ex-type strains. Novel sequences are printed in bold.

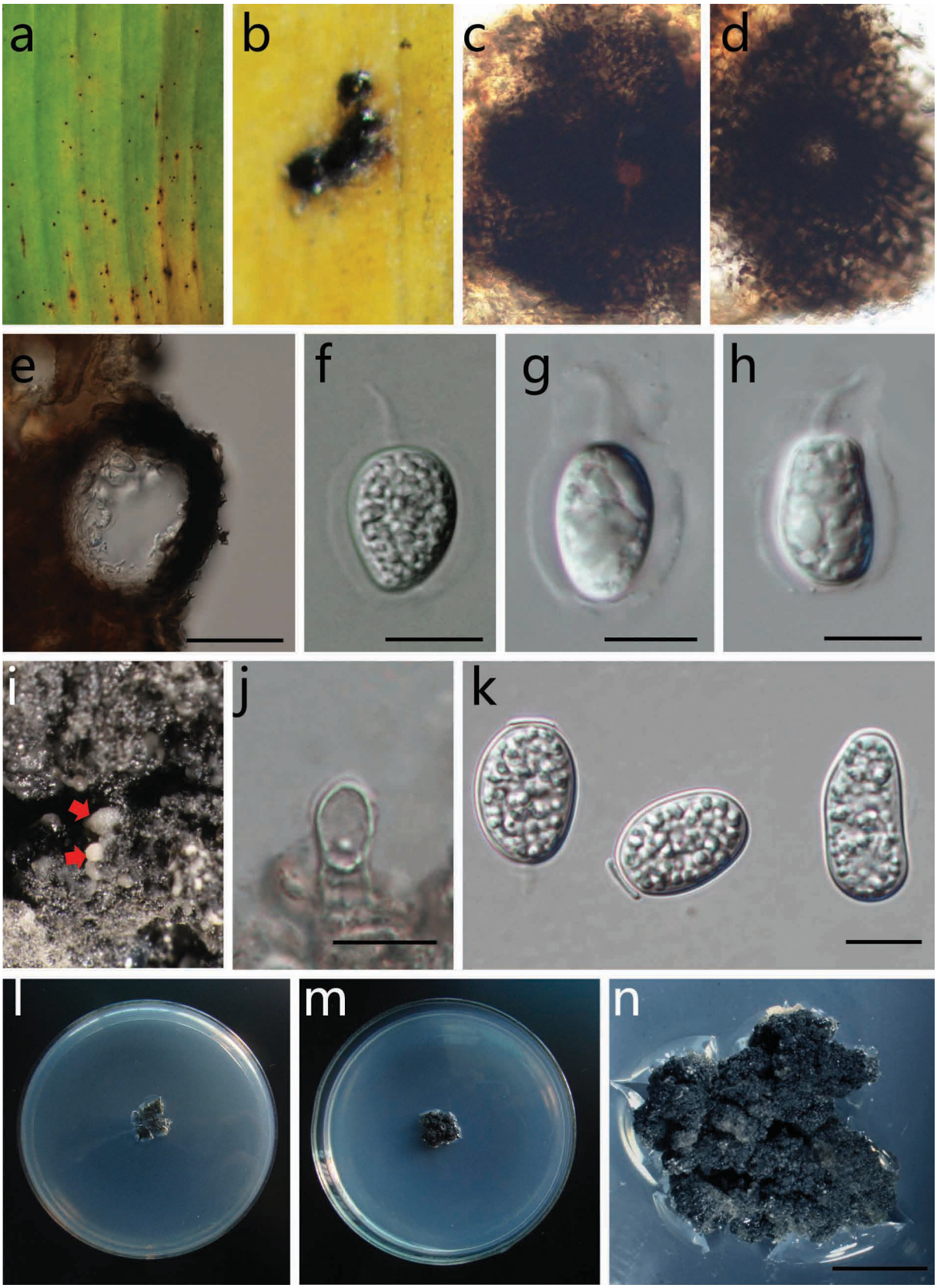

FIGURE 3.*Phyllosticta musaechinensis* (CQ12097, holotype).a. Symptom of disease. b. Pycnidia on infected leaf of *Musa* sp. c, d. Ostiole of pycnidium. e. Pycnidium and conidiogenous cell. f–h. Conidia. i. Conidia on colony. j. Conidiogenous cell in culture. k. Conidia from culture. l–n. Culture on PDA. Scale bars: e = 50µm, f–h, j, k = 20 µm, n = 5 mm.

Taxonomy

Phyllosticta musaechinensis S.P. Wu, Z.Y. Liu & K.D. Hyde, *sp. nov.* (Fig 3) MycoBank MB 806057.

Phyllosticta musaechinensis is weakly pathogenic on leaves of *Musa* sp. It grows slowly on PDA. Sexual and spermatial states were not observed.

Type: —CHINA. Chongqing Municipality: Yubei District, Longtou Temple Park, on leaves of *Musa* sp., 21 September 2012, *Shiping Wu*, (CQ12097, holotype); ex-type living culture = GZAAS6.1247 = MFLUCC13-0907 = ICMP 20111).

Etymology:—From *Musa,* the host and *chinensis*, in reference to the first collection of the species on a banana host in China.

Weakly pathogenic on leaves of *Musa* sp., slightly discoloring leaves yellow, with black, shiny conidiomata forming on healthy green, or yellowing parts of leaves. Sexual state: Unknown. Asexual state: Pycnidia 45–145 µm ($\bar{x}$ = 93 µm) diam. subcuticular to erumpent, solitary or clustered in small groups, black, shiny, globose or subglobose, with a rounded ostiole at the center. Conidiogenous cells cylindrical or conical. Conidia 14–18 × 8–12 µm ($\bar{x}$ = 17 × 10 µm), hyaline, aseptate, coarsely guttulate, ellipsoidal or clavate, thin- and smooth-walled, surrounded by a mucilaginous sheath 0.5–3.5 µm thick, apex tapering, straight to curved, appendage 4.0–18.5 µm ($\bar{x}$ = 12 µm) long. Spermatial state: unknown.

Colony on PDA bluish black to black, without aerial mycelium, irregular, raised to about 0.7 mm, reaching 14.2–12.5 mm diam after 60 d at 28°C. Pycnidiasolitary or aggregated in colony, black. Conidia 15.5–22.5 × 8.5–13 µm ($\bar{x}$= 18 × 11 µm), hyaline, aseptate, coarsely guttulate, ellipsoidal, clavate or irregular, thin- and smooth-walled, surrounded by a mucilaginous sheath or not, apex tapering, straight to curved 4–18 µm ($\bar{x}$ = 11.6 µm). Spermatia not formed.

Known distribution:—China.

Host:—*Musa* sp.

Additional material examined:—CHINA. Chongqing Municipality: Yubei District, Longtou Temple Park, on leaves of *Musa* sp., 3 August 2013, *Shiping Wu* (CQ13018); living culture = GZAAS6.1384.

Discussion

After phylogenetic analysis based on four gene loci (Fig. 2), our taxon displayed a close relationship with *P. musarum*, *P. maculata* and *P. cavendishii* with strong statistical support, but formed an independent branch. All of these species are known from *Musa* sp. Additionally, four other species of *Phyllosticta/Guignardia* have also been reported from banana (van der Aa & Vanev 2002, Wulandari *et al*. 2010, Wong *et al*. 2012). Thus, we compare our taxon with these seven species. The asexual morphs of *G. stevensii* and *G. sydowiana* have never been reported (Wulandari *et al.* 2010). However, only the asexual states have been found for our new species. The new species shows greater variability than other related *Phyllosticta* spp. in pycnidia size, and the conidia are longer than those of *P. capitalensis*, *P. musicola*, and *P. cavendishii*. The conidial appendage of *P. musaechinensis* is shorter than in *P. maculata* and *P. musarum.* Thus, *P. musaechinensis* is distinguished from other *Phyllosticta* spp. from banana in pycnidia size, conidia size, mucilaginous sheath and appendage. The detailed information about morphological comparison is shown in Table 2.

Phyllosticta musaechinensis caused disease symptom similar to freckle disease, however, this weak pathogen mainly attacked older leaves which rapidly became yellow around the leaf spots. Additionally, leaves could not be infected by artificial inoculation.

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TABLE 2. Conidia morphology of *Phyllosticta / Guignardia* spp. described from *Musa*.

Taxa	Pycnidia size (µm)	Conidia size (µm)	Mucilaginous sheath thickness (µm)	Appendage length (µm)
P. cavendishii	78–137	12–17 × 8–10	1–3	8–20
P. maculata	84–137	15–21 × 9–13	2–6	12–37
P. musarum	69–118	12–20 × 7–11	1–3	14–20
G. stevensii (no asexual state)	–	–	–	–
G. sydowiana (no asexual state)	–	–	–	–
G. musicola	90–125	12–17 × 8–11	2–4	10–15
P. capitalensis	300	10–14 × 5–7	2–4	6–8
P. musaechinensis	45–145	15.5–22.5 × 8.5–13	0.5–3.5	4–18.5

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