Fungal Diversity (2012) 56:173–187
DOI 10.1007/s13225-012-0182-9

Phyllosticta species associated with freckle disease of banana

Mee-Hua Wong · Pedro W. Crous · Juliane Henderson · Johannes Z. Groenewald · Andre Drenth

Received: 19 March 2012 / Accepted: 6 June 2012 / Published online: 21 August 2012

Abstract The identity of the casual agent of freckle disease of banana was investigated. The pathogen is generally referred to in literature under its teleomorphic name, *Guignardia musae*, or that of its purported anamorph, *Phyllosticta musarum*. Based on morphological and molecular data from a global set of banana specimens, several species were found associated with freckle disease. *Phyllosticta maculata* (from Southeast Asia and Oceania) is introduced as a new name for *Guignardia musae*, and an epitype is designated from Australia. *Phyllosticta musarum* (from India and Thailand) is shown to represent a distinct species, and the name is fixed by designation of an epitype from India. *Guignardia stevensii* is confirmed as distinct species from Hawaii, while *Guignardia musicola* from northern Thailand is shown to contain different taxa and is regarded as *nomen confusum*. *Phyllosticta cavendishii* is described as a new, widely distributed species, appearing primarily on Cavendish, but also on non-Cavendish banana cultivars.

Keywords Freckle disease of *Musa* · *Phyllosticta* · Taxonomy

M.-H. Wong
School of Agriculture and Food Sciences,
The University of Queensland,
St. Lucia, Queensland 4072, Australia

M.-H. Wong · J. Henderson · A. Drenth (✉)
Centre for Plant Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland,
Ecosciences Precinct, GPO Box 46, Brisbane, Queensland 4001, Australia
e-mail: a.drenth@uq.edu.au

M.-H. Wong
Department of Agriculture, Sarawak,
Agriculture Research Centre, Semongok,
P.O. Box 977, 93720, Kuching, Sarawak, Malaysia

P. W. Crous · J. Z. Groenewald
CBS-KNAW Fungal Biodiversity Centre,
P.O. Box 85167, 3508 AD, Utrecht, The Netherlands

P. W. Crous
Laboratory of Phytopathology, Wageningen University and Research Centre (WUR),
Droevendaalsesteeg 1,
6708 PB, Wageningen, The Netherlands

P. W. Crous
Microbiology, Department of Biology, Utrecht University,
Padualaan 8,
3584 CH, Utrecht, The Netherlands

Introduction

Freckle disease of banana, a name that describes the rough, freckle-like spots that occur on infected fruit and leaves, is common in Southeast and East Asia, especially on *Musa* clones in the Cavendish and Plantain subgroups, as well as *M. acuminata* ssp. *banksii* and *M. schizocarpa* (Jones 2000) (Fig. 1). The disease was first reported from Hawaii in 1917 (Carpenter 1919) and a few years later in the Philippines (Lee 1922). Freckle disease is generally accepted to be caused by the fungus *Guignardia musae* Racib., and its purported anamorph, *Phyllosticta musarum* (Cooke) Van der Aa. The abolishment of dual nomenclature for fungi (Hawksworth et al. 2011; Wingfield et al. 2012) gives priority to the older name *Phyllosticta* (1818) over that of *Guignardia* (1892) (Glienke et al. 2011; Wikee et al. 2011). For the remainder of this paper, we give preference to the name *Phyllosticta*.

Freckle disease has become one of the major constraints in the production of high quality wholesome banana fruit. For countries such as Taiwan and the Philippines that produce

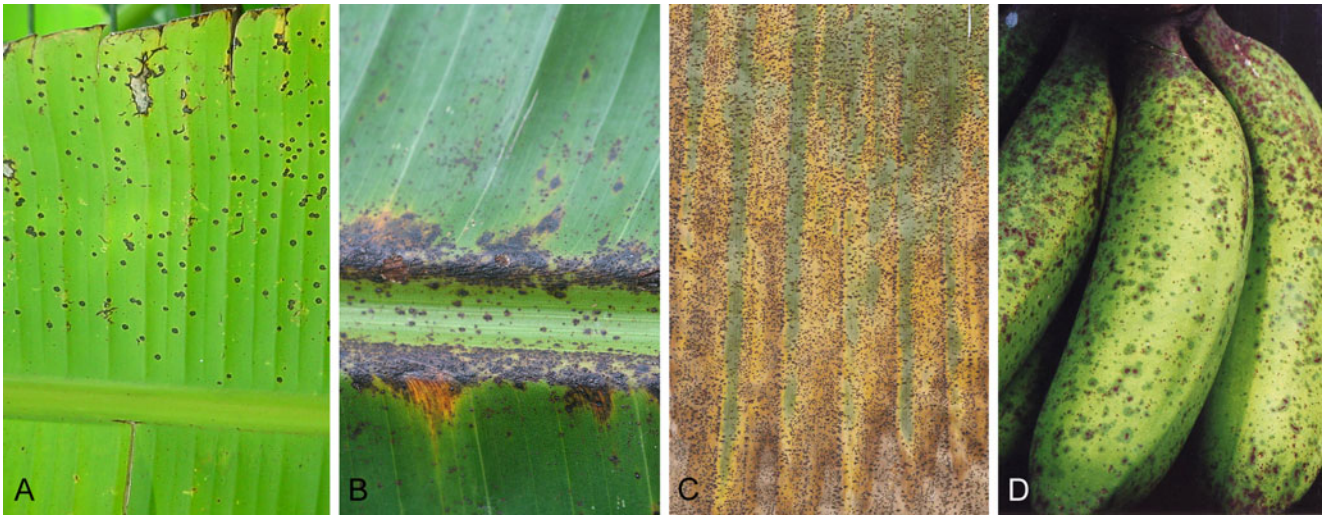

Fig. 1 Foliar and fruit disease symptoms associated with freckle disease on banana. **a**. Initial stage of infection with freckle spots on green leaf. **b**. Freckle spots aggregated on mid-rib of leaf. **c**. Late stage of leaf infection. **d**. Water-soaked lesions on banana fruit. Photo's courtesy of **a**. J. Henderson, **c**. S. van Brunschot, **b**, **d**. Department of Agriculture, Sarawak

bananas for export, the economic significance of freckle disease lies in the fact that blemished fruit is not accepted by importing countries (Jones 2000; Corcolon and Raymundo 2008). In Taiwan, freckle has replaced black Sigatoka (Arzanlou et al. 2007, 2010) as the major foliar disease (Tsai et al. 1993) and in the Philippines it is becoming a serious problem in plantations of Cavendish banana (Jones 2000).

In Australia, freckle disease was first identified on Saibai Island in the Torres Strait in 1979 (Jones and Alcorn 1982) and presently occurs in the northern parts of Queensland and the Northern Territory. The disease affects banana cultivars of the AAB and ABB genomic groups including Lady Finger (AAB) and Bluggoe (ABB) but the AAA genomic group of Cavendish subgroup has not shown disease symptoms. The observed link between geographic and host differentiation indicates that the freckle pathogen may consists of more than one fungal taxon.

Species of *Phyllosticta* generally cause leaf spots, but also infect fruits and stems of a wide range of host plants (van der Aa 1973; van der Aa and Vanev 2002). Most species are endophytes, some are saprobes, but many are plant pathogens (Baayen et al. 2002; Okane et al. 2003; Wulandari et al. 2009; Crous et al. 2011; Glienke et al. 2011; Wikee et al. 2011; Wang et al. 2012). In addition to freckle disease of banana, other important *Phyllosticta* diseases include black spot disease of citrus caused by *P. citricarpa*, *P. citriasiana* and *P. citrichinaensis* (Wulandari et al. 2009; Wang et al. 2012), black rot disease of grapes caused by *P. ampelicida* (Kuo and Hoch 1996), as well as many other diseases on ornamentals, shrubs and trees (van der Aa and Vanev 2002). A particular problem when isolating species of *Phyllosticta* is that many species co-occur in the same lesion, and endophytic species are frequently easier to cultivate than pathogens, leading to incorrect identification of species based on cultures (Baayen et al. 2002; Glienke et al. 2011).

In a recent morphological study, Wulandari et al. (2010) provided a detailed overview of all the species of *Phyllosticta* and *Guignardia* that have been described from *Musa*. In contrast to the species occurring on citrus, where substantial molecular work has been carried out (Baayen et al. 2002; Rodrigues et al. 2004; Wulandari et al. 2009; Glienke et al. 2011; Wang et al. 2012), molecular data on the *Phyllosticta* complex of banana has mostly been lacking. In a preliminary study employing DNA sequence data from the ITS nuclear rDNA of a global set of *Phyllosticta* specimens from banana, three clades could be resolved (M.H. Wong unpubl. data).

The aims of the present study were to (i) identify *Phyllosticta* species associated with freckle disease on banana based on morphological features and molecular analysis; (ii) determine if morphological differences exist among the phylogenetic clades and if possible, correlate clades to existing species, and (iii) investigate the relationship of *Phyllosticta* isolates from banana to others in the genus.

Materials and methods

Isolates

Symptomatic green banana leaves with expanding lesions were selected for isolation. Leaves were cut into pieces approximately 3×5 cm, and surface-sterilised in 70% ethanol for 1 min, and allowed to dry. Isolations were performed using two different laboratory methods. Single fruiting bodies were removed under a stereo microscope with a scalpel,

and plated onto 2% potato-dextrose agar (PDA) with streptomycin sulphate (Crous et al. 2009). Alternatively, the oozing conidial mass was transferred onto plates containing half-strength PDA amended with streptomycin sulphate. Plates were incubated at 25 °C, and after 3–4 weeks the first growth was observed. Once growing, colonies were subcultured onto PDA. Cultures were grown on oatmeal agar (OA; Crous et al. 2009), with or without sterilised pine needle or banana leaf, and incubated at 25 °C under near-ultraviolet light to promote sporulation.

Six cultures of *P. capitalensis* from *Musa* spp. were obtained from the Centraalbureau voor Schimmelcultures (CBS-KNAW Fungal Biodiversity Centre), Utrecht, The Netherlands, and included in this study. Reference cultures and specimens are deposited at the CBS, and the Plant Pathology Herbarium, Biosecurity Queensland, Dutton Park, Queensland, Australia (Table 1).

DNA isolation, amplification and phylogeny

Genomic DNA was extracted from fungal cultures grown on PDA using the UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, USA) according to the manufacturer's protocol. For genomic DNA extracted from herbarium leaf specimens, lesions with fruiting bodies were ground in liquid nitrogen with glass beads (Sigma, USA) followed by DNA extraction using a NucleoSpin Plant II (Macherey-Nagel, Germany) isolation kit according to the manufacturer's instructions.

Amplification reactions were performed under two different conditions depending on the DNA source, primers used and the laboratory where these reactions were performed. The first PCR condition was carried out with primer pairs, ITS5 and ITS4 (White et al. 1990) or LROR (Rehner and Samuels 1994) and LR5 (Vilgalys and Hester 1990) with genomic DNA from culture as template. Each reaction mixture with a total volume of 12.5 µl comprised of 1× PCR buffer, 2 mM $MgCl_2$, 40 µM dNTPs (Bioline, Germany), 5.6% DMSO (v/v) (Sigma, USA), 0.2 µM of each forward and reverse primers and 0.25 U *Taq* DNA polymerase (BioTaq DNA polymerase, Bioline, Germany). The amplification conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 45 s, 48 °C for 30 s and 72 °C for 1 min 30 s and final extension at 72 °C for 10 min. PCR products were checked by electrophoresis in 1% agarose gels stained with GelRed (Biotium, USA). Amplicons were sequenced using the same primers and a BigDye Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, USA) and sequences were analysed on an ABI 3730xl DNA Sequencer (Applied Biosystems, USA) at CBS, Utrecht, The Netherlands.

For the second condition, PCR was carried out using primer pair GmF1 (5'-GTGCGCACCTCTCGAAC-3') and GmR2 (5'-ACCTGATCCGAGGTCAAC-3') (J. Henderson and S. van Brunschot, unpubl. data) with genomic DNA extracted from herbarium leaf specimen as template. This primer pair amplifies partial ITS of the nuclear rRNA operon, including the ITS1 region, 5.8S rRNA gene and the ITS2 region. The reaction volume of 20 µl consisted of 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs (Promega, USA), 0.5 µM of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase (AmpliTaq Gold DNA Polymerase, Applied Biosystems, USA). The cycle conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were subjected to electrophoresis in 1.5–3% agarose gels stained with GelRed (Biotium, USA). Amplified products of the expected size were cut from the gels and the DNA was purified with a Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions. Purified DNA was re-amplified with the same primer pair and under the same amplification conditions. Re-amplified products were again purified with the Wizard SV Gel and PCR Clean-Up System. Amplicons were sequenced using the same primer pair and a BigDye Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, USA) and sequences were analysed on an ABI 3730xl DNA Sequencer (Applied Biosystems, USA) at the Australian Genome Research Facility, Brisbane, Australia.

DNA sequences were assembled using SeqMan Pro software (Lasergene 8, DNASTAR) or Vector NTI (Invitrogen). For the ITS alignment, representative sequences from earlier studies (Wulandari et al. 2009; Glienke et al. 2011) were downloaded from NCBI's GenBank nucleotide database and representative sequences from the reference tree of Crous et al. (2006) were downloaded for the LSU alignment. Sequences generated were automatically aligned using MAFFT v. 6 (http://mafft.cbrc.jp/alignment/server/index.html) and the alignments were corrected by eye using MEGA v. 5.05 (Tamura et al. 2011).

The phylogenetic analysis of the aligned sequences were conducted using PAUP (Phylogentic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) which comprised of neighbour-joining analysis with the uncorrected ('p'), the Kimura 2-parameter and the HKY85 substitution models. Sites containing alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony trees were constructed using the heuristic search option with 100 random additions of simple taxa and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The confidence

Table 1 Cultures and herbarium specimens of *Phyllosticta* spp. on banana used for morphological and molecular studies

Species	Herbarium specimen no	Culture accession no.[1]	Country (state/province/island)	Host species/cultivar (genomic group)	Collector(s)	GenBank accession no. [2]	
						ITS	LSU
Phyllosticta cavendishii	Bft26; CBS H-20918 (holotype); BRIP 55419	—	Taiwan (Pingtung)	*Musa* cv. Formosana (AAA)	C.P. Chao	JQ743562	—
	Bfn39; BRIP 55420	—	Indonesia (Sumatra)	*Musa* cv. Cavendish (AAA)	J. Ray	JQ743563	—
	Bfv40; BRIP 55421	—	Viet Nam (Thua Thien Hue)	*Musa* cv. Cavendish (AAA)	T.T. Nguyen & N.A. Doan	JQ743564	—
	BRIP 45872	—	East Timor	*Musa* sp.	P. Stephens	JQ771317	—
	Bfs12; BRIP 55422	—	Malaysia (Sarawak)	*Musa* cv. Keling (AAB)	M.-H. Wong	JQ743561	—
	IBFREC8; BRIP 55423	—	USA (Hawaii)	*Musa* sp. (AAB/ABB)	I. Buddenhagen	JQ771318	—
	PDD 50952*	—	Micronesia	*Musa* sp. (AAB/ABB)	G.V.H. Jackson	JQ743565	—
	PDD 34914*	—	Tonga	—	S.R. Pennycook	JQ743566	—
Phyllosticta maculata	PS1; BRIP 46622	PS1; BRIP 46622; CPC 18342; CPC 19949; CBS 132578	Australia (Northern Territory)	*Musa* cv. Lady finger (AAB)	P. Stephens	JQ743567	JQ743590
	PS3; BRIP 46620	PS3; BRIP 46620; CPC 18343; CBS 132579	Australia (Northern Territory)	Cooking banana	P. Stephens	JQ743568	JQ743591
	Bf001; BRIP 55424	Bf001; CPC 18346 CPC 19952; CBS 132580; BRIP 55424	Australia (Queensland)	*Musa* cv. Blue Java (ABB)	M. Berridge	JQ743569	JQ743592
	Bf002; CBS H-20919 (epitype); BRIP 55425	Bf002; CPC 18347; CPC 19953; CBS 132581; BRIP 55425	Australia (Queensland)	*Musa* cv. Goly-goly pot-pot (ABB)	M. Berridge	JQ743570	JQ743593
	Bf003; BRIP 55426	Bf003; CPC 18348; CPC 19954; CBS 132582; BRIP 55426	Australia (Queensland)	*Musa* cv. Bluggoe (ABB)	C. Pearce	JQ743571	JQ743594
	BRIP 55427	Bf004; CPC 18349; CBS 132583; BRIP 55427	Australia (Queensland)	*Musa* cv. Bluggoe (ABB)	C. Pearce	JQ743572	JQ743595
	—	Bf005; CPC 18350; CBS 132584; BRIP 55428	Australia (Queensland)	*Musa* cv. Blue Java (ABB)	C. Pearce	JQ743573	JQ743596
	Bf006; BRIP 55429	Bf006; CPC 18351; CPC 19955; CBS 132585; BRIP 55429	Australia (Queensland)	*Musa* cv. Blue Java (ABB)	C. Pearce	JQ743574	JQ743597
	Bf007; BRIP 55430	Bf007; CPC 18352; CPC 19956; BRIP 55430	Australia (Queensland)	*Musa* cv. Simoi (ABB)	C. Pearce	JQ743575	JQ743598
	Bfs20; BRIP 55431	—	Malaysia (Sarawak)	*Musa* cv. Tandok (AAB)	M.-H. Wong	JQ743576	—
	CBS H-20920; BRIP 55432; IBFREC10 allele a1	—	Indonesia (Sulawasi)	*Musa* cv. Sepatin maluhusi (probably BBB)	I. Buddenhagen	JQ743577	—
	CBS H-20920; BRIP 55432; IBFREC10 allele a2	—	Indonesia (Sulawasi)	*Musa* cv. Sepatin maluhusi (probably BBB)	I. Buddenhagen	JQ743578	—
	CBS H-20920; BRIP 55432; IBFREC10 allele a3	—	Indonesia (Sulawasi)	*Musa* cv. Sepatin maluhusi (probably BBB)	I. Buddenhagen	JQ743579	—
	CBS H-20920; BRIP 55432; IBFREC10 allele a4	—	Indonesia (Sulawasi)	*Musa* cv. Sepatin maluhusi (probably BBB)	I. Buddenhagen	JQ743580	—
	MPW1390; CBS H-20921; BRIP 55433	—	Papua New Guinea (Daru)	*Musa* cv. Mysore (AAB)	M. Weinert	JQ743581	—
	PDD 55765*	—	American Samoa	*Musa* sp. (AAB/ABB)	E.H.C. McKenzie	JQ743582	—
Phyllosticta musarum	Bfd34; CBS H-20922 (epitype); BRIP 55434	—	India (Tamil Nadu)	Hill banana (AAB)	R. Thangavelu	JQ743584	—
	Bfh44; CBS H-20923; BRIP 55435	—	Thailand (Nakorn Ratchasima)	*Musa* cv. Nam Wa (ABB)	M. Kanjanamaneesa-thian	JQ743583	—
Phyllosticta capitalensis	—	CPC 18122	Malaysia (Sarawak)	*Musa* sp.	M.-H. Wong	JQ743585	JQ743599
	—	CPC 13013; CBS 119720	USA (Hawaii)	*Musa* sp.	I. Buddenhagen	FJ538340	JQ743600
	—	CBS 123404	Thailand	*Musa paradisiaca*	N.F. Wulandari	JQ743586	JQ743601
	—	CBS 123405	Thailand	*Musa acuminata*	N.F. Wulandari	FJ538334	JQ743602
	—	CBS 117118	Indonesia	*Musa acuminata*	I. Buddenhagen	FJ538339	JQ743603
	—	CBS 123373	Thailand	*Musa paradisiaca*	N.F. Wulandari	JQ743587	JQ743604

[1] BRIP: Plant Pathology Herbarium, Biosecurity Queensland, Dutton Park, Queensland, Australia; CBS: Centaalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous housed at CBS; PDD: The New Zealand Fungal Herbarium, Landcare Research, Auckland, New Zealand

[2] ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; LSU: partial 28S nrDNA

* Specimen originated from Landcare Research, New Zealand

limit of the resulting trees was estimated by bootstrap analysis with 1000 replications (Hillis and Bull 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with Geneious v. 5.5.4 (Drummond et al. 2011) and layout was done in Adobe Illustrator CS 5.1. Novel sequence data were deposited in GenBank (Table 1) and the alignment in TreeBASE (www.treebase.org).

Morphology

Fungal structures were mounted on glass slides in clear lactic acid for microscopic examination. Due to poor sporulation, descriptions were based on structures taken from herbarium materials except where stated otherwise. The 95% confidence levels were based on 30 spore measurements, and extremes are given in parentheses. Observations were made with a Zeiss V20 Discovery stereo microscope, and with a Zeiss Axio Imager 2 light microscope using differential interference contrast (DIC) illumination and an AxioCam MRc5 camera and software.

Fruiting bodies were sectioned at a thickness of 10 μm using a freezing microtome (Leica CM1100, Leica Microsystems, Germany), with sections mounted in clear lactic acid. Observations were made with a Nikon SMZ1000 stereo microscope, and with a Nikon Eclipse 80i light microscope using DIC illumination and a Nikon DS-Fi camera and software.

Colony colours (surface) were determined using the colour charts of Rayner (1970) after 1 month at 25 °C under near-ultraviolet light. Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org; Crous et al. 2004).

Results

Phylogenetic analyses

The LSU sequences of the 15 isolates of *Phyllosticta* species isolated from banana were aligned with representative sequences of various genera in the *Botryosphaeriaceae* obtained from NCBI's GenBank nucleotide database. The manually adjusted LSU sequence alignment matrix consisted of 51 sequences (including the outgroup) and 799 characters (including alignment gaps) that were used in the phylogenetic analysis. Of these, 148 were parsimony-informative, 44 were variable and parsimony uninformative and 607 were constant. The Neighbour-joining analysis using three substitution models on the sequence alignment data generated trees with identical topologies and which support the same terminal clades as obtained from the parsimony analysis. The parsimony analysis of the sequence alignment data generated 30 equally most parsimonious trees (Fig. 2; TL=395 steps; CI=0.613; RI=0.886; RC=0.543).

The phylogenetic tree of the LSU region revealed the various *Phyllosticta* species to form a monophyletic lineage sister to *Botryosphaeriaceae*. It confirms that in spite of its different culture characteristics, *P. maculata,* which is associated with freckle disease on banana, is a member of *Phyllosticta s.str.*

The ITS sequences of the 29 isolates of *Phyllosticta* species isolated from banana were aligned with sequences of various known *Phyllosticta* species (including the outgroup) obtained from NCBI's GenBank nucleotide database. Due to the inclusion of shorter (missing a part of the 5' side of the first internal transcribed spacer) sequences from the herbarium specimens, it was not possible to use full-length ITS sequences in the analyses. The manually adjusted ITS sequence alignment matrix consisted of 53 sequences (including the outgroup) and 446 characters (including alignment gaps; see TreeBASE) were used in the phylogenetic analysis. Of these, 115 were parsimony-informative, 53 were variable and parsimony uninformative and 278 were constant. The neighbour-joining analysis using three substitution models on the sequence alignment data generated trees with identical topologies and which support the same terminal clades as obtained from the parsimony analysis. The parsimony analysis of the sequence alignment data generated 20 equally most parsimonious trees (Fig. 3; TL=316 steps; CI=0.747; RI=0.937; RC=0.700).

The phylogenetic tree of the ITS region showed that all isolates from banana clustered in four clades. Twenty-three isolates did not group with any other species of *Phyllosticta* but formed three individual clades while the remaining six isolates clustered with *P. capitalensis* to form the fourth clade. Clade 1 consists of isolates from non-Cavendish cultivars from countries in Southeast Asia and Oceania and is described below as *P. maculata*. Clade 2 consists of isolates that infect both the Cavendish and non-Cavendish cultivars from countries in Asia and Oceania and is described as a novel species, *P. cavendishii*. Clade 3 contains isolates from India and Thailand on non-Cavendish cultivars, and is identified as *P. musarum*. This latter species was originally described from a specimen collected in India. Clade 4 consists of six isolates from various host cultivars and clustered with *P. capitalensis*, indicating the presence of this common endophyte on *Musa*.

Taxonomy

Several *Phyllosticta* species associated with banana freckle disease were examined in the present study, and these are treated below.

Phyllosticta cavendishii M.H. Wong & Crous, **sp. nov.** Fig. 4

MycoBank MB564747.

Etymology Named after William Cavendish, 6th Duke of Devonshire after whom the *Musa* cultivar Cavendish was named.

Leaf spots on adaxial leaf surface, raised, rough, circular or irregular. *Fruiting bodies* black, solitary or aggregated, on

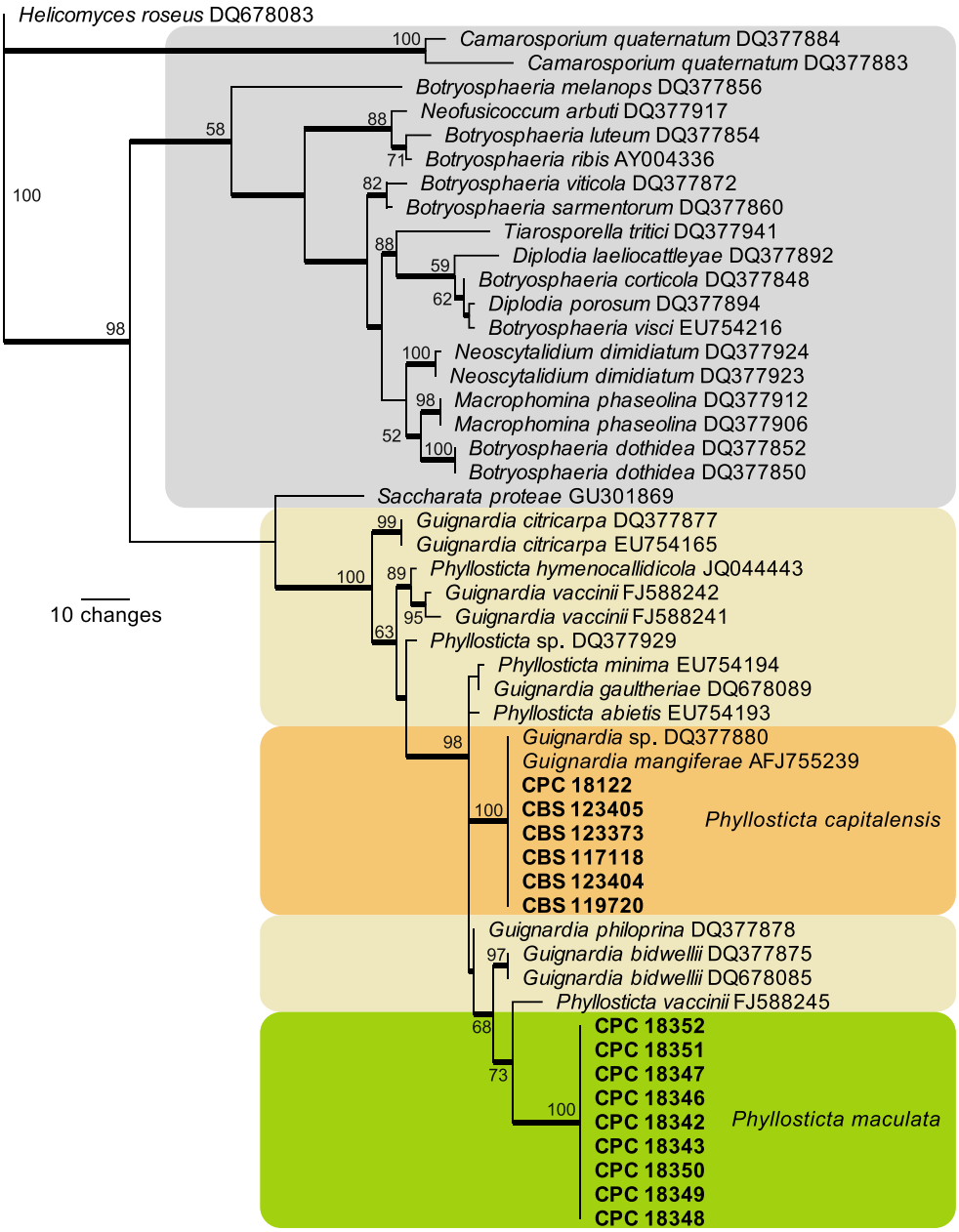

Fig. 2 The first of 30 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the LSU sequence alignment. The scale bar shows 10 changes, and bootstrap support values from 1 000 replicates are shown at the nodes. Novel sequences are printed in bold inside the coloured boxes. Branches present in the strict consensus tree are thickened and the tree was rooted to a sequence of *Helicomyces roseus* (GenBank accession DQ678083). Species specifically treated in this manuscript, as well as representative species from *Phyllosticta*/*Guignardia* and *Botryosphaeriaceae*, are indicated in colour-coded boxes

brownish black or orange-brown lesions, erumpent and subepidermal, globose to subglobose, unilocular, apex papillate or non-papillate with a central, apical ostiole; all walls of *textura angularis*. *Ascomata* 78–129 µm diam, 68–119 µm high, ostiole 9–13 µm diam, upper ascomatal wall composed of two regions: outer region 15–30 µm thick, of 3–6 layers of thick-walled, brown cells and inner region 7–15 µm, of multiple compressed layers of thin-walled, hyaline cells; peridium 14–27 µm thick, consisting of an outer region of 2–3 layers of thick-walled, pale brown cells, and inner region of multiple layers of thin-walled, compressed, hyaline cells. *Asci* bitunicate, ocular chamber prominent, 4–5 µm diam, 8-spored, clavate or pyriform to narrowly ovoid, 45–77×19–27 µm, broadly rounded at the apex, truncate base tapering to a pedicel (up to 15 µm long) attached to basal peridium wall. *Ascospores* oblong, ends obtuse, (12–)14–17(–18)×(7–)8–9(–10) µm, hyaline, aseptate, biseriate, overlapping, guttulate, mucilaginous caps not present once released, but visible in asci, up to 4 µm diam, rather inconspicuous. *Conidiomata* pycnidial, 78–137 µm diam, 65–104 µm high, ostiole central, round, 10–14 µm diam, upper conidiomatal wall composed of two regions: outer region 15–24 µm thick, of 3–5 layers of thick-walled, dark brown cells and inner region 4–11 µm thick, of multiple layers of hyaline, thin-walled cells; peridium 14–23 µm thick, consisting of two regions: outer region of 2–3 layers of pale brown, thick-walled cells, and inner region of multiple layers of thin-walled, compressed, hyaline cells. *Conidiogenous cells* doliiform or subcylindrical, 8–12×4–

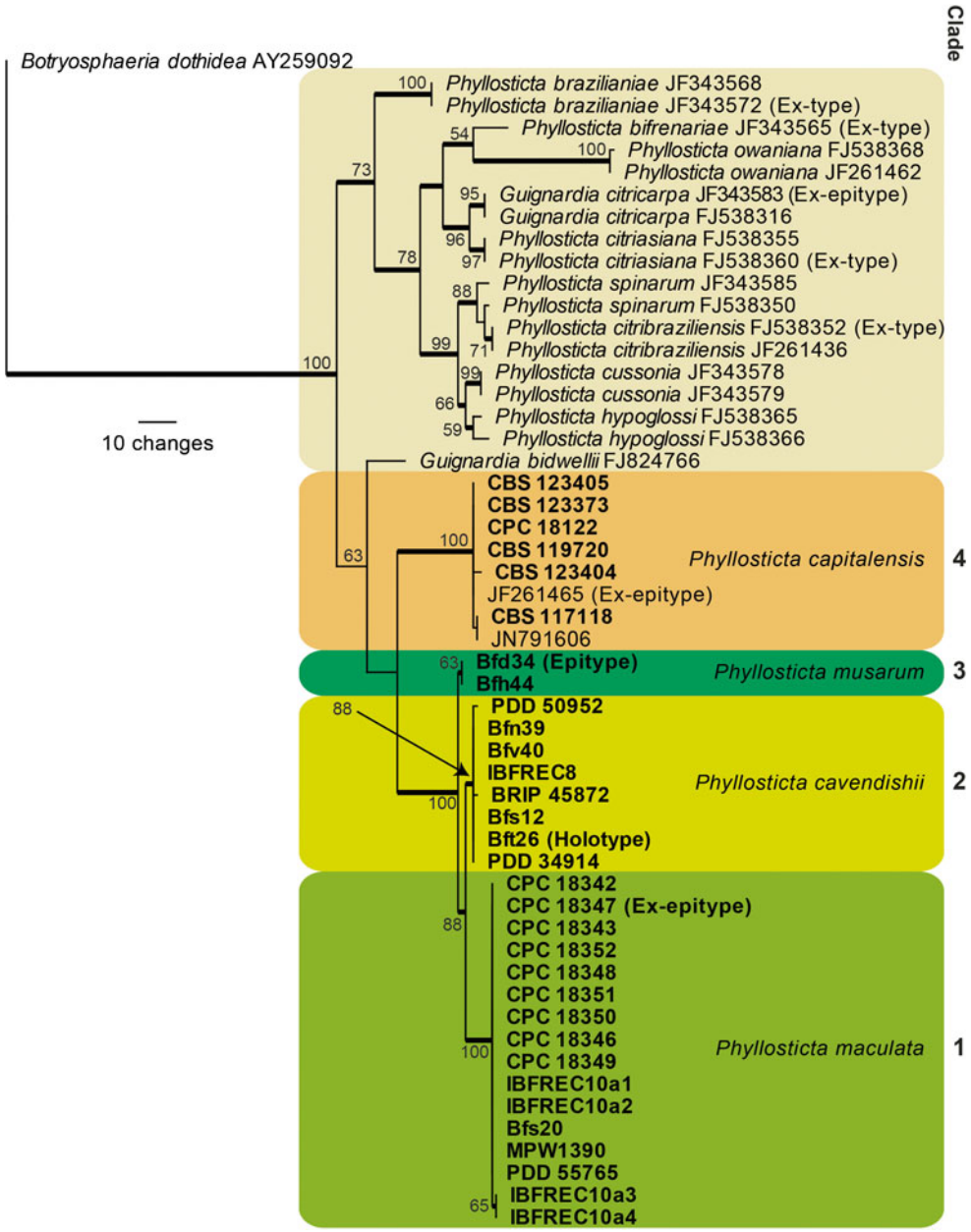

Fig. 3 The first of 20 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes, and bootstrap support values from 1 000 replicates are shown at the nodes. Novel sequences are printed in bold inside the coloured boxes. Branches present in the strict consensus tree are thickened and the tree was rooted to a sequence of *Botryosphaeria dothidea* (GenBank accession AY259092). Species specifically treated in this manuscript, as well as representative species from *Phyllosticta*/*Guignardia*, are indicated in colour-coded boxes

5 μm, solitary, hyaline, proliferating 1–2 times percurrently near apex. *Conidia* oblong or ellipsoid, apex broadly rounded or obtuse, base truncate or obtuse, (12–)(13–16)(–17)×8–9 (–10) μm, solitary, hyaline, aseptate, coarsely guttulate, with a large central guttule, thin- and smooth-walled, surrounded by a mucilaginous sheath 1–3 μm thick, with an apical mucilaginous appendage, straight to curved, (8–)(11–16)(–20) μm long, tapering towards an acute apex. *Spermagonia* 67–127 μm diam, 51–90 μm high. *Spermatia* dumb-bell shaped, 6–7(–8)×(1–)2 μm, hyaline, aseptate, biguttulate.

Culture characteristics Colonies at 25 °C under near-ultraviolet light after 1 month stromatic, coralloid, undulating, superficial, salmon-pink to rosy-buff, with shades of hazel-brown at the edge, or interspersed within the colony, very slow growing, lacking aerial mycelium.

Specimens examined **Taiwan**, TBRI, Pingtung, on leaves of *Musa* sp., 24 Mar. 2010, CP Chao, **holotype** CBS H-20918, **isotype** BRIP 55419 (anamorph present). **Federated States of Micronesia**, Yap, on leaves of *Musa* sp. 30 May 1986, G.V.H. Jackson, PDD 50952. **Tonga**, Tongatapu, on leaves of *Musa* sp., 8 Aug. 1975, S.R. Pennycook, PDD 34914 (both anamorph and teleomorph present).

Note All isolates studied from *Musa* cv. Cavendish cluster in this clade, indicating that this species is the cause of freckle disease on this banana subgroup.

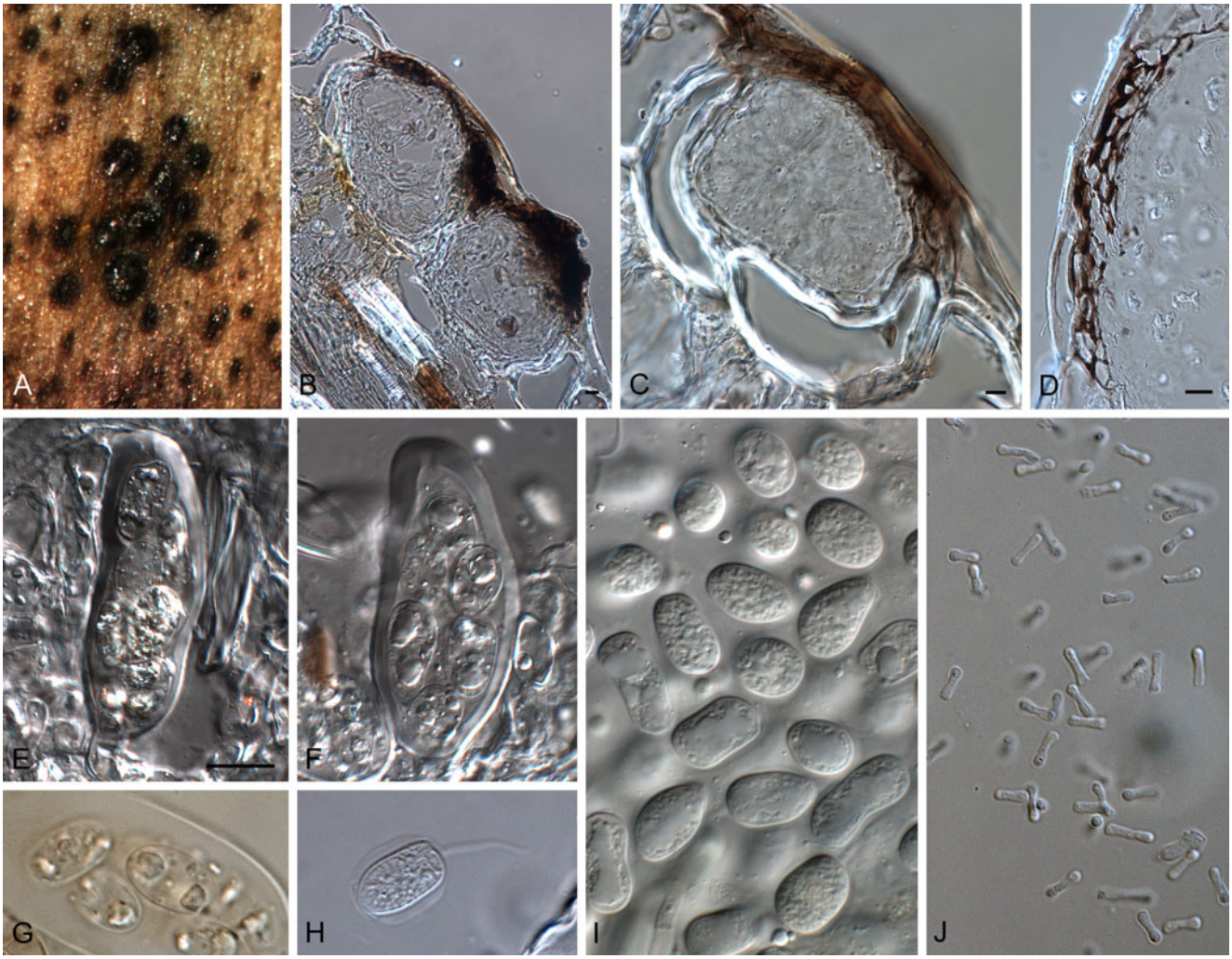

Fig. 4 *Phyllosticta cavendishii.* **a**. Fruiting bodies on leaf. **b**. Vertical section through two adjoining ascomata. **c**. Vertical section through spermatogonium. **d**. Section through upper wall of pycnidium. **e**–**g**. Asci with ascospores. **h**, **i**. Conidia. **j**. Spermatia. (**a**–**g**=PDD50952, **h**–**j**=CBS H-20918). Scale bars=10 μm

Phyllosticta maculata M.H. Wong & Crous, **nom. nov.** Fig. 5

≡ *Guignardia musae* Racib., *Bull. int. Acad. Sci. Lett. Cracovie,* Cl. Sci. math. Nat. Sér. B, sci. nat. **3**: 388 (1909), non *Phyllosticta musae* F. Stevens & E. Young, 1925.

= *Phomatospora musae* Yen, *Bull. Soc. mycol. Fr.* **88**: 224 (1972).

MycoBank MB564748.

Etymology Named after the characteristic freckle leaf disease of *Musa* spp., which is most commonly associated with this species.

Leaf spots on adaxial leaf surface, raised, rough, circular to irregular. *Fruiting bodies* black, solitary or aggregated, on patches of orange-brown lesions, erumpent and subepidermal, globose to subglobose, unilocular, apex papillate or non-papillate with central apical ostiole; wall layers all of *textura angularis*. *Ascomata* 84–187 μm diam, 71–170 μm high, ostiole 12–15 μm diam, upper ascomatal wall composed of two regions: outer region 13–25 μm thick, of 3–6 layers of thick-walled, brown cells and inner region 6–20 μm, of multiple compressed layers of thin-walled, hyaline cells; peridium 11–29 μm thick, consisting of an outer region of 2–3 layers of thick-walled, pale brown cells, and inner region of multiple layers of thin-walled, compressed, hyaline cells. *Asci* bitunicate, 8-spored, clavate to pyriform or narrowly ovoid, with visible ocular chamber, 3–5 μm diam, 47–85×18–31 μm, apex broadly rounded, base truncate, tapering to a pedicel attached to basal peridium. *Ascospores* oblong or ovoid, with obtuse ends, (17–)19–23(–24)×(8–)9–11(–13) μm, hyaline, aseptate, biseriate, overlapping, guttulate, mucilaginous caps mostly absent, inconspicuous, 3–4 μm diam when present. *Conidiomata* pycnidial, 84–137 μm diam, 68–132 μm high, ostiole central, round, 9–12 μm diam, upper conidiomatal wall composed of two regions: outer region 11–23 μm thick, of 3–5

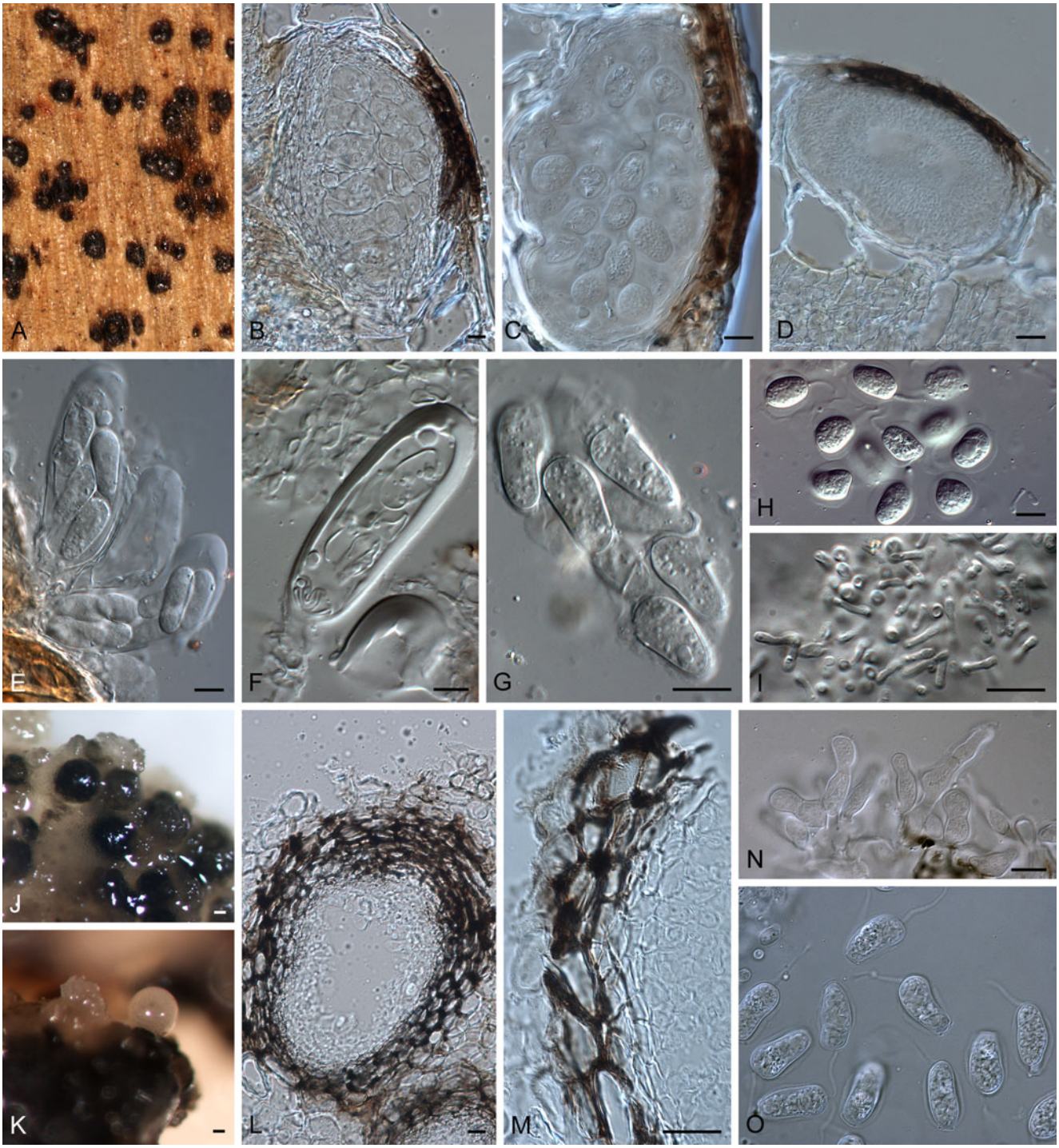

Fig. 5 *Phyllosticta maculata*. **a**. Fruiting bodies on leaf. **b**. Vertical section through ascoma, showing developing asci. **c**. Vertical section through pycnidium. **d**. Vertical section through spermatogonium. **e**–**g**. Asci and ascospores. **h**. Conidia, **i**. Spermatia. **j**–**o**. Sporulation on OA. **j**, **k**. Conidiomata oozing spore masses. **l**. Vertical section through pycnidium. **m**. Vertical section through pycnidial wall. **n**. Conidiogenous cells. **o**. Conidia with mucilaginous sheaths and apical appendages. (**a**–**i**=CBS H-20920, **j**–**o**=BRIP 46622). Scale bars=10 μm, except **j**, **k**=20 μm

layers of thick-walled, dark brown cells and inner region 3–11 μm thick, of multiple layers of hyaline, thin-walled cells; peridium 9–18 μm thick, consisting of two regions: outer region of 2–3 layers of pale brown, thick-walled cells, and inner region of multiple layers of thin-walled, compressed, hyaline cells. *Conidiogenous cells* subcylindrical to doliiform, 9–12×4–5 μm, solitary, hyaline, proliferating 1–2 times percurrently near apex. *Conidia* oblong or obovoid to subclavate,

apex broadly rounded or obtuse, base truncate, becoming somewhat obtuse with age, (15–)16–19(–21)×(9–)10–12(–13) μm, solitary, hyaline, aseptate, coarsely guttulate with a large central guttule, thin- and smooth-walled, surrounded by a mucilaginous sheath 2–4(–6) μm thick, and apical mucilaginous appendage, straight to curved, (12–)15–26(–37) μm long, tapering towards an acute apex. *Spermagonia* 78–115 μm diam, 63–99 μm high. *Spermatogenous cells* hyaline, cylindrical, 10–15×3–4 μm, with visible periclinal thickening. *Spermatia* cylindrical with acutely rounded ends, becoming dumb-bell shaped with obtuse ends with age, (10–)11–13(–14)×(1–)2 μm, hyaline, aseptate, biguttulate.

Morphology on OA (BRIP 46622) Pycnidia black, reflective, solitary or aggregated, superficial, globose to subglobose, exuding a colourless, slimy conidial mass. *Pycnidia* (75–)91–141(–171)×(92–)107–152(–174) μm, ostiole central, 8–10 μm diam, pycnidial wall composed of two regions: outer region 12–38 μm thick of several layers of thick-walled dark brown cells of *textura angularis* to *globularis*, and inner region 7–19 μm thick of multiple, thin-walled, hyaline cells, *Conidiogenous cells* doliiform or subcylindrical, frequently with a mucoid coating, 6–15×3–6 μm, solitary, hyaline, proliferating 1–2 times percurrently near apex. *Conidia* oblong or obovoid, apex obtuse, base truncate or obtuse, (18–)20–23(–25)×(8–)9–10(–11) μm, solitary, hyaline, aseptate, coarsely guttulate with a large central guttule, thin- and smooth-walled, surrounded by a mucilaginous sheath, 1–3 μm thick, an apical appendage, straight to curved, (18–)20–33(–50) μm long, that tapers towards an acute apex.

Culture characteristics Colonies at 25 °C under near-ultraviolet light after 1 month stromatic, coralloid, undulating, extending, superficial, hazel-brown and violaceous black, interspersed with rosy-buff sectors, very slow growing, lacking aerial mycelium.

Specimens examined **American Samoa**, Manua, on leaves of *Musa* sp., 27 Aug. 1989, E.H.C. McKenzie, PDD 55765 (both anamorph and teleomorph present). **Australia**, Queensland, Cairns, Sugar World, on leaves of *Musa* sp., 29 July 2009, M. Berridge, **epitype designated here** CBS H-20919 (**isoepitype** BRIP 55425), culture ex-epitype CPC 18347=CPC 19953=CBS 132581. Northern Territory, on leaves of *Musa* sp., 14 June 2005, P.M. Stephens, BRIP 46622, culture CPC 18343=CBS 132579. **Indonesia**, Bogor, on leaves of *Musa acuminata*, M. Raciborski, holotype of *G. musae* Racib., KRA 063561; Sulawasi, on leaves of *Musa* sp., 30 July 2005, I. Buddenhagen, CBS H-20920. **Papua New Guinea**, Daru, on leaves of *Musa* sp., 19 July 2005, M.P. Weinert, CBS H-20921 (anamorph present).

Notes The name *Guignardia musae* Racib. has been incorrectly linked to the anamorph *Phyllosticta musarum* (Cooke) van der Aa (Van der Aa 1973; Punithalingam 1974). Van der Aa (1973) did not examine type material, but based the anamorph/telemorph connection on observations made from secondary collections, and Punithalingam (1974) based his conclusions on cultures from North Borneo (IMI 147360) and Jamaica (IMI 165061a). The latter's description of the colonies as "greenish grey" and homothallic on OA, clearly distinguished these strains from authentic cultures of *P. maculata*, which are hazel-brown to violaceous black, interspersed with sectors of rosy-buff. It is difficult to cultivate *P. maculata*, and it is probable that Punithalingham isolated a common *Phyllosticta* endophyte that also occur on *Musa* leaves with freckle disease symptoms. This is further supported by the different ascus and ascospore morphology (IMI 147360, figs 26, 27) (Punithalingam 1974) to that of *P. maculata*.

Phomatospora musae (Yen 1972), which was described from Cavendish banana in Taiwan, is listed here as a synonym based on ascus and ascospore morphology and dimensions. However, the *Phyllosticta* anamorph described by Yen (1972) does not compare to that of *P. maculata*, and we suspect that this was not the anamorph of *Phomatospora musae*, but represented a co-occurring species. As we have been unable to locate the type material of *Phomatospora musae* in Paris (PC), the status of this unnamed *Phyllosticta* anamorph remains unresolved.

Phyllosticta musae-sapientium Gonz. Frag. & Cif., *Boln Real Soc. Españ. Hist. Nat.*, Biologica **27**: 170 (1927).

Note This species represents the *Asteromella* state of *Mycosphaerella musae* (Arzanlou et al. 2008) (*Asteromella paradisiaca*), which occurs on the same material (van der Aa and Vanev 2002).

Phyllosticta musarum (Cooke) Van der Aa, *Stud. Mycol* **5**: 72 (1973). Fig. 6
≡ *Sphaeropsis musarum* Cooke, *Grevillea* **8**: 93 (1880).
≡ *Phoma musae* (Cooke) Sacc., *Syll. fung.* (Abellini) **3**: 163 (1884).
≡ *Macrophoma musae* (Cooke) Berl. & Vogl., *Atti Soc. Veneto-Trent. Sci. Nat.* **10**(1): 187. 1886.
≡ *Phyllostictina musarum* (Cooke) Petr., *Annls mycol.* **29**(3/4): 268 (1931).

Leaf spots on adaxial leaf surface, raised, rough in texture, circular or irregular. *Fruiting bodies* black, solitary or aggregated in tight clusters within a black stroma, erumpent and subepidermal, globose to subglobose, unilocular, apex papillate or non-papillate, with a central apical ostiole; all walls of *textura angularis*. *Ascomata* 102–118 μm diam, 70–102 μm high, ostiole central, 9–13 μm diam, upper ascomatal wall composed

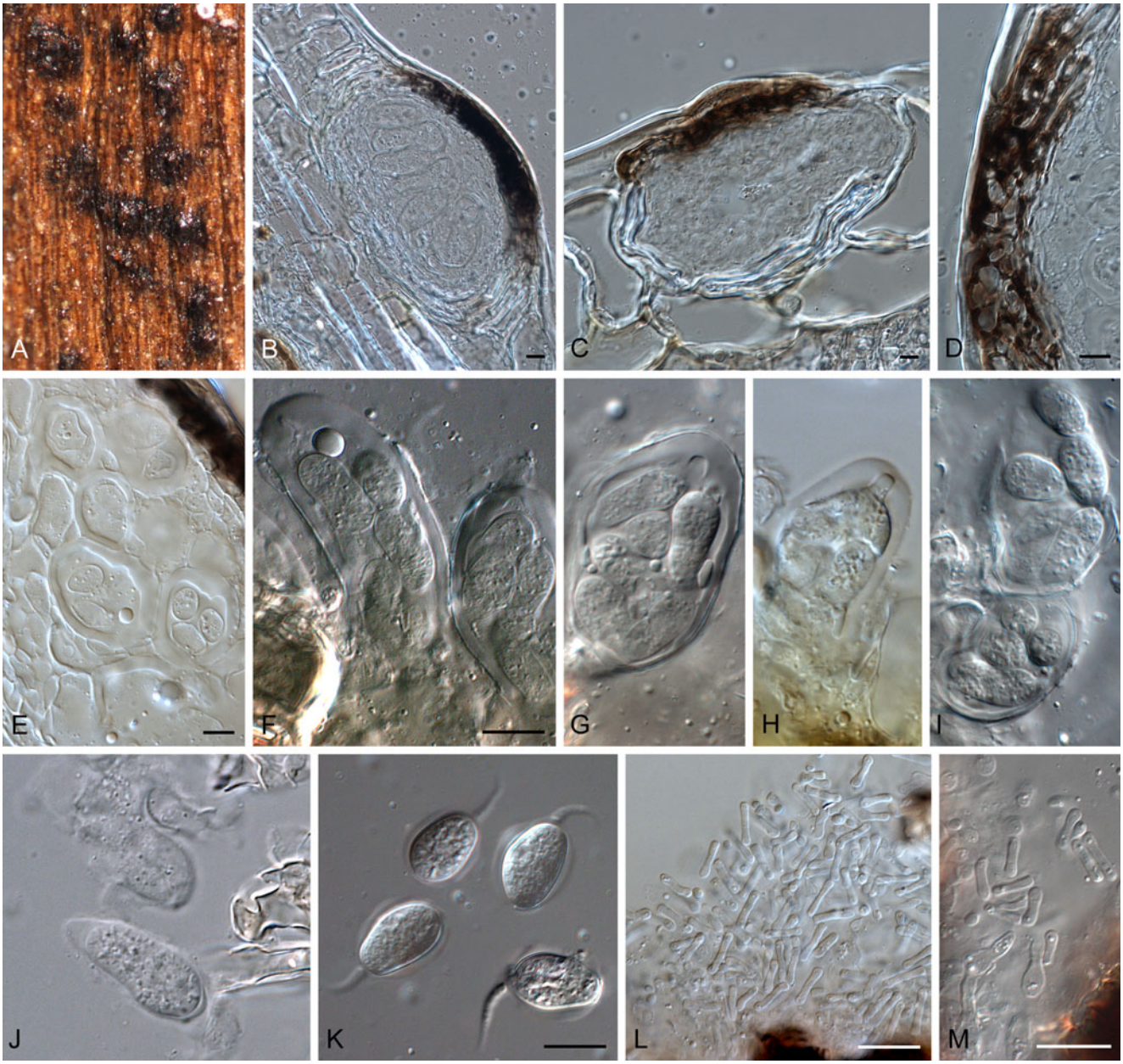

Fig. 6 *Phyllosticta musarum*. **a**. Fruiting bodies on leaf. **b**. Vertical section through ascoma, showing developing asci. **c**. Vertical section through spermatogonium. **d**. Vertical section through upper wall of ascoma. **e**. Vertical section through ascoma, showing developing asci. **f**–**i**. Asci with ascospores. **j**. Ascospore with apical mucilaginous appendage. **k**. Conidia with mucilaginous sheaths and apical appendages. **l**. Spermatia. **m**. Spermatogenous cells giving rise to spermatia. (**a**–**m**=CBS H-20923). Scale bars=10 μm

of two regions: outer region 10–24 μm thick, of 3–6 layers of thick-walled, dark brown cells; inner region 4–16 μm thick, of multiple layers of thin-walled, hyaline cells; peridium 16–36 μm thick, consisted of an outer region of thick-walled, pale brown cells, and an inner region of thin-walled, compressed, hyaline cells. *Asci* bitunicate, 8-spored, clavate or pyriform, 51–78×20–30 μm, broadly rounded at apex, with visible ocular chamber, 3–5 μm diam; truncate at base, tapering to a pedicel attached to the basal peridium. *Ascospores* oblong with obtuse ends, (14–)16–18(–21)×7–8(–9) μm, hyaline, aseptate, biseriate, overlapping, guttulate, with prominent flame-like mucilaginous caps present at both ends, 3–4 μm diam, up to 7 μm long. *Conidiomata* pycnidial, 69–118 μm diam, 52–80 μm high, ostiole central, 8–14 μm diam; upper conidiomatal wall composed of two regions: outer region 11–21 μm thick, of 3–5 layers of thick-walled, dark brown cells; inner region 3–13 μm thick, of multiple layers of thin-walled, hyaline cells; peridium 14–29 μm thick, consisting of an outer region of 2–3 layers of thick-walled, pale brown cells, and an inner region of multiple layers of thin-walled, compressed, hyaline cells. *Conidiogenous cells* subcylindrical

to allantoid, 10–15×3–5 μm, solitary, hyaline, proliferating 1–2 times percurrently near apex. *Conidia* oblong, obovoid or ellipsoid, apex broadly rounded or obtuse, base truncate, becoming somewhat obtuse with age, (12–)13–16(–20)×(7–)9–10(–11) μm, solitary, hyaline, aseptate, coarsely guttulate, with a large central guttule, thin- and smooth-walled, surrounded by a mucilaginous sheath 1–3 μm thick, and mucilaginous apical appendage, straight to curved, (12–)14–18(–20) μm long, tapering towards an acute apex. *Spermagonia* 54–111 μm diam, 46–78 μm high. *Spermatia* dumb-bell shaped, (6–)7–8(–9)×(1–)2 μm, hyaline, aseptate, biguttulate.

Specimens examined **India**, Belgaum, on leaves of *Musa paradisiaca*, 1879, J. Hobson, **holotype** K(M 173237); Tamil Nadu, on leaves of *Musa* sp., 18 June 2010, R. Thangavelu, **epitype designated here** CBS H-20922 (**isoepitype** BRIP 55434). **Thailand**, Nakorn Ratchasima, on leaves of *Musa* sp., 8 Sept. 2010, M. Kanjanamaneesathian, CBS H-20923.

Note Conidia on the holotype specimen closely match that of other collections from India (not on Cavendish banana) that cluster in the clade labelled *P. musarum* (Fig. 3), having conidia 13–20×8–11 μm in size, with a 1–3 μm thick sheath, and apical appendages up to 20 μm long.

Guignardia musicola N.F. Wulandari, L. Cai & K.D. Hyde, *Cryptogam. Mycol*. **31**: 412 (2010).

Notes The description of *G. musicola* contains elements of two different species, as the teleomorph (cylindro-clavate asci, and ellipsoid to clavate ascospores, figs 23–25) does not belong to the anamorph (figs 26–28), which represents the common endophyte, *Phyllosticta capitalensis* (Glienke et al. 2011), as does the ex-type culture CBS 123405. We therefore consider this taxon as a *nomen confusum*.

Guignardia stevensii Wulandari & K.D. Hyde, *Cryptogam. Mycol*. **31**: 406 (2010). (nom. nov.).
≡ *Guignardia musae* F. Stevens, *Bull. Bernice P. Bishop Mus. Honolulu* **19**: 101 (1925).

Note Guignardia stevensii, which is associated with speckle disease symptoms of banana in Hawaii (BISH 596860), is distinct from other taxa on *Musa* in that it has inequilaterally ellipsoid ascospores that are flattened on the one side (14–17×5–6 μm; Wulandari et al. 2010). The connection with the purported anamorph, *P. musae* F. Stevens & E. Young, has not been proven in culture. Punithalingam (1974) examined the type specimen and concluded that there were two different *Phyllosticta* species present, the taxonomy of which in the absence of cultures could not be resolved.

Guignardia sydowiana Trotter, in Saccardo, *Syll. Fung*. **24**: 788 (1928).
≡ *Guignardia musae* Syd. & P. Syd., *Annls mycol*. **10**: 80 (1912) (*nom. inval*.)

Notes Type material of this species (on *Musa*, Democratic Republic of Congo, S 10753) was examined by Wulandari et al. (2010), but no conclusion could be reached as the material was in poor condition.

Discussion

The main aim of the present study was to determine which *Phyllosticta* species caused freckle disease of banana, and if any of the three phylogenetic clades delineated in this study (Fig. 3) correlated to names previously introduced for species occurring on banana. To date literature has chiefly associated freckle disease with *Guignardia musae*, and its purported anamorph *Phyllosticta musarum* (Jones 2000). However, a recent study by Wulandari et al. (2010) showed that the situation was more complicated, and that several species were present on banana, though not necessarily associated with freckle disease. The latter study, however, lacked molecular data, making conclusions difficult. Several workers have erroneously linked the common endophyte, *P. capitalensis*, with *Guignardia* teleomorphs occurring on banana, assuming them to be the same species (Yen 1972; Punithalingam 1974; Wulandari et al. 2010). Given the difficulty experienced in this study to isolate and cultivate *Phyllosticta* species associated with freckle disease, and the ease to obtain cultures of the common co-occurring endophytic *P. capitalensis* (Okane et al. 2003; Pandey et al. 2003; Glienke et al. 2011), it is reasonable to assume that some species previously described as new probably represent *P. capitalensis* (Baayen et al. 2002; Wulandari et al. 2010).

Since freckle disease was first reported from Hawaii and the Philippines, the disease has been found in many banana producing countries including: Australia, Papua New Guinea (Jones and Alcorn 1982), Taiwan (Hwang 1991), Sri Lanka (Abayasekera et al. 1993), China (Pu et al. 2008), India, Malaysia, Indonesia, Thailand, Vietnam and the Pacific Islands (Jones 2000). Outside the Asia-Pacific region, only the Democratic Republic of Congo and the Dominican Republic have reported the presence of this disease (Wardlaw 1961). The pathogen may have spread later to Central America (CABI 1990). All these records have attributed the disease to a single organism, *G. musae*.

The fungus may infect the banana leaf during any stage of plant growth, and the disease severity increases over time. Disease symptoms on the leaf initially appear as individual reddish brown spots, which later turn black and coalesce to form patches or streaks of lesions (Jones 2000). The

protruding fruiting bodies from the lesions give the infected surface a sandpaper-like texture. Infected leaves senesce prematurely, causing significant yield loss (Tsai et al. 1993). The pathogen may infect fruit as early as few weeks after bud emergence, and the disease progresses as the fruit matures (Corcolon and Raymundo 2008). Freckle spots on fruit appear reddish brown to black with a dark green water-soaked halo, and spots may completely cover the fruit at harvest (Punithalingam and Holliday 1975). Although spots found on the fruit surface do not go deeper than the exocarp (Ocfemia 1927), their presence would adversely affect the cosmetic value of the fruits, rendering them unfit for export.

A recent morphological study on *Guignardia/Phyllosticta* species on banana by Wulandari et al. (2010) reported three species. Although *P. maculata* (as *G. musae* Racid.) was acknowledged as one of the main causal agents of freckle disease, it was distinguished morphologically from *G. stevensii* by the inequilaterally ellipsoid ascospores of the latter, rejecting the synonymy proposed by Punithalingam (1974). Wulandari et al. (2010) introduced a third species from banana in Thailand, *G. musicola*, although we regard this species as a *novem confusum* for reasons discussed above.

Results from our study have confirmed through morphology and molecular data that at least three closely related, but different species of *Phyllosticta* are commonly associated with freckle disease. *Phyllosticta cavendishii* is introduced as a new species for the taxon that has strong pathogenicity towards commonly grown Cavendish banana cultivars of the AAA genomic group as well as cultivars of AAB and ABB genomic groups, while epitypes are designated for *P. maculata* (a nom. nov. for *G. musae*), and *P. musarum* which are presently known to occur on banana cultivars of AAB and ABB genomic groups.

Based on the apomorphic traits of asci (clavate to pyriform and stipitate) and oblong ascospores, it is clear that these species shared a common ancestor. Of interest, is the difference in host cultivar range and geographic distribution. Although these species can infect the non-Cavendish cultivars, only *P. cavendishii* is capable of infecting the Cavendish cultivars. Furthermore, while *P. maculata* and *P. cavendishii* have wide global distribution, this is not the case for *P. musarum*, which appears to have a more restricted distribution. *Phyllosticta maculata*, a new name introduced for *G. musae*, appears to be a relatively common species, and is presently known from Australia, Malaysia, Indonesia, Papua New Guinea, Philippines and the South Pacific Islands.

The newly described species, *Phyllosticta cavendishii,* is interesting in that all specimens thus far obtained from *Musa* cv. Cavendish seem to cluster in this clade (Fig. 3), indicating a strong host-cultivar preference. All incidences of freckle disease on Cavendish appear to be associated with *P. cavendishii*. Although it appears to be widely distributed along with Cavendish cultivars, this species is presently known from countries such as Ceylon, East Timor, Hawaii, India, Indonesia, Malaysia, Philippines, Taiwan, the South Pacific Islands, and Vietnam.

For many years *Phyllosticta musarum* (from *Musa paradisiaca*, India) was incorrectly assumed to represent the anamorph of *Guignardia musae* (= *P. maculata*). Morphologically it correlates to specimens that reside in the clade labelled *P. musarum* (Fig. 3), that also originate from India, as well as Thailand.

This study has demonstrated that in addition to classical morphological methods, modern molecular techniques are required to resolve the identity of these closely related taxa, as well as to understand the phylogenetic relationship of these species within the genus *Phyllosticta*. The importance of molecular data in delineating cryptic taxa in species complexes was highlighted by Crous and Groenewald (2005). The discriminating power of molecular techniques has enabled differentiation of cryptic species that are morphologically similar, but genetically distinct, as found in the present study. The fact that these species differ in host cultivar range and geographic distribution adds further value to using this method of taxon discrimination, as it heralds important implications for trade in agricultural produce. With the ever-increasing generation of DNA sequence data for novel and existing species of plant pathogenic fungi, further revision and reclassification of taxa are anticipated.

Key to species causing freckle disease of banana*

1. Ascospores inequilaterally ellipsoid, flattened on one side, 14–17×5–6 μm …. ***G. stevensii***
1. Ascospores oblong to ovoid with obtuse ends …… 2
2. Ascospores (17–)19–23(–24)×(8–)9–11(–13) μm; conidia (15–)16–19(–21)×(9–)10–12(–13) μm …… ***P. maculata***
2. Ascospore and conidial mean range smaller than above …….. 3
3. Ascospores (14–)16–18(–21)×7–8(–9) μm; occurring on non-Cavendish cultivars ……. ***P. musarum***
3. Ascospores shorter and wider than above, (12–)14–17(–18)×(7–)8–9(–10) μm; occurring on Cavendish and non-Cavendish cultivars …… ***P. cavendishii***

**In vivo*.

Acknowledgements MHW is supported by a postgraduate scholarship from the Sarawak State Government, Malaysia. The Mort Johnston Professional Development scholarship is acknowledged for financial support to MHW during part of this study conducted at

CBS. We are grateful to many colleagues and researchers in various parts of the world who collected specimens for this study, and to the curator of Kew, who made specimens available for comparison. We also thank the technical staff, A. van Iperen (cultures), and M. Vermaas (photo plates) for their invaluable assistance.

References

Abayasekera C, Karunaratne AM, Adikaram, NKB (1993) Freckle disease of banana. In: Champ BR, Highlay CE, Johnson GI (eds) Proceedings of an International Conference on Postharvest Handling of Tropical Fruits, Chiang Mai, Thailand. ACIAR Proceedings No. 50, pp 458–459

Arzanlou M, Groenewald JZ, Gams W, Braun U, Shin H-D, Crous PW (2007) Phylogentic and morphotaxonomic revision of *Ramichloridium* and allied genera. Stud Mycol 58:57–93

Arzanlou M, Groenewald JZ, Fullerton RA, Abeln ECA, Carlier J, Zapater MF, Buddenhagen IW, Viljoen A, Crous PW (2008) Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. Persoonia 20:19–37

Arzanlou M, Crous PW, Zwiers LH (2010) Evolutionary dynamics of mating-type loci of *Mycosphaerella* spp. occurring on banana. Eukaryot Cell 9:164–172

Baayen RP, Bonants PJM, Verkley G, Carroll GC, van der Aa HA, de Weerdt M, van Brouwershaven IR, Schutte GC, Maccheroni W Jr, Glienke de Blanco C, Azevedo JL (2002) Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). Phytopathology 92:464–477

CABI (1990) *Guignardia musae* Racib. Distribution Maps of Plant Diseases, No. 263. CAB International, Wallingford, UK

Carpenter CW (1919) Banana freckle or black spot disease. Hawaii Agr Exp Stat 1918:36–40

Corcolon BM, Raymundo AD (2008) Estimating yield losses in banana due to freckle disease caused by *Phyllosticta musarum* (Cke.) van der Aa. Philippine J Crop Sci 33(2):75–85

Crous PW, Groenewald JZ (2005) Host, species and genotypes: opinions versus data. Australas Plant Pathol 34:463–470

Crous PW, Gams W, Stalpers JA, Robert V, Stegehuis G (2004) MycoBank: an online initiative to launch mycology into the 21st century. Stud Mycol 50:19–22

Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Phillips AJL, Alves A, Burgess T, Barber P, Groenewald JZ (2006) Phylogenetic lineages in the *Botryosphaeriaceae*. Stud Mycol 55:235–253

Crous PW, Verkleij GJM, Groenewald JZ, Samson RA (eds) (2009) Fungal biodiversity. CBS Laboratory Manual Series 1. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

Crous PW, Summerell BA, Shivas RG, Romberg M, Mel'nik VA, Verkley GJM, Groenewald JZ (2011) Fungal planet description sheets: 92-106. Persoonia 27:130–162

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious v5.4, Available from http://www.geneious.com/

Glienke C, Pereira OL, Stringari D, Fabris J, Kava-Cordeiro V, Galli-Terasawa L, Cunnington J, Shivas RG, Groenewald JZ, Crous PW (2011) Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. Persoonia 26:47–56

Hawksworth D, Crous PW, Redhead SA, Reynolds DR, Samson RA, Seifert KA, Taylor JW, Wingfield MJ (2011) The Amsterdam Declaration on fungal nomenclature. IMA Fungus 2(1):105–112

Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol 42:182–192

Hwang SC (1991) Status of banana diseases in Taiwan. In: Proceedings of a Regional Technical Meeting on Disease Affecting Banana and Plantain in Asia and the Pacific, Brisbane, Australia. INIBAP-ASPNET, Los Banos (PHL), pp 73–83

Jones DR (2000) Fungal diseases of the foliage. In: Jones DR (ed) Diseases of banana, abaca and enset. CABI Publishing, CAB International, UK, pp 37–141

Jones DR, Alcorn JL (1982) Freckle and black Sigatoka diseases of banana in far North Queensland. Australas Plant Pathol 11:7–9

Kuo KC, Hoch HC (1996) The parasitic relationship between *Phyllosticta ampelicida* and *Vitis vinifera*. Mycologia 88(4):626–634

Lee HA (1922) Banana freckle in the Philippine Islands. Phytopathology 12:101–102

Ocfemia GO (1927) *Macrophoma musae* (Cke.) Berl. and Vogl. and *Phoma musae* Carpenter. Philipp Agric 15:467–469

Okane I, Lumyong S, Nakagiri A, Ito T (2003) Extensive host range of an endophytic fungus, *Guignardia endophyllicola* (anamorph: *Phyllosticta capitalensis*). Mycoscience 44:353–363

Pandey AK, Reddy MS, Suryanarayanan TS (2003) ITS-RFLP and ITS sequence analysis of a foliar endophytic *Phyllosticta* from different trees. Mycol Res 107(4):439–444

Pu J, Xie Y, Zhang X, Qi Y, Zhang C, Liu X (2008) Preinfection behaviour of *Phyllosticta musarum* on banana leaves. Australas Plant Pathol 37:60–64

Punithalingam E (1974) Studies on Sphaeropsidales in culture II. Mycological Papers 136:1–63

Punithalingam E, Holliday P (1975) *Guignardia musae*. CMI descriptions of pathogenic fungi and bacteria No. 467. CABI Publishing, CAB International, UK

Rayner RW (1970) A mycological colour chart. CMI and British Mycological Society, Kew, Surrey, England

Rehner SA, Samuels GJ (1994) Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequence. Mycol Res 98(6):625–634

Rodrigues KF, Sieber TN, Grünig CR, Holdenrieder O (2004) Characterization of *Guignardia mangiferae* isolated from tropical plants based on morphology, ISSR-PCR amplifications and ITS1-5.8S-ITS2 sequences. Mycol Res 108(1):45–52

Swofford DL (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods) version 4. Sinauer Associates, Sunderland, Massachusetts

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol BiolEvol 28(10):2731–2739

Tsai YP, Chen HP, Liu, SH (1993) Freckle disease of banana in Taiwan. In: Proceedings of the International Symposium on Recent Developments in Banana Cultivation Technology, Pingtung, Taiwan. INIBAP-ASPNET, Los Banos (PHL), pp 298–307

van der Aa HA (1973) Studies in Phyllosticta I. Stud Mycol 5:1–110

van der Aa HA, Vanev S (2002) A revision of the species described in *Phyllosticta*. CBS, Utrecht, The Netherlands

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172(8):4238–4246

Wang X, Chen G, Huang F, Zhang J, Hyde KD, Li H (2012) *Phyllosticta* species associated with citrus disease in China. Fungal Diversity 52(1):209–224

Wardlaw CW (1961) Freckle or black spot disease. In: Banana diseases including plantain and abaca. Longmans, Green and Co. Ltd., pp 428–430

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols A guide to methods and applications. Academic, San Diego, pp 315–322

Wikee S, Udayanga D, Crous PW, Chukeatirote E, McKenzie EHC (2011) *Phyllosticta* – an overview of current status of species recognition. Fungal Diversity 51(1):43–61

Wingfield MJ, De Beer WZ, Slippers B, Wingfield BD, Groenewald JZ, Lombard L, Crous PW (2012) One fungus, one name promotes progressive plant pathology. Mol Plant Pathol. doi:10.1111/J.1364-3703.2011.00768.X

Wulandari NF, To-Anun C, Hyde KD, Duong LM, de Gruyter J, Meffert JP, Groenewald JZ, Crous PW (2009) *Phyllosticta citriasiana* sp. nov., the cause of Citrus tan spot of *Citrus maxima* in Asia. Fungal Diversity 34:23–39

Wulandari NF, To-Anun C, Cai L, Abd-Elsalam KA, Hyde KD (2010) *Guignardia*/*Phyllosticta* species on banana. Cryptogam Mycol 31 (4):403–418

Yen J-M (1972) Étude sur les champignons parasites du sud-est Asiatique. XVIII. Maladies des taches foliaires des bananiers provoquées, á Formose, par deux nouveaux champignons. III. Bulletin de la Société mycologique de France 88:221–225