Phyllosticta citriasiana sp. nov., the cause of Citrus tan spot of *Citrus maxima* in Asia

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Guignardia citricarpa, the causal agent of Citrus Black Spot, is subject to phytosanitary legislation in the European Union and the U.S.A. This species is frequently confused with *G. mangiferae*, which is a non-pathogenic, and is commonly isolated as an endophyte from citrus fruits and a wide range of other hosts. Recently, necrotic spots similar to those caused by *G. citricarpa* were observed on fruit of *Citrus maxima* intercepted in consignments exported from Asia. In these spots, pycnidia and conidia of a *Guignardia* species closely resembling *G. citricarpa* were observed, and therefore measures were taken for the consignments in line with the European Union legislation for *G. citricarpa*. To determine the identity of the causal organism on this new host, fungal isolates were subjected to DNA sequence analysis of the internal transcribed spacer region (ITS1, 5.8S, ITS2), translation elongation factor 1-alpha (TEF1) and actin genes. A combined phylogenetic tree resolved three species correlating to *G. citricarpa*, *G. mangiferae* and a previously undescribed species, *Phyllosticta citriasiana* sp. nov., closely related to *G. citricarpa*. Morphologically *P. citriasiana* can be distinguished from *G. citricarpa* by having larger conidia, longer conidial appendages, and in not producing any diffuse yellow pigment when cultivated on oatmeal agar (OA). Furthermore, it is distinguishable from *G. mangiferae* by having smaller conidia, with a narrower mucoid sheath. In culture, colonies of *P. citriasiana* can also be distinguished from *G. citricarpa* and *G. mangiferae* by being darker shades of grey and black on OA, malt extract agar (MEA), potato-dextrose agar, and cornmeal agar. Furthermore, cultures of *P. citriasiana* achieved optimal growth after 2 weeks at 21–27°C, and ceased to grow at 30–33°C. In contrast, colonies of *G. citricarpa* and *G. mangiferae* achieved optimal growth at 27–30°C, and ceased to grow at 30–36°C. Colonies of *P. citriasiana* also grew faster than those of *G. citricarpa* and *G. mangiferae* on OA and MEA. *Phyllosticta citriasiana* appears to be a harmful pathogen of *Citrus maxima*, causing a tan spot on fruit, underlining the need for further surveys and research to determine its distribution and host range.

Key words: Citrus Black Spot, *Guignardia*, quarantine, molecular phylogeny, *Phyllosticta*, systematics.

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Introduction

Where known, anamorphs of *Guignardia* (*Botryosphaeriaceae*) reside in *Phyllosticta* (Van der Aa, 1973; Punithalingam, 1974; Van der Aa and Vanev, 2002). *Guignardia* species have often been recorded as endophytes, plant pathogens and saprobes (Baayen *et al*., 2002; Glienke-Blanco *et al*., 2002; Rodrigues *et al*., 2004; Huang *et al*., 2008; Sánchez Márquez *et al*., 2008; Thongkantha *et al*., 2008). Many *Guignardia* species cause leaf blotch and black spots on fruits of various plants (Raabe *et al*., 1981; Glienke-Blanco *et al*., 2002). Few studies, however, have focused on the phylogenetic relationships among *Guignardia* and

Phyllosticta species, and uncertainty remains pertaining to species boundaries and host ranges of most taxa (Okane *et al*., 2001; Baayen *et al*., 2002; Crous *et al*., 2006). Species of *Guignardia* are commonly known only from their anamorphs. The identification of *Phyllosticta* anamorphs is highly problematic, as few characters are available to separate different species. Sometimes a single difference in conidial morphology, such as the thickness of the mucoid layer surrounding the conidium, or appendage shape and size, resulted in authors describing different species. Van der Aa and Vanev (2002) revised the 2936 taxa described in the genus *Phyllosticta*, accepting 141 species. Many other *Phyllosticta* species were combined into genera such as *Ascochyta*, *Coleophoma*, *Fusicoccum*, *Leptodothiorella*, *Phoma* and *Phomopsis*, to name but a few.

Recent studies on *Guignardia* and *Phyllosticta* have illustrated the confusion that still exists surrounding species concepts in literature. For instance, both *Guignardia endophyllicola* and *G. mangiferae* have been linked to *P. capitalensis* as anamorph (Okane *et al*., 2001). This matter was resolved by Baayen *et al*. (2002), who successfully used ITS DNA sequence data to show that *G. endophyllicola* was conspecific with *G. mangiferae*. To date the ITS domain has chiefly been used for species discrimination in this group (Everett and Rees-George, 2006), and further work remains to determine if a multigene phylogenetic approach would not resolve more cryptic taxa.

Citrus Black Spot, caused by *Guignardia citricarpa* (anamorph *Phyllosticta citricarpa*) is regulated as a quarantine pest in the European Union and the U.S.A. It occurs in a number of countries in Southeast Asia, Africa, South America, and Australia, and can be disseminated by means of infected fruit or vegetative plant material. Two distinct *Guignardia* species are associated with citrus, namely the pathogen *G. citricarpa*, and the endophyte *G. mangiferae* (Meyer *et al*., 2001; Baayen *et. al*., 2002; Everett & Rees-George, 2006; Baldassari *et al*., 2008). Based on the ITS sequences, several PCR detection methods have been developed for detection of *G. citricarpa* (Bonants *et al*., 2003; Meyer *et al*., 2006; Peres *et al*., 2007; Van Gent-Pelzer *et al*., 2007). Recently, necrotic spots similar to those caused by *G. citricarpa* were observed on fruit of *Citrus maxima*, intercepted in consignments exported from Asia. In these spots, pycnidia and conidia of a *Guignardia* species, closely resembling *G. citricarpa*, were observed and therefore, measures were taken for the consignments in line with the EU legislation for *Guignardia citricarpa*.

By testing several PCR methods developed for detection of *G. citricarpa* on lesions of *Citrus maxima* and isolates at the Dutch Plant Protection Service, the real-time PCR method developed by Van Gent-Pelzer *et al*. (2007) failed to provide any amplification, which was due to sequence divergence in the ITS region. Therefore, a conventional PCR assay is normally recommended for the diagnosis of *G. citricarpa* on *C. maxima* fruit. This was confirmed in a comparative study with a conventional PCR assay developed by Bonants *et al*. (2003) on isolates obtained from *C. maxima* (I.R. Heurneman-van Brouwershaven, unpubl. data).

To determine the identity of the *Guignardia* species associated with tan spot of *Citrus maxima* fruit intercepted from Asia, fungal isolates were subjected to DNA sequence analysis of the internal transcribed spacer (ITS1, 5.8S, ITS2) region and partial translation elongation factor 1-alpha (TEF1) and actin (ACT) gene sequences. Further aims were to investigate the species boundaries of Asian isolates compared to isolates identified as *G. citricarpa* and *G. mangiferae*, and to determine which genes are useful in distinguishing isolates of *Guignardia* at the species level.

Material and Methods

Isolates

Cultures were obtained from the CBS Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands, and the Plant Protection Service (PD), Wageningen, The Netherlands. To supplement the dataset with more species, single ascospore and conidial isolates were respectively made from *Guignardia* and *Phyllosticta* fruiting bodies occurring on leaves and fruit of diverse host plants (Table 1). Colonies were established on 2 % malt extract agar plates (MEA; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) by using the

techniques as explained in Cheewangkoon *et al*. (2008).

Molecular phylogeny

DNA extraction was done using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to manufacturer's protocol. The primers V9G (de Hoog and Gerrits van den Ende, 1998) and ITS4 (White *et al*., 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5' end of the 28S rRNA gene. To resolve taxa in the *G. mangiferae* and *G. citricarpa* complex the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-α gene (TEF1) and the primers ACT-512F and ACT-783R (Carbone and Kohn, 1999) were used to amplify part of the actin gene (ACT). Amplification conditions followed Arzanlou *et al*. (2008). Amplicons were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analysed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA).

Sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Se-Al; Rambaut, 2002) by inserting gaps. Phylogenetic analyses of the aligned sequence data were performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2003) as explained by Arzanlou *et al*. (2007). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistenc yindex (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page 1996). New sequences were lodged in GenBank and the alignment and phylogenetic tree inTreeBASE (www.treebase.org). *Botryosphaeria obtusa* (ITS = AY972105, TEF1 = DQ280419, ACT = AY972111) was used as outgroup in the phylogenetic analyses.

Morphology

Cultures were grown on 2% tap-water agar supplemented with sterile pine needles (WAP) (Crous *et al*., 2006), for microscopic examination. Preparations were mounted in cotton-blue lactophenol or clear lactic acid, and studied by means of a light microscope (× 1000 magnification). The 95 % confidence intervals were derived from 30 observations of spores formed on WAP, with extremes given in parentheses. All cultures obtained in this study are maintained in the culture collection of the CBS (Table 1). Colony colours (surface and reverse) were assessed after growth on four different media, potato-dextrose agar (PDA), cornmeal agar (CMA), MEA and oatmeal agar (OA, Gams *et al*., 2007) using the colour charts of Rayner (1970). Radial cultural growth rate was determined after 2 wk at 27 °C in the dark. Cardinal temperature requirements for growth were determined after 2 wk by incubating representative strains (three strains per species at each temperature, respectively) at 12 different temperatures (from 6–39°C in 3°C intervals) in the dark. The nomenclatural novelty and description was deposited in MycoBank (www.MycoBank.org).

Results

Phylogeny

The manually adjusted combined (ITS, TEF1 and ACT) alignment contained 60 taxa (including the outgroup sequence) and, of the 1092 characters used in the phylogenetic analysis, 280 were parsimony-informative, 301 were variable and parsimony-uninformative, and 511 were constant. Neighbour-joining analysis using the three substitution models on the sequence data yielded trees with identical topology and similar bootstrap values. Only the first 1000 equally most parsimonious trees were saved from the heuristic search and one of these is shown in Fig. 1 (TL = 945, CI = 0.847, RI = 0.971, RC = 0.822). Individual gene trees resolved the same clades presented in Fig. 1 and only differed with regard to the placement of *P. hypoglossi*, *P. spinarum*, *G. vaccinii* and *Guignardia* sp. strain CBS 100098 (data not shown). Three well-supported clades representing the citrus isolates could be resolved. Clade 1 consisted of isolates identified

Table 1. Details of *Guignardia* and *Phyllosticta* isolates investigated during this study.

Species	Original identification	Strain no.[1]	Substrate	Country	Collector	GenBank no. (ITS, TEF1, ACT)[2]
Guignardia citricarpa		CBS 102345	*Citrus aurantium* (Rutaceae), lesions on peel	Brazil	—	FJ538311, FJ538369, FJ538427
Guignardia citricarpa		CBS 102373; PD 99/911383	*Citrus aurantium* (Rutaceae), black spot on fruit	Brazil	—	FJ538312, FJ538370, FJ538428
Guignardia citricarpa		CBS 102374; PD 99/911519	*Citrus aurantium* (Rutaceae), black spot on fruit	Brazil	—	FJ538313, FJ538371, FJ538429
Guignardia citricarpa		CBS 111.20; DSM 3514	—	—	—	FJ538314, FJ538372, FJ538430
Guignardia citricarpa		CBS 120489; PD 04/01844897	*Citrus limon* (Rutaceae)	Brazil	J. de Gruyter	FJ538315, FJ538373, FJ538431
Guignardia citricarpa		CBS 122384	*Citrus limon* (Rutaceae)	South Africa	M. Truter	FJ538316, FJ538374, FJ538432
Guignardia citricarpa		CBS 122482; CPC 14848	*Citrus sinensis* (Rutaceae), lesions on fruit	Zimbabwe	L. Huisman	FJ538317, FJ538375, FJ538433
Guignardia citricarpa		CBS 828.97	*Citrus aurantium* (Rutaceae), fruits and leaves	Brazil	C. Glienke	FJ538318, FJ538376, FJ538434
Guignardia mangiferae	*Guignardia heveae*	CBS 101228	*Nephelium lappaceum* (Sapindaceae), discoloured spinters	USA: Hawaii	K.A. Nishijima	FJ538319, FJ538377, FJ538435
Guignardia mangiferae	*G. citricarpa*	CBS 100175	*Citrus* sp. (Rutaceae), healthy leaves	Brazil	C. Glienke	FJ538320, FJ538378, FJ538436
Guignardia mangiferae	*G. citricarpa*	CBS 100176	*Citrus* sp. (Rutaceae), healthy leaves	Brazil	C. Glienke	FJ538321, FJ538379, FJ538437
Guignardia mangiferae		CBS 115046	*Myracrodruon urundeuva* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538322, FJ538380, FJ538438
Guignardia mangiferae		CBS 115047	*Aspidosperma polyneuron* (Apocynaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538323, FJ538381, FJ538439

Table 1 (continued). Details of *Guignardia* and *Phyllosticta* isolates investigated during this study.

Species	Original identification	Strain no.[1]	Substrate	Country	Collector	GenBank no. (ITS, TEF1, ACT)[2]
Guignardia mangiferae		CBS 115049	*Bowdichia nitida* (Fabaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538324, FJ538382, FJ538440
Guignardia mangiferae		CBS 115051	*Spondias mombin* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538325, FJ538383, FJ538441
Guignardia mangiferae		CBS 115052	*Spondias mombin* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538326, FJ538384, FJ538442
Guignardia mangiferae		CBS 115053	*Myracrodruon urundeuva* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538327, FJ538385, FJ538443
Guignardia mangiferae		CBS 115056	*Anacardium giganteum* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538328, FJ538386, FJ538444
Guignardia mangiferae		CBS 115057	*Anacardium giganteum* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodriques	FJ538329, FJ538387, FJ538445
Guignardia mangiferae		CBS 115313	*Myracrodruon urundeuva* (Anacardiaceae), leaf or bark	Brazil	K.F. Rodrigues	FJ538330, FJ538388, FJ538446
Guignardia mangiferae		CBS 115345	*Bowdichia nitida* (Fabaceae), leaf or bark	Brazil	K.F. Rodrigues	FJ538331, FJ538389, FJ538447
Guignardia mangiferae	*Guignardia* sp.	CBS 123374; NFW-220	*Citrus aurantium* (Rutaceae)	Thailand	N.F. Wulandari	FJ538332, FJ538390, FJ538448
Guignardia mangiferae	*Guignardia* sp.	CBS 123404; NFW-219	*Musa paradisiaca* (Musaceae)	Thailand	N.F. Wulandari	FJ538333, FJ538391, FJ538449
Guignardia mangiferae	*Guignardia* sp.	CBS 123405; NFW-154	*Musa acuminata* (Musaceae)	Thailand	N.F. Wulandari	FJ538334, FJ538392, FJ538450
Guignardia mangiferae	*G. citricarpa*	CBS 173.77; CECT 2874	*Citrus aurantiifolia* (Rutaceae), fruit	New Zealand	—	FJ538335, FJ538393, FJ538451
Guignardia mangiferae	*P. capitalensis*	CBS 226.77; IFO 32914	*Paphiopedilum callosum* (Orchidaceae), leaf spot	Germany	—	FJ538336, FJ538394, FJ538452

Table 1 (continued). Details of *Guignardia* and *Phyllosticta* isolates investigated during this study.

Species	Original identification	Strain no.[1]	Substrate	Country	Collector	GenBank no. (ITS, TEF1, ACT)[2]
Guignardia mangiferae		ATCC 32757; PD 04/01844926	*Citrus limon* (Rutaceae), leaf	Taiwan	J. de Gruyter	FJ538337, FJ538395, FJ538453
Guignardia mangiferae		CBS 120490; PD 04/01844942	*Citrus paradisi* (Rutaceae), fruit	USA, Florida	J. de Gruyter	FJ538338, FJ538396, FJ538454
Guignardia mangiferae	*P. musarum*	CBS 117118	*Musa acuminata* (Musaceae)	Indonesia	I. Buddenhagen	FJ538339, FJ538397, FJ538455
Guignardia mangiferae	*G. musae*	CBS 119720; CPC 13013	*Musa* sp. (Musaceae)	USA: Hawaii	I. Buddenhagen	FJ538340, FJ538398, FJ538456
Guignardia mangiferae	*G. musae*	CBS 123373; NFW-221	*Musa paradisiaca* (Musaceae)	Thailand	N.F. Wulandari	FJ538341, FJ538399, FJ538457
Guignardia mangiferae	*G. philoprina*	CBS 356.52; ATCC 11368	*Ilex* sp. (Aquifoliaceae)	—	—	FJ538342, FJ538400, FJ538458
Guignardia mangiferae	*G. philoprina*	CBS 373.54	*Ilex* sp. (Aquifoliaceae)	—	—	FJ538343, FJ538401, FJ538459
Guignardia mangiferae	*G. sansevieriae*	CBS 120428; PD 04/01543402	*Sansevieria* sp. (Dracaenaceae)	Netherlands	J. de Gruyter	FJ538344, FJ538402, FJ538460
Guignardia mangiferae	*Guignardia capsici*	CBS 111638	*Capsicum* sp. (Solanaceae), fruit	Dominican Republic	G. Carroll	FJ538345, FJ538403, FJ538461
Guignardia mangiferae	*Guignardia* sp.	CMU 131	*Magnolia liliifera* (Magnoliaceae), leaf endophyte	Thailand	L.M. Duong	FJ538346, FJ538404, FJ538462
Guignardia mangiferae	*Guignardia* sp.	CMU 139	*Magnolia liliifera* (Magnoliaceae), leaf endophyte	Thailand	L.M. Duong	FJ538347, FJ538405, FJ538463
Guignardia mangiferae	*Guignardia* sp.	CMU 142	*Magnolia liliifera* (Magnoliaceae), leaf endophyte	Thailand	L.M. Duong	FJ538348, FJ538406, FJ538464
Guignardia mangiferae	*G. vaccinii*	CBS 114751	*Vaccinium* sp. (Ericaceae), leaf	New Zealand	T. Fluher	FJ538349, FJ538407, FJ538465
Guignardia mangiferae	*G. philoprina*	CBS 937.70	*Hedera helix* (Araliaceae), leaf litter	Italy	W. Gams	FJ538350, FJ538408, FJ538466
Guignardia psidii		CBS 100250	*Psidium guajava* (Myrtaceae), fruits	Brazil	C. Glienke	FJ538351, FJ538409, FJ538467

Table 1 (continued). Details of *Guignardia* and *Phyllosticta* isolates investigated during this study.

Species	Original identification	Strain no.[1]	Substrate	Country	Collector	GenBank no. (ITS, TEF1, ACT)[2]
Guignardia sp.		CBS 100098	*Citrus* sp. (Rutaceae), healthy leaves	Brazil	C. Glienke	FJ538352, FJ538410, FJ538468
Guignardia vaccinii		CBS 126.22; IFO 32911	*Oxycoccus macrocarpus* (Ericaceae)	USA	—	FJ538353, FJ538411, FJ538469
Phyllosticta citriasiana	*P. citricarpa*	CBS 120488; PD 05/02436019	*Citrus maxima* (Rutaceae)	Thailand	J. de Gruyter	FJ538354, FJ538412, FJ538470
Phyllosticta citriasiana	*P. citricarpa*	CBS 123370; PD 08/04453736	*Citrus maxima* (Rutaceae)	Vietnam	J. de Gruyter	FJ538355, FJ538413, FJ538471
Phyllosticta citriasiana	*P. citricarpa*	CBS 123371; PD 08/04454173	*Citrus maxima* (Rutaceae)	Vietnam	J. de Gruyter	FJ538356, FJ538414, FJ538472
Phyllosticta citriasiana	*P. citricarpa*	CBS 123372; PD 08/04454191	*Citrus maxima* (Rutaceae)	Vietnam	J. de Gruyter	FJ538357, FJ538415, FJ538473
Phyllosticta citriasiana	*P. citricarpa*	CBS 123393; PD 08/04453728	*Citrus maxima* (Rutaceae)	Vietnam	J. de Gruyter	FJ538358, FJ538416, FJ538474
Phyllosticta citriasiana	*P. citricarpa*	CBS 120427; PD 05/01654890	*Citrus maxima* (Rutaceae)	China	J. de Gruyter	FJ538359, FJ538417, FJ538475
Phyllosticta citriasiana	*P. citricarpa*	CBS 120486; PD 05/01969753	*Citrus maxima* (Rutaceae)	Thailand	J. de Gruyter	FJ538360, FJ538418, FJ538476
Phyllosticta citriasiana	*P. citricarpa*	CBS 120487; PD 05/03081053	*Citrus maxima* (Rutaceae)	China	K. Rosendahl-Peters	FJ538361, FJ538419, FJ538477
Phyllosticta citriasiana	*P. citricarpa*	CBS 120491; PD 06/03125095	*Citrus maxima* (Rutaceae)	China	K. Rosendahl-Peters	FJ538362, FJ538420, FJ538478
Phyllosticta citriasiana	*P. citricarpa*	CBS 120485; PD 06/03125116	*Citrus maxima* (Rutaceae)	China	K. Rosendahl-Peters	FJ538363, FJ538421, FJ538479

Table 1(continued). Details of *Guignardia* and *Phyllosticta* isolates investigated during this study.

Species	Original identification	Strain no.[1]	Substrate	Country	Collector	GenBank no. (ITS, TEF1, ACT)[2]
Phyllosticta citriasiana	*P. citricarpa*	CBS 120426; PD 06/03125132	*Citrus maxima* (Rutaceae)	China	K. Rosendahl-Peters	FJ538364, FJ538422, FJ538480
Phyllosticta hypoglossi		CBS 101.72; IFO 32916	*Ruscus aculeatus* (Ruscaceae), living leaves	Italy	W. Gams	FJ538365, FJ538423, FJ538481
Phyllosticta hypoglossi		CBS 167.85	*Ruscus hypoglossum* (Ruscaceae)	Italy	W. Gams	FJ538366, FJ538424, FJ538482
Phyllosticta hypoglossi		CBS 434.92	*Ruscus aculeatus* (Ruscaceae), dead cladodes	Italy	W. Gams	FJ538367, FJ538425, FJ538483
Phyllosticta owaniana		CBS 776.97; CPC 1009	*Brabejum stellatifolium* (Proteaceae), leaf spot	South Africa	A. den Breeÿen	FJ538368, FJ538426, FJ538484

[1]ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMU: Microbiology Section, Chiang Mai University (MSCMU), Department of Biology, Faculty of science, Chang Mai University, Thailand; CPC: Culture collection of Pedro Crous, housed at CBS; CECT: Coleccion Española de Cultivos Tipo, University of Valencia, Valencia, Spain; DSM: DSMZ, Deutsche Sammlung von Mikrorrganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO: Institute For Fermentation, Osaka, Japan; NFW: Culture collection of Nilam F. Wulandari; PD: Plant Protection Service, Wageningen, The Netherlands.
[2]ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; TEF1: partial translation elongation factor 1-alpha gene; ACT: partial actin gene.

as *G. citricarpa* (Citrus Black Spot). *Guignardia citricarpa* was originally described from Australia. Although the single Australian isolate found in this clade (CBS 111.20) proved to be sterile, it still produced the characteristic yellow pigment when cultivated on OA. Clade 2 represented the new species, described here as *Phyllosticta citriasiana*, and thus far known from citrus cultivated in China, Thailand and Vietnam. Clade 3 represented isolates identified as *G. mangiferae*, including strains from a wide host range, namely Rutaceae (*Citrus* spp.), Musaceae (*Musa* sp.), Myrtaceae (*Psidium guajava*), Anacardiaceae (*Mangifera indica*), Solanaceae (*Capsicum annuum*), Dracaenaceae (*Sansevieria* sp.), and Orchidaceae (Orchid). Based on these data, several species including *G. capsici*, *G. capitalensis*, *G. endophyllicola*, *G. heveae*, *G. mangiferae*, *G. musae*, *G. philoprina*, *G. sansevieriae*, and *G. vaccinii* were shown to belong to the *G. mangiferae* complex. These data support the findings of Baayen *et al*. (2002), leading to the conclusion that *G. mangiferae* is a cosmopolitan species that frequently occurs in lesions with other, plant pathogenic species. A further curious result was the fact that isolate CBS 100098 (from a *Citrus* sp. in Brazil) clustered separately, appearing more closely related to *G. spinarum* CBS 937.70 and *G. vaccinii* CBS 126.22, suggesting that there are yet more unresolved species that occur on *Citrus*.

Taxonomy

Three well-defined species were delineated on *Citrus* in the present study. These include *Guignardia mangiferae* (anamorph *Phyllosticta capitalensis*), *Guignardia citricarpa* (anamorph *Phyllosticta citricarpa*), and a *Phyllosticta* species that is morphologically distinct, and does not correlate with any other known species presently known in GenBank or our own DNA sequence databases. For these reasons this species is newly described below.

Phyllosticta citriasiana Wulandari, Crous & Gruyter, **sp. nov.** Fig. 2.

MycoBank: MB508387.

Teleomorph: Unknown.

Spermatial state: *Leptodothiorella* sp.

Etymology: Named after its host, *Citrus*, and continent of origin, Asia.

Phyllostictae citricarpae similis, sed conidiis maioribus, 10–16 × 5–8 µm.

On WAP. *Pycnidia* immersed to erumpent, globose, subglobose to ellipsoidal. Exuding spore-masses varied per culture medium, being colourless and glossy on CMA and MEA, grey and opaque on PDA, and colourless and opaque on OA and WAP. *Pycnidia* 120–240 × 125–225 µm; pycnidial wall consisting of several layers, 25–70 µm thick; outer wall of pale brown to brown, thickened cells of *textura angularis* to *globularis*; inner wall consisting of one to two pale brown cell layers, that become hyaline toward interior, *textura angularis*. *Ostiole* single, central, 7–8 µm wide, 30–32 µm deep, appearing cylindrical in section, consisting of thickened, dark-brown cells. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells or branched from a supporting basal cell, 7–25 × 3–6 µm. *Conidiogenous cells* terminal, subcylindrical to ampulliform or somewhat doliiform, hyaline, smooth, coated in a thin mucoid layer, inconspicuously proliferating once or twice percurrently near apex, 7–17 × 3–5 µm. *Conidia* (10–)12–14(–16) × (5–)6–7(–8) µm, solitary, hyaline, aseptate, thin- and smooth-walled, coarsely guttulate, ellipsoidal to obovoid, tapering toward a narrowly truncate base, enclosed in a thin mucilaginous sheath, 1 µm thick, and bearing a hyaline, mucoid apical appendage, 7–10(–14) × 1–2 µm, straight to flexible, unbranched, tapering towards an acutely rounded tip. *Spermatia* at times forming in conidial conidiomata, hyaline, bacilliform to somewhat ellipsoid, 3–5 × 1–2 µm.

Specimens examined: THAILAND, on peel fruit of *Citrus maxima* (Rutaceae) as black spot, 20 Oct. 2005, J. de Gruyter, CBS H-20185, holotypus, culture ex-type CBS 120486 = PD 05/01969753); CHINA, on fruit of *Citrus maxima*, 2 Dec. 2005, K. Rosendahl-Peters, CBS 120487 = PD 06/03125095).

Cultural characteristics: Colonies on MEA flat, regular, with entire edge; surface leaden-grey in centre, lavender-grey at margin, and leaden-black underneath. On PDA flat, spreading, with feathery margin, fluffy; surface dark slate-blue, and olivaceous-black underneath. On CMA flat, irregular, with lobed-edge; surface greenish black in centre,

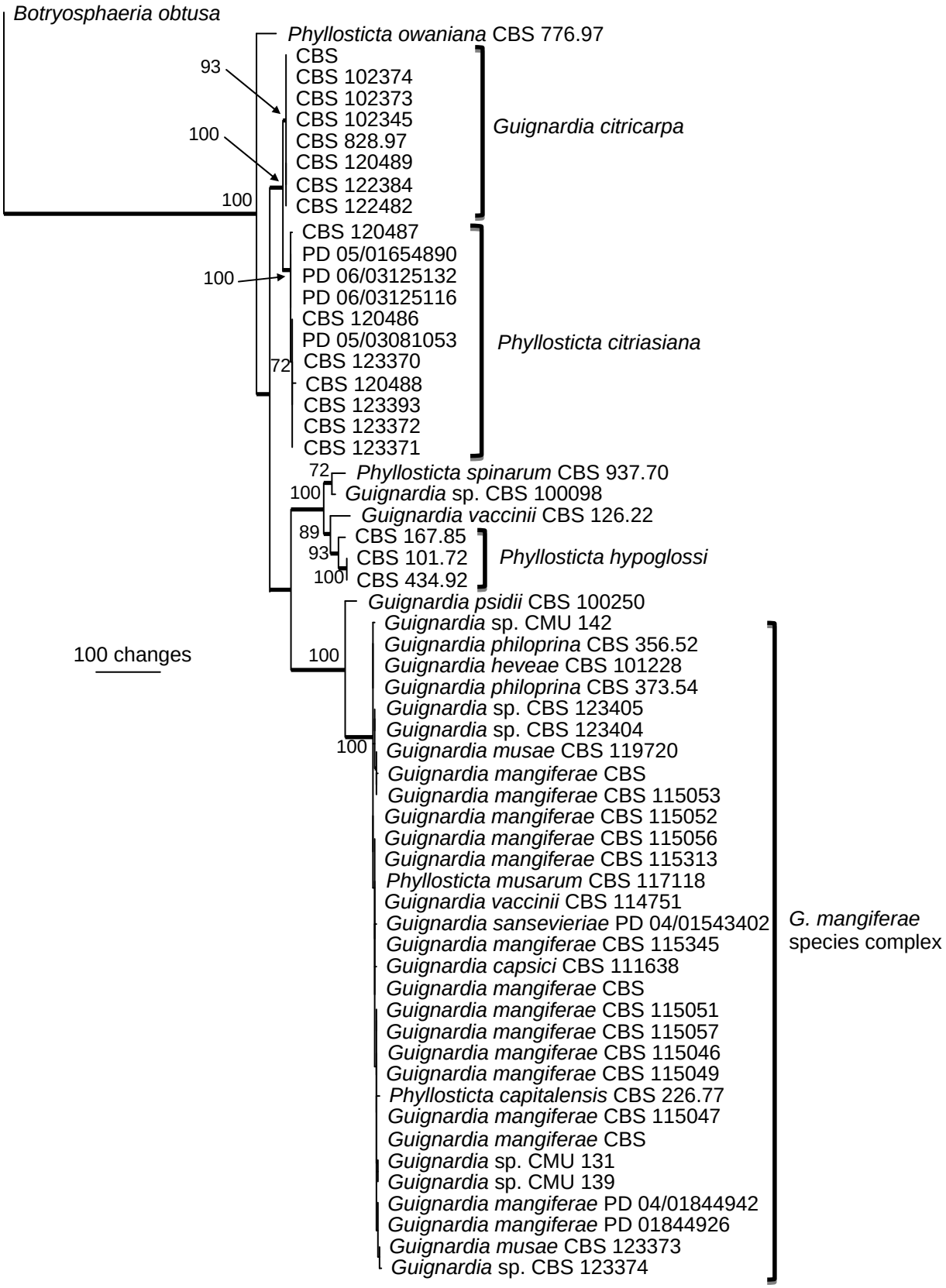

Fig. 1. One of 1000 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined sequence alignment using PAUP v. 4.0b10. The scale bar shows 100 changes, and bootstrap support values higher than 70 % from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and the tree was rooted to *Botryosphaeria obtusa* (ITS = AY972105, TEF1 = DQ280419, ACT = AY972111).

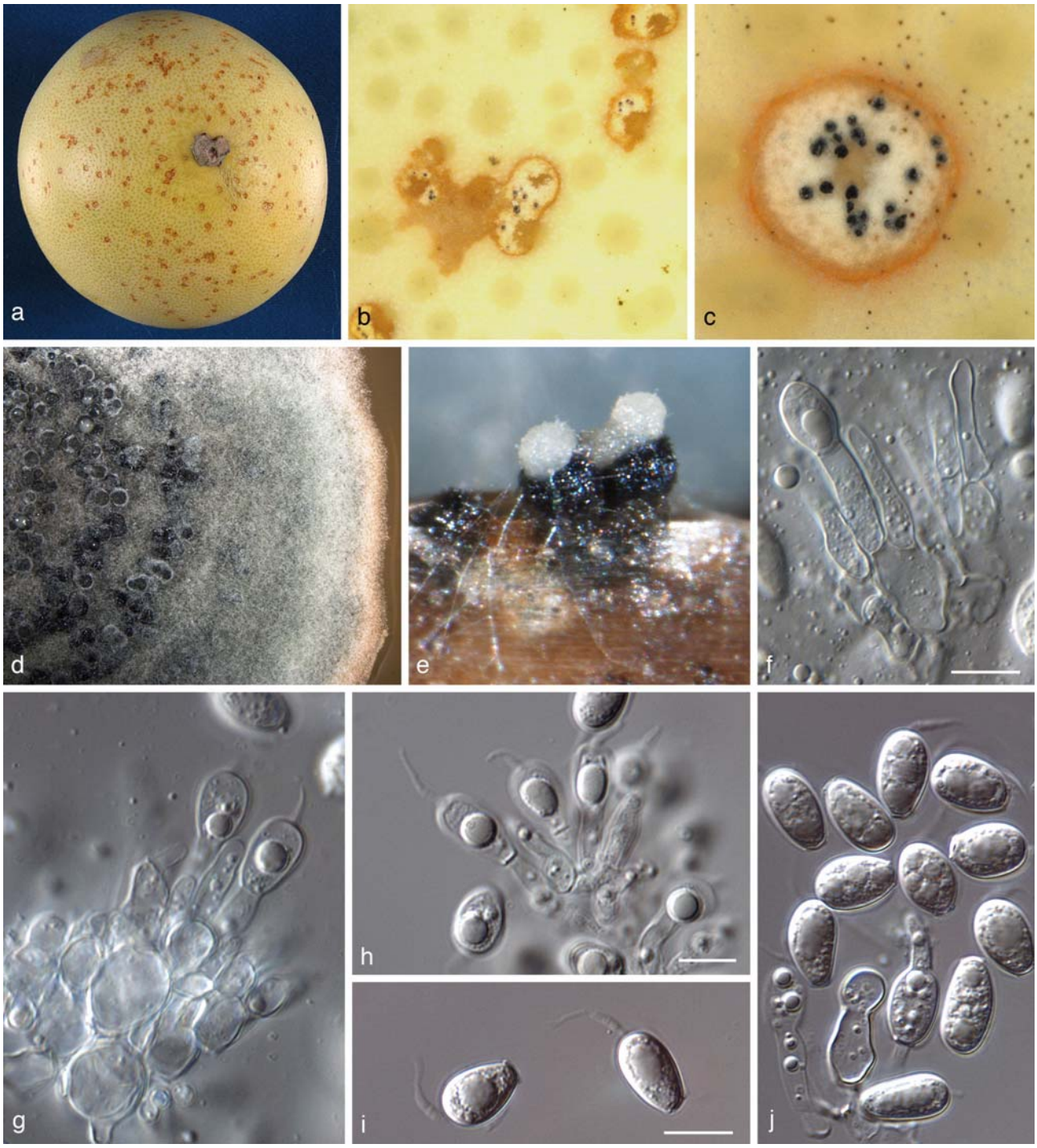

Fig. 2. *Phyllosticta citriasiana*. a–c. Symptoms on fruit of *Citrus maxima*. d. Colony on malt extract agar. e. Pycnidia sporulating on sterile pine needles on tap-water agar. f–h. Conidiogenous cells giving rise to solitary conidia. i, j. Conidia with mucoid sheath and apical mucilaginous appendage visible. Scale bars = 10 µm.

pale olivaceous-grey at margin, and leaden-black underneath. On OA flat, irregular, with entire to feathery margin, wooly; surface leaden-black in centre, olivaceous-black at margin, and leaden-black to leaden-grey underneath.

Cardinal temperatures: After 2 wk in the dark the optimum growth rate was observed at 30°C on MEA, CMA and OA (22 mm); on PDA this occurred at 27°C (43 mm). Minimum growth rate was observed at 15°C on MEA (5 mm), PDA (15 mm), CMA (5 mm) and OA (6 mm). Maximum growth rate was at 33°C on MEA, CMA and OA (17 mm); on PDA this occurred at 36°C (3.5 mm).

Notes: *Phyllosticta citriasiana* differs from the two other species occurring on citrus in its conidial dimensions, culture characteris-

tics and cardinal temperature growth requirements. This species has larger conidia when compared to *Guignardia citricarpa*, and thus far is only known from its *Phyllosticta* state. The conidial sheath is intermediate between that of *G. citricarpa* and *G. mangiferae*. The sheath itself is rather thin, being more similar to *G. citricarpa* than *G. mangiferae*, whereas the apical appendage is again longer than in *G. citricarpa*. In culture colonies are darker than that of the other two species, being shades of leaden-grey to leaden-black in all media tested. The maximum temperature for growth occurred at 30–33°C, whereas for the other species this was at 30–36°C. Lastly, *P. citriasiana* can be distinguished from *G. citricarpa* by not producing a diffuse yellow pigment on OA. Phylogenetically, *P. citriasiana* can easily be distinguished from *G. citricarpa* and *G. mangiferae* based on all three gene regions sequenced. Between *P. citriasiana* and *G. citricarpa*, 12 fixed nucleotide changes and 1 indel were observed over 602 nucleotides (identity of 97.84 %) for ITS; whereas TEF1 contained 7 fixed nucleotide changes and 2 indels over 271 nucleotides (identity of 96.68 %) and ACT had only 2 fixed nucleotide changes over 257 nucleotides (identity of 99.22 %) (Table 2).

Disease symptoms on fruits

Fruit symptoms are similar to those produced by *G. citricarpa*, the causal agent of Citrus Black Spot. They mainly consist of shallow lesions with a small central grey to tan crater usually with a dark brown rim, 3–10 mm diam. This symptom usually appears after the fruit has started to ripen. Often, but not always, pycnidia can be seen inside the spots as tiny and slightly elevated black dots in the grey to tan field. A magnifying glass or dissecting microscope is needed to see these clearly.

Another symptom that can sometimes be observed after harvest, consists of small (1–3 mm diam), slightly depressed spots. These spots may be grey to tan, or reddish, or brownish, or not discoloured at all. Often they have a dark red or brown rim. Pycnidia are only incidentally present in these lesions. Many intermediates occur between these spots and the previous type.

Discussion

Guignardia citricarpa and *G. mangiferae* are two well-established species. *Guignardia citricarpa* is confined to *Citrus* species, and is of importance in view of phytosanitary requirements (Glienke-Blanko *et al*., 2002). *Guignardia mangiferae* has been recorded on many hosts and is a common endophyte of diverse woody host plants (Baayen *et al*., 2002). There has, however, been considerable confusion about the identification of these species. Morphologically these two species are distinct. *Guignardia citricarpa* differs from *G. mangiferae* in ascospore size, anamorph characters and pathogenicity. Ascospores of *G. citricarpa* (8–17 × 3.5–8 μm) are usually larger than those of *G. mangiferae* (10–12 × 4–5 μm). Conidia of *P. citricarpa* (9–10 × 6–7 μm) are larger than those of *P. capitalensis* (8–10 × 4–5 μm) (anamorph of *G. mangiferae*), and also have a thinner sheath. However misidentification of the two fungi has often occurred (Everett and Rees-George, 2006).

In what has proven to be a pivotal paper on *Guignardia* taxonomy, Baayen *et al*. (2002) used ITS sequences to analyse *Guignardia* isolated from *Citrus* spp. from various locations to investigate the distinction between pathogenic *G. citricarpa* and nonpathogenic *G. mangiferae*. They divided the isolates into two different groups of *Guignardia* based on morphology and ITS sequence data. The first group comprised strains isolated from black spots and the second group comprised strains from *Citrus* spp. and 18 other hosts. ITS analyses of the strains from these two groups including reference *Guignardia* sequences resulted in strains from the first group being considered to be *Guignardia citricarpa sensu stricto*; the strains from the second group being conspecific with *Phyllosticta capitalensis*. The strains from the 18 hosts other than *Citrus* were generally isolated from healthy leaves, where the fungus was present as an endophyte, and coincidentally from spotted leaves. None of the isolates of the second group came from fruits with classical black spots. They concluded that *G. mangiferae* is present as endophyte in many different hosts of various plant families. The morphological distinction between the

Table 2. Nucleotide differences and their base positions observed in three loci between *Guignardia citricarpa* and *Phyllosticta citriasiana*. Sequences of *Guignardia citricarpa* strain CBS 111.20 were used as references to calculate base positions, which do not include spaces caused by alignment gaps. Nucleotides in bold print are identical to the reference sequence and bases in round parentheses were not considered as fixed nucleotide changes specific to a species. See Table 1 for the definition of the strain and locus abbreviations and for complete strain information.

Species	**Strain**	**ITS1**												**ITS2**
		68[a]	**77/78**[c]	**83**[b]	**98**[a]	**129**[a]	**172**[a]	**187**[a]	**189**[b]	**191**[a]	**234**[b]	**245**[a]	**275**[a]	**554**[a]
Guignardia citricarpa	CBS 111.20	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 828.97	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 102345	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 102373	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 102374	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 120489	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 122384	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Guignardia citricarpa	CBS 122482	**G**	**-**	**T**	**G**	**T**	**C**	**C**	**C**	**A**	**C**	**C**	**A**	**G**
Phyllosticta citriasiana	CBS 120486	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	CBS 120487	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	CBS 120488	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	CBS 123370	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	CBS 123371	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	CBS 123372	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	CBS 123393	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	PD 05/01654890	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	PD 05/03081053	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	PD 06/03125116	A	G	G	A	C	T	T	A	G	A	T	G	A
Phyllosticta citriasiana	PD 06/03125132	A	G	G	A	C	T	T	A	G	A	T	G	A

[a] Transition.
[b] Transversion.
[c] Insertion / duplication of leading nucleotide.

Table 2 (continued). Nucleotide differences and their base positions observed in three loci between *Guignardia citricarpa* and *Phyllosticta citriasiana*. Sequences of *Guignardia citricarpa* strain CBS 111.20 were used as references to calculate base positions, which do not include spaces caused by alignment gaps. Nucleotides in bold print are identical to the reference sequence and bases in round parentheses were not considered as fixed nucleotide changes specific to a species. See Table 1 for the definition of the strain and locus abbreviations and for complete strain information.

Species	Strain	TEF1											
		32[b]	(49)[b]	74[b]	80[c]	107[a]	129[a]	150[a]	173[c]	218[a]	228[a]	(263)[a]	(270)[a]
Guignardia citricarpa	CBS 111.20	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	**T**	**C**
Guignardia citricarpa	CBS 828.97	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	**T**	T
Guignardia citricarpa	CBS 102345	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	**T**	T
Guignardia citricarpa	CBS 102373	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	**T**	T
Guignardia citricarpa	CBS 102374	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	**T**	T
Guignardia citricarpa	CBS 120489	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	C	T
Guignardia citricarpa	CBS 122384	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	C	T
Guignardia citricarpa	CBS 122482	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**A**	**T**	C	T
Phyllosticta citriasiana	CBS 120486	T	**A**	A	-	T	G	C	-	G	C	**T**	**C**
Phyllosticta citriasiana	CBS 120487	T	**A**	A	-	T	G	C	-	G	C	**T**	T
Phyllosticta citriasiana	CBS 120488	T	**A**	A	-	T	G	C	-	G	C	C	T
Phyllosticta citriasiana	CBS 123370	T	**A**	A	-	T	G	C	-	G	C	C	T
Phyllosticta citriasiana	CBS 123371	T	**A**	A	-	T	G	C	-	G	C	C	T
Phyllosticta citriasiana	CBS 123372	T	**A**	A	-	T	G	C	-	G	C	C	T
Phyllosticta citriasiana	CBS 123393	T	**A**	A	-	T	G	C	-	G	C	C	T
Phyllosticta citriasiana	PD 05/01654890	T	T	A	-	T	G	C	-	G	C	**T**	**C**
Phyllosticta citriasiana	PD 05/03081053	T	T	A	-	T	G	C	-	G	C	**T**	**C**
Phyllosticta citriasiana	PD 06/03125116	T	**A**	A	-	T	G	C	-	G	C	**T**	**C**
Phyllosticta citriasiana	PD 06/03125132	T	**A**	A	-	T	G	C	-	G	C	**T**	**C**

[a] Transition.
[b] Transversion.
[c] Insertion / duplication of leading nucleotide.

Table 2 (continued). Nucleotide differences and their base positions observed in three loci between Guignardia citricarpa and Phyllosticta citriasiana. Sequences of Guignardia citricarpa strain CBS 111.20 were used as references to calculate base positions, which do not include spaces caused by alignment gaps. Nucleotides in bold print are identical to the reference sequence and bases in round parentheses were not considered as fixed nucleotide changes specific to a species. See Table 1 for the definition of the strain and locus abbreviations and for complete strain information.

Species	Strain	ACT						
		(11)[a]	(55)[b]	(101)[b]	118[b]	(190)[a]	204[a]	(209)[b]
Guignardia citricarpa	CBS 111.20	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 828.97	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 102345	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 102373	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 102374	T	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 120489	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 122384	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Guignardia citricarpa	CBS 122482	**C**	**C**	**T**	**G**	**G**	**T**	**G**
Phyllosticta citriasiana	CBS 120486	**C**	**C**	**T**	T	A	C	**G**
Phyllosticta citriasiana	CBS 120487	**C**	**C**	**T**	T	**G**	C	**G**
Phyllosticta citriasiana	CBS 120488	**C**	A	G	T	A	C	C
Phyllosticta citriasiana	CBS 123370	**C**	**C**	**T**	T	A	C	**G**
Phyllosticta citriasiana	CBS 123371	**C**	**C**	**T**	T	A	C	**G**
Phyllosticta citriasiana	CBS 123372	**C**	**C**	**T**	T	A	C	**G**
Phyllosticta citriasiana	CBS 123393	**C**	**C**	**T**	T	A	C	**G**
Phyllosticta citriasiana	PD 05/01654890	**C**	**C**	**T**	T	**G**	C	**G**
Phyllosticta citriasiana	PD 05/03081053	**C**	**C**	**T**	T	A	C	**G**
Phyllosticta citriasiana	PD 06/03125116	**C**	**C**	**T**	T	**G**	C	**G**
Phyllosticta citriasiana	PD 06/03125132	**C**	**C**	**T**	T	**G**	C	**G**

[a] Transition.
[b] Transversion.
[c] Insertion / duplication of leading nucleotide.

anamorphs of *G. citricarpa* and *G. mangiferae* is found in the thickness of the conidial mucilaginous sheath. In vitro *G. citricarpa* strains produce a distinct yellow pigment on OA. However, molecular tools are required to rapidly identify these two species. The isolates obtained from *Citrus maxima* clearly represent a different taxon, for which the name *P. citriasiana* was introduced. *Phyllosticta citrisiana* can be distinguished from *G. mangiferae* by having smaller conidia, with a narrower mucoid sheath. Furthermore, it is distinguishable from *G. citricarpa* by having larger conidia, longer conidial appendages, and not producing any diffuse yellow pigment when cultivated on OA. In culture, colonies of *P. citriasiana* are also darker shades of grey and black on OA, MEA, PDA, and CMA than observed in the other two species.

In the present study, three strains isolated from *Citrus* spp. (CBS 100175, CBS 100176 and CBS 173.77) although these sporulating poorly on OA, produce yellow pigments in culture, and are seen as *G. citricarpa sensu stricto*. However, in this molecular study these isolates clustered in the *G. mangiferae* complex (Fig. 1). The present study was based on the available cultures at the CBS and the Dutch Plant Protection Service culture collections, and some fresh isolates obtained from Asia. Numerous species of *Guignardia* and *Phyllosticta* require further study, as few have been compared thus far on a molecular basis. Although preliminary, our data suggest that the ITS locus is insufficient for separating all cryptic taxa in *Guignardia* (*Phyllosticta*). However, ITS (97.84 % identity between *P. citriasiana* and *G. citricarpa*) and TEF1 (96.68 % identity between *P. citriasiana* and *G. citricarpa*) gave a better species resolution between *P. citriasiana* and *G. citricarpa* than ACT (99.22 % identity between *P. citriasiana* and *G. citricarpa*) in this study. More transitions than transversions were observed (Table 2) for ITS and TEF1 and an almost equal frequency for ACT. None of the three genes in this study revealed significant variation in the *G. mangiferae* complex (data not shown; near

vertical line in Fig. 1). Further investigation is thus called for, to determine if other loci support the morphological variation observed among isolates in *G. mangiferae*. The apparent synonymy of numerous taxa under this epithet, therefore (Baayen *et al*., 2002), should be accepted with some reservation.

Our study has revealed two species of *Guignardia* to cause diseases of *Citrus*. *Guignardia citricarpa* causes Citrus Black Spot in Southeast Asia, Africa, South America and Australia, while *P. citriasiana* is presently known only from Asia, where it causes a Citrus Tan Spot on *Citrus maxima* fruit. Because there are several *Guignardia* species with a similar conidial morphology that cause disease on a range of cultivated plants, it is unclear whether these could represent either *G. citricarpa* or a teleomorph of *P. citriasiana*. Further surveys, pathogenicity studies and molecular analyses are thus required, to resolve the distribution, host range and importance of these two species. A survey may also answer the question whether there is a teleomorph of *P. citriasiana* occurring in orchards in the Asian area of origin.

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