Nonpathogenic Isolates of the Citrus Black Spot Fungus, *Guignardia citricarpa*, Identified as a Cosmopolitan Endophyte of Woody Plants, *G. mangiferae* (*Phyllosticta capitalensis*)

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ABSTRACT

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The population structure of *Guignardia citricarpa* sensu lato (anamorph: *Phyllosticta citricarpa*), a fungus of which strains pathogenic to citrus are subject to phytosanitary legislation in the European Union and the United States, was investigated. Internal transcribed spacer sequences revealed two phylogenetically distinct groups in *G. citricarpa*. This distinction was supported by amplified fragment length polymorphism analysis that also supported the exclusion of two isolates that had apparently been misclassified as *G. citricarpa*. On cherry decoction agar, but not on other media, growth rates of group I isolates were lower than those of group II isolates. Conidial dimensions were similar, but group I isolates formed conidia with barely visible mucoid sheaths, whereas those of group II formed conidia with thick sheaths. Cultures of isolates

belonging to group I produced rare infertile perithecia, whereas fertile perithecia were formed by most isolates of group II. Colonies of isolates belonging to group I were less dark than those of group II, with a wider translucent outer zone and a lobate rather than entire margin. On oatmeal agar, exclusively group I isolates formed a yellow pigment. Group I harbored strains from citrus fruits with classical black spot lesions (1 to 10 mm in diameter) usually containing pycnidia. Group II harbored endophytic strains from a wide range of host species, as well as strains from symptomless citrus fruits or fruits with minute spots (<2-mm diameter) without pycnidia. These observations support the historic distinction between slowly growing pathogenic isolates and morphologically similar fast-growing, nonpathogenic isolates of *G. citricarpa*. The latter proved to belong to *G. mangiferae* (*P. capitalensis*), a ubiquitous endophyte of woody plants with numerous probable synonyms including *G. endophyllicola*, *G. psidii*, *P. anacardiacearum*, and *P. theaeorum*. *G. mangiferae* occurs in the European Union and the United States on many host species including citrus, and does not cause symptoms of citrus black spot, justifying its exclusion from quarantine measures.

Citrus black spot, caused by *Guignardia citricarpa* Kiely sensu lato, is a fruit disease that affects the rind of *Citrus* fruit but does not cause internal decay (9-12,30,32,33). Heavy infection near the pedicel of the developing fruit may lead to premature fruit drop. Losses may be substantial because affected fruits are no longer suited for the fresh fruit market, and yield losses and costs for chemical control can be significant (11,32).

Benson (2) first reported black spot in citrus orchards near Sydney, Australia, and shortly after McAlpine described the causal agent as *Phoma citricarpa* McAlpine (14). The teleomorph, *G. citricarpa* Kiely was described in 1948 (9). The anamorph was later reclassified as *Phyllosticta citricarpa* (McAlpine) van der Aa (36). The spermatial state is in *Leptodothiorella* (36); however, the synanamorph has not been formally described.

The disease cycle was originally described from Australia by Kiely (9,10), and was subsequently studied in southern Africa

(11,17,18,22,23), where the disease had been introduced with bud wood imported from Australia (5) and had developed to epidemic levels. Perithecia develop on fallen infected leaves on the orchard floor, and from these, ascospores are released that serve as primary inoculum of leaves and fruits. After a period of latency, leaf spots and pycnidia develop on the infected leaves, and various lesion types (hard spot, virulent spot, freckle spot, and false melanose), and often pycnidia, develop on infected fruits (9,10,12,32). Symptom development on mature fruit is enhanced by high temperatures, high light intensity, drought, and poor vigor (12). After harvest, storage of fruits in the light at 20°C further promotes the formation of pycnidia in the lesions (3). The role of pycnidia in the disease cycle is not clear. Attempts to inoculate plants with conidia have failed so far, suggesting that the ascospores are the prime source of infection (12,17,22). Perithecia do not develop on leaves or fruits still on the tree, or on harvested fruits, but only on fallen and already decaying infected leaves (12). Ascospore production and host infection require warm and moist conditions (12). Indeed, the disease has thus far only been found in subtropical regions of the world that are subject to summer rainfall such as Argentina, eastern Australia, Brazil, China, Hong Kong, Indonesia, Japan, Kenya, Mozambique, Nigeria, Peru, Philippines, northeastern South Africa, Swaziland, Taiwan, Uruguay, Vene-

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zuela, and Zimbabwe (8,12,31,34). Black spot is unknown in the citrus-producing countries or regions subjected to winter rainfall such as Chile, Greece, Israel, Italy, Spain, Turkey, the Cape Province of South Africa, and California.

Kiely (9) found that the fungus not only occurs on *Citrus* spp., but also on the leaves of various native Australian species growing near citrus groves (e.g., *Telopea speciosissima*) and on garden plants. On some of these plants, especially on the old leaves, restricted lesions up to 2 mm in diameter sometimes developed. Kiely concluded that such infections serve as a reservoir for the black spot fungus (9). In contrast, McOnie (16,19–21) did not find any evidence that any plant besides citrus plays a role in the epidemiology of the black spot disease. In the South African Cape Province, the fungus is commonly present in indigenous plants as well as in *Citrus* spp. (38), in line with the observations of Kiely (9). However, black spot symptoms did not occur in the Cape at all. Upon a closer scrutiny, isolates from the Cape Province differed in behavior from regular isolates from fruits with black spot symptoms; they grew faster, easily produced perithecia on culture media (whereas black spot-associated isolates remained sterile), and rarely produced pycnidia on culture media (whereas black spot-associated isolates produced pycnidia abundantly) (16). Subsequent inoculation trials revealed that the isolates from the Cape, although morphologically identical to *G. citricarpa* from black spot-affected fruits, were consistently nonpathogenic to oranges and grapefruits in infection trials (16,20). McOnie (16) concluded that *Citrus* and many other host species might harbor a latent *Guignardia* sp. that is morphologically similar to, but pathogenically different from, *G. citricarpa*. Well before McOnie's classical studies, Chiu (4) had reported the same two types of strains from Taiwan as well as their apparent association with the presence (absence) of black spot symptoms. Lee (13) subsequently demonstrated that Taiwanese isolates of the first group (those growing slowly and having a lobed margin) caused freckle and virulent spot on Ponkan fruit (*Citrus tankan*), whereas those of the second group (those growing rapidly and having an entire margin) penetrated the fruits but did not induce any black spot. The distinction between slowly growing pathogenic *G. citricarpa* and fast-growing nonpathogenic *Guignardia* sp. (also referred to as nonpathogenic strains of *G. citricarpa*) has been followed widely (8,12,32). The host range of the nonpathogenic *Guignardia* sp. currently includes a wide array of families of wild and cultivated plants (8,32,34). Its geographic distribution is much wider than that of pathogenic *G. citricarpa*, including important black spot-free citrus regions such as Florida, Spain, Sicily, and Israel (20,32).

The European Union and the United States consider pathogenic strains of *G. citricarpa* a serious phytosanitary risk and have regulated the import of fruit consignments. Consignments with black spot symptoms are refused. Quarantine legislation in the European Union is restricted to pathogenic strains of *G. citricarpa* (1) in order to exclude strains of the nonpathogenic *Guignardia* sp. reported by McOnie (16). Recent interceptions of black spot-infected fruit consignments have shown that the distinction is not very practicable. Black spot is traditionally diagnosed from classical fruit symptoms such as hard spot lesions containing pycnidia of *P. citricarpa*. The diagnosis becomes troublesome when no pycnidia are present in the lesions, which is often the case when only freckle spot or false melanose symptoms are found. In contrast to hard spot and virulent spot, freckle spot and false melanose are not very distinctive and can be easily confused with symptoms of other *Citrus* diseases such as true melanose (caused by *Diaporthe citri*), greasy spot (caused by *Mycosphaerella citri*), or lesions caused by *Colletotrichum* spp. (12). Such indistinct symptoms are commonly seen at import inspection. Culturing of the fungus is then required to verify that *G. citricarpa* is associated with the lesions, and that the fungus is a strain pathogenic to citrus. However, the fungus is difficult to culture because of

competition with common endophytic fungi of citrus fruit such as *Colletotrichum* spp., and subtle differences in cultural characteristics require the study of pure cultures. Given the slow growth of the fungus (the formation of mature pycnidia may require 14 days) and the rapidly decreasing value of consignments held for quarantine testing, culturing is highly impracticable. Moreover, isolates in the authors' collections seemed to represent a continuum between the two groups described by McOnie (16), thus raising doubts about the validity of their distinction. The present study was undertaken to investigate whether the distinction is justified and at what level, and if so, whether more robust methods (morphological, cultural, or molecular) could be developed for differentiating pathogenic *G. citricarpa* from nonpathogenic *Guignardia* sp. during quarantine diagnosis.

MATERIALS AND METHODS

Fungal strains. Investigated isolates of *G. citricarpa* sensu lato from *Citrus* spp. and from associated host species are listed in Table 1. Reference isolates of *Guignardia* and *Phyllosticta* spp. are listed in Table 2.

DNA extraction, amplification, and sequencing. Strains were grown in potato dextrose broth (Difco Laboratories, Detroit) at room temperature for 3 weeks, after which the mycelium was harvested, freeze-dried, and ground (10 to 50 mg) in microcentrifuge tubes with sterile sand and a pestle. DNA was isolated using the Puregene kit (Gentra/Biozym, Landgraaf, the Netherlands) according to the instructions of the manufacturer. Internal transcribed spacer-polymerase chain reaction (ITS-PCR) was performed with primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCCTCCGCTTATTGATATGC) as described by White et al. (41). Cycle sequencing of PCR products was performed according to Werres et al. (40). PCR products (550 bp) were sequenced on an automatic sequencer (ABI3700; Perkin-Elmer, Nieuwerkerk a/d IJssel, the Netherlands). Sequences were aligned, and phylograms were constructed with the MEGALIGN module of the DNASTAR software (DNASTAR Inc., Madison, WI). Representative ITS1 and ITS2 sequences are deposited as GenBank Accession Nos. AY042907 to AY042934.

In a number of cases, DNA was isolated and sequenced according to an alternative method described by Winton et al. (42). PCR reaction mixtures were prepared with the following components: 30 µl of betaine (4 M), 10 µl of MgCl₂ (25 mM), 10 µl of PCR buffer, 8 µl of dNTP's (2.5 mM), 2.5 µl of bovine serum albumin (10 mg/ml), 2.0 µl of *Taq* polymerase, and 27.5 µl of deionized water. The total volume (90 µl) was divided into 9-µl aliquots and to each was added 0.4 µl of the forward primer (10 µM), 0.4 µl of the reverse primer (10 µM), and 0.2 µl of genomic DNA. PCR reactions were carried out in a RapidCycler (Idaho Technology, Salt Lake City, UT) in capillary tubes according to the following program: hold: 94°, 15 s; cycle: denature 94°, 1 s; anneal 55°, 3 s; elongation, 72°, 15 s (slope 2.0, 39 cycles); hold: 72°, 1 min. Following completion of the thermocycling program, a 1.5% agarose gel (electrophoresis grade) prepared with a narrow comb was loaded with 5 µl of sample in 5 µl of loading buffer (total volume 10 µl) and run in Tris-borate-EDTA buffer at 70 V for 30 min with a 200 bp standard in the far left lane. Following electrophoresis, the entire gel was stained with SYBAR Gold (Molecular Probes, Eugene, OR) at 1:10,000 dilution for 15 min. The gel was viewed on a with blue light and an amber-colored filter. Sequencing was carried out on an automated sequencer using a dye terminator sequencing kit (ABI 377; Perkin-Elmer Applied Biosystems, Foster City, CA).

Amplified fragment length polymorphism analysis. DNA (250 ng) was digested in a 50-µl reaction volume with *Eco*RI (10 units) and *Msp*I (10 units) for 5 h at 37°C in restriction ligation buffer (10 mM Tris/HAc at pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM dithiothreitol, and bovine serum albumin at

50 ng/μl), and adapters were ligated overnight to the restriction fragments in 40 μl of reaction product (10 μl for gel) at 10 to 12°C. Final concentrations were 2.4 units of T4 DNA ligase (Pharmacia, Uppsala, Sweden), 0.1 μM *EcoRI* adapter (5' CTCG-TAGACTGCGTACC/CATCTGACGCATGGTTAA 5'), 1.0 μM *MspI* adapter (5' GACGATGAGTCCTGAT/CTACTCAGGACT-AGC 5'), and 0.2 mM ATP. Nonselective amplification was performed with 5 μl of 10× diluted ligation product added to 20 μl of buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂) with 60 μM dNTP, primers *Eco00* (5' GACTGCGTA-

CCAATTC) and *Msp00* (5' GATGAGTCCTGATCGG) at 5 ng/μl, and 1 unit of *Taq* DNA polymerase (Boehringer GmbH, Mannheim, Germany). Reactions were performed in a thermocycler (PTC200; MJ Research, Watertown, MA) programmed as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C, and 90 s at 72°C; and final extension of 10 min at 72°C and cooling to 4°C. Amplicons were checked on 1.0% agarose gels and visualized with ethidium bromide and UV illumination.

Selective PCR was performed on 5 μl of 20× diluted amplicons in a 20-μl final reaction volume of the buffer mentioned above but

TABLE 1. Characteristics and identity of isolates received as *Guignardia citricarpa* arranged according to internal transcribed spacer (ITS) group and host^a

Host	Isolate no.	Other codes ^b	Source	Symptoms	Origin	ITS group	AFLP type	Colony diameter	Yellow pigment	Identity
ITS group I										
Rutaceae										
<i>Citrus aurantium</i>	2	CBS 828.97	Fruit	BS	Brazil	1	1	25	Present	<i>G. citricarpa</i>
<i>Citrus aurantium</i>	37	IMI 304799	Fruit	Unknown	India	1	1	26	Present	<i>G. citricarpa</i>
<i>Citrus limettioides</i>	39	PPRI 1568	Fruit	BS; pycnidia present	South Africa	1	1	27	Present	<i>G. citricarpa</i>
<i>Citrus limon</i>	35	PPRI 5029	Fruit	BS; pycnidia present	South Africa	1	1	29	Present	<i>G. citricarpa</i>
<i>Citrus limon</i>	46	PPRI 6526	Fruit	BS; pycnidia present	Brazil	1	1	24	Present	<i>G. citricarpa</i>
<i>Citrus limon</i>	51	...	Fruit	Unknown	South Africa	1	1	31	Present	<i>G. citricarpa</i>
<i>Citrus limon</i>	54	PPRI 1569	Fruit	BS; pycnidia present	Argentina	1	1	33	Present	<i>G. citricarpa</i>
<i>Citrus reticulata</i>	40	PPRI 5278	Leaf	BS; no pycnidia	South Africa	1	1	27	Absent	<i>G. citricarpa</i>
<i>Citrus reticulata</i>	41	PPRI 5276	Fruit	BS; pycnidia present	South Africa	1	1	26	Present	<i>G. citricarpa</i>
<i>Citrus reticulata</i>	56	PPRI 5277	Unknown	BS; pycnidia present	Zimbabwe	1	NT	16	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	3	...	Fruit	BS; no pycnidia	Brazil	1	1	29	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	4	...	Fruit	BS; no pycnidia	Brazil	1	1	29	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	5	...	Fruit	BS; pycnidia present	Brazil	1	1	23	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	6	...	Fruit	BS; pycnidia present	Brazil	1	1	29	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	9	...	Fruit	BS; no pycnidia	Brazil	1	1	25	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	22	...	Fruit	BS; pycnidia present	South Africa	1	1	30	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	23	...	Fruit	BS; pycnidia present	South Africa	1	1	28	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	24	...	Fruit	BS; pycnidia present	South Africa	1	1	28	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	25	...	Fruit	BS; pycnidia present	South Africa	1	1	27	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	26	...	Fruit	BS; pycnidia present	South Africa	1	1	28	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	27	...	Fruit	BS; pycnidia present	South Africa	1	1	29	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	28	...	Fruit	BS; pycnidia present	South Africa	NT	NT	20	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	29	...	Fruit	BS; pycnidia present	South Africa	1	1	31	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	45	PPRI 5350	Fruit	BS; pycnidia present	South Africa	1	1	24	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	55	PPRI 6514	Leaf	BS; no pycnidia	South Africa	1	1	25	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	59	GCS JB2PSQ2	Fruit	BS; pycnidia present	South Africa	1	1	28	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	62	GCS RV3	Fruit	BS; pycnidia present	South Africa	1	1	28	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	63	GCS B10	Fruit	BS; pycnidia present	South Africa	1	1	28	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	64	GCS B0	Fruit	BS; pycnidia present	South Africa	1	1	29	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	67	GCS JBmonshR	Fruit	BS; pycnidia present	South Africa	1	1	25	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	68	GCS FVZB1SSR	Fruit	BS; pycnidia present	South Africa	1	1	25	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	69	GCS TLMensyR	Fruit	BS; pycnidia present	South Africa	1	1	21	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	70	GCS TR	Fruit	BS; pycnidia present	South Africa	1	1	27	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	71	GCS TS	Fruit	BS; pycnidia present	South Africa	1	1	24	Present	<i>G. citricarpa</i>
<i>Citrus sinensis</i>	72	GCS JBF5R5	Fruit	BS; pycnidia present	South Africa	1	1	30	Present	<i>G. citricarpa</i>
<i>Citrus</i> sp.	1	CBS 111.20	Fruit	BS	China ^c	1	1	20	Present	<i>G. citricarpa</i>
<i>Citrus</i> sp.	7	...	Fruit	BS; pycnidia present	Brazil	1	1	24	Present	<i>G. citricarpa</i>
<i>Citrus</i> sp.	8	...	Fruit	BS; pycnidia present	Brazil	1	1	29	Present	<i>G. citricarpa</i>
ITS group II										
Anacardiaceae										
<i>Mangifera indica</i>	89	PD 21003195	Leaf	Leaf spot	Ghana	2	NT	46	Absent	<i>G. mangiferae</i>
<i>Rhus crenata</i>	112	GCC RHUCRE 1-2	Leaf	Absent	South Africa	2	NT	34	Absent	<i>G. mangiferae</i>
Araliaceae										
<i>Schefflera</i> sp.	91	GCC SCHEFSP 1-1	Leaf	Absent	Costa Rica ^d	2	NT	50	Absent	<i>G. mangiferae</i>
Celastraceae										
<i>Putterlickia verrucosa</i>	92	GCC PUTVER 1-1	Leaf	Absent	South Africa	2	NT	37	Absent	<i>G. mangiferae</i>

(continued on next page)

^a Colony diameters (millimeters) were measured after 7 days growth on cherry decoction agar (CHA). Production of yellow pigment was evaluated after 7 days growth on oatmeal agar (OA). The thickness of the mucoid conidial sheath was assessed from conidia from approximately 14-day-old CHA cultures. BS, citrus black spot; NT, not tested.

^b ATCC, American Type Culture Collection, Rockville, MD; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; DAR, Plant Pathology Herbarium (HERB-DAR), Orange, NSW, Australia; GCC, G. C. Carroll, Eugene, Oregon; GCS, G. C. Schutte, Nelspruit, South Africa; IMI, former International Mycological Institute, Egham, U.K.; MEP, M. E. Palm, Beltsville, MD; PD, Plant Protection Service, Wageningen, the Netherlands; and PPRI, Plant Protection Research Institute, Pretoria, South Africa.

^c Intercepted in New South Wales, Australia, on citrus peel from China; isolated and identified by W. A. Birmingham in 1920.

^d Intercepted in California by USDA-APHIS in 1999.

^e New South Wales.

^f Torres Strait Islands.

^g This is fast-growing perithecia-producing isolate PC-1 of Wang and Tsai (39).

with 200 µM dNTP and 5 ng of Cy5-labeled fluorescent *Eco*20 primer (5' GACTGCGTACCAATTCGC) and 30 ng of *Msp*15 primer (5' GATGAGTCCTGATCGGCA). Reactions were performed under the following conditions: 1 cycle of 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C; 13 cycles of 72°C (annealing temperature was lowered by 0.7°C during each cycle); followed by 23 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C; and a final extension of 10 min at 72°C and cooling to 4°C. Products were run on an AFLexpress automatic sequencer (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) with a 50-bp ladder (Amer-

sham) as a reference. Amplified fragment length polymorphism (AFLP) patterns were analyzed qualitatively with Imagemaster 1D software (Amersham). Presence or absence of bands was converted to binary data and incorporated in the analysis. A similarity matrix was constructed according to the method of Nei and Li (25). Unweighted pair group method cluster analysis of binary data was performed with Treecon software (35) and a similarity dendrogram was constructed with a distance scale.

Growth rate, cultural characteristics, and morphology. Culture media were prepared according to Gams et al. (6). Colony

TABLE 1. (continued from preceding page)

Host	Isolate no.	Other codes ^b	Source	Symptoms	Origin	ITS group	AFLP type	Colony diameter	Yellow pigment	Identity
Cornaceae										
<i>Curtisia dentata</i>	93	GCC CURDEN 1-1	Leaf	Absent	South Africa	2	NT	39	Absent	<i>G. mangiferae</i>
Euphorbiaceae										
<i>Clusia natalensis</i>	94	GCC CLUNA 1-1	Leaf	Absent	South Africa	2	NT	31	Absent	<i>G. mangiferae</i>
Lecythidaceae										
<i>Barringtonia racemosa</i>	95	GCC BARRA 1-2	Leaf	Absent	South Africa	2	NT	59	Absent	<i>G. mangiferae</i>
Meliaceae										
<i>Trichilia emetica</i>	96	GCC TRIEM 1-2	Leaf	Absent	South Africa	2	NT	32	Absent	<i>G. mangiferae</i>
Moraceae										
<i>Artocarpus</i> sp.	97	GCC ARTOSP 1-1	Leaf	Absent	Thailand ^d	2	NT	25	Absent	<i>G. mangiferae</i>
Musaceae										
<i>Musa</i> sp.	44	DAR 19970	Leaf	Leaf spot	Australia ^c	2	2	50	Absent	<i>G. mangiferae</i>
Pittosporaceae										
<i>Pittosporum hawaiiense</i>	98	GCC PITHA 1-16	Leaf	Absent	Hawaii	2	NT	59	Absent	<i>G. mangiferae</i>
Rubiaceae										
<i>Coprosma ernodeoides</i>	99	GCC COPERN 1-7	Leaf	Absent	Hawaii	2	NT	30	Absent	<i>G. mangiferae</i>
Rutaceae										
<i>Citrus aurantiifolia</i>	85	PPRI 1562	Unknown	Unknown	Brunei	2	NT	42	Absent	<i>G. mangiferae</i>
<i>Citrus jambhiri</i>	53	DAR 66105a	Leaf	Leaf spot	Australia ^f	2	2	54	Absent	<i>G. mangiferae</i>
<i>Citrus limon</i>	10	...	Fruit	Minute spots; no pycnidia	South Africa	2	2	32	Absent	<i>G. mangiferae</i>
<i>Citrus limon</i>	11	...	Fruit	Minute spots; no pycnidia	South Africa	2	2	36	Absent	<i>G. mangiferae</i>
<i>Citrus limon</i>	12	...	Fruit	Minute spots; no pycnidia	Argentina	2	2	55	Absent	<i>G. mangiferae</i>
<i>Citrus limon</i>	13	...	Fruit	Minute spots; no pycnidia	Argentina	2	2	47	Absent	<i>G. mangiferae</i>
<i>Citrus limon</i>	48	ATCC 32757; PC-1 ^g	Leaf	Unknown	Taiwan	2	2	57	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	14	...	Fruit	Minute spots; no pycnidia	Florida	2	2	51	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	15	...	Fruit	Minute spots; no pycnidia	Florida	2	2	55	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	16	...	Fruit	Minute spots; no pycnidia	Florida	2	2	53	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	17	...	Fruit	Minute spots; no pycnidia	Florida	2	2	55	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	18	...	Fruit	Minute spots; no pycnidia	Florida	2	2	52	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	19	M.E.P. 1445	Fruit	Minute spots; no pycnidia	Florida	2	2	50	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	20	M.E.P. 1446	Fruit	Symptomless peel	Florida	2	2	54	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	21	M.E.P. 1447	Fruit	Symptomless peel	Florida	2	2	45	Absent	<i>G. mangiferae</i>
<i>Citrus paradisi</i>	73	...	Fruit	Minute spots; no pycnidia	Florida	2	2	59	Absent	<i>G. mangiferae</i>
<i>Citrus reticulata</i>	36	JW738E2	Fruit	Unknown	Hong Kong	NT	NT	57	Absent	<i>G. mangiferae</i>
<i>Citrus reticulata</i>	86	PPRI 1563	Unknown	Unknown	Hong Kong	2	NT	18	Absent	<i>G. mangiferae</i>
<i>Citrus reticulata</i>	87	PPRI 1565	Unknown	Unknown	Hong Kong	2	NT	45	Absent	<i>G. mangiferae</i>
<i>Citrus sinensis</i>	47	PPRI 1572	Fruit	Unknown	Brazil	2	2	45	Absent	<i>G. mangiferae</i>
<i>Citrus</i> sp.	88	PPRI 1567	Fruit	Unknown	Japan	2	NT	21	Absent	<i>G. mangiferae</i>
<i>Zanthoxylum martinense</i>	100	GCC ZANMA 1-11	Leaf	Absent	Puerto Rico	2	NT	39	Absent	<i>G. mangiferae</i>
Sapindaceae										
<i>Allophylus africanus</i>	101	GCC ALLAF 1-1	Leaf	Absent	South Africa	2	NT	48	Absent	<i>G. mangiferae</i>
Smilacaceae										
<i>Smilax kraussiana</i>	90	GCC SMIKRA 1-2	Leaf	Absent	South Africa	2	NT	39	Absent	<i>G. mangiferae</i>
Stangeriaceae										
<i>Stangeria eriopus</i>	102	GCC STER 6-1	Leaf	Absent	South Africa	2	NT	38	Absent	<i>G. mangiferae</i>
Theaceae										
<i>Camellia japonica</i>	103	GCC CAMJA 1-1	Leaf	Absent	Louisiana	2	NT	42	Absent	<i>G. mangiferae</i>
Viscaceae										
<i>Viscum obscurum</i>	104	GCC VISCOB 1-3	Leaf	Absent	South Africa	2	NT	16	Absent	<i>G. mangiferae</i>
Vitaceae										
<i>Rhoicissus tomentosa</i>	105	GCC RHOTO 1-3	Leaf	Absent	South Africa	2	NT	33	Absent	<i>G. mangiferae</i>
Zamiaceae										
<i>Encephalartos ferox</i>	106	GCC ENFE 3-2	Leaf	Absent	South Africa	2	NT	22	Absent	<i>G. mangiferae</i>
<i>Encephalartos latifrons</i>	107	GCC ENLAT 2-1	Leaf	Absent	South Africa	2	NT	30	Absent	<i>G. mangiferae</i>
<i>Yamia integrifolia</i>	108	GCC ZAMIN 1-2	Leaf	Absent	Florida	2	NT	47	Absent	<i>G. mangiferae</i>
ITS group III										
Proteaceae										
<i>Telopea speciosissima</i>	52	DAR 60749	Leaf	Leaf spot	Australia ^f	3	4	58	Absent	<i>P. telopeae</i>
ITS group IV										
Unknown host	38	IMI 070028	Unknown	Unknown	Japan	4	3	16	Absent	<i>P. spinarum</i>

diameters of strains were measured in duplicate after 7 days growth in darkness at 22°C on cherry decoction agar (CHA). For a subset of strains, additional single measurements were carried out after 7 days growth in darkness at 22°C on CHA, malt extract agar (MEA), oatmeal agar (OA), and potato dextrose agar (PDA; Oxoid, London). Cultural characteristics were assessed, and the color of upper and lower sides of cultures was evaluated according to Rayner (28). Pigmentation was assessed after 7 days growth in darkness at 22°C on OA. For six slowly growing isolates and six fast-growing ones, the diameter of 10 pycnidia and the length and width of 30 conidia were determined from cultures that had been grown for 12 days in darkness at 22°C on CHA, followed by 2 days under near-UV illumination (8 h per 24 h). The thickness of the mucoid conidial sheath was assessed from CHA cultures using differential interference contrast optics. Finally, a detailed morphological analysis including measurements of fungal organs and assessment of cultural morphology and color was made of the subset of slowly growing isolates (35, 37, 46, and 70) and fast-growing isolates (12, 19, 36, 44, 48, and 73) mentioned above after incubation at 18°C, under 12 h near-UV light and 12 h darkness.

RESULTS

ITS sequence analysis. ITS sequence analysis revealed two major groups among the investigated isolates of *G. citricarpa* sensu lato (including isolates presumably belonging to nonpathogenic *Guignardia* sp. from citrus and associated host species) (Table 1; Figs. 1 and 2). Group I comprised 38 isolates from *Citrus aurantium*, *C. limettoides*, *C. limon*, *C. reticulata*, and *C. sinensis* from Argentina, Brazil, China, India, South Africa, and Zimbabwe. These isolates were obtained mainly from citrus fruits with classical black spot symptoms, generally including the presence of pycnidia in the lesions. Black spot symptoms varied from hard spot, often with a halo of freckle spot lesions, to virulent spot. Lesions were generally 2 to 10 mm in diameter, although satellite freckle spot lesions surrounding hard spot lesions tended to be smaller. Group I included isolate 1, a reference strain (CBS 111.20) of *G. citricarpa*. ITS1 sequences placed group I isolates close to *P. hypoglossi* and *P. spinarum* (Fig. 1). ITS2 sequences did not resolve their relationships to other species (Fig. 2).

Group II comprised 18 isolates from *C. aurantiifolia*, *C. jambhiri*, *C. limon*, *C. paradisi*, *C. reticulata*, and *C. sinensis* from Argentina, Australia, Brazil, Brunei, Hong Kong, Japan, South Africa, Taiwan, and the United States (Florida), an isolate from *Zanthoxylum martinence* (Rutaceae), and 21 isolates from 18 other host families (Table 1). Isolates from other host species

generally originated from healthy leaves, where the fungus had been present endophytically, and coincidentally from spotted leaves. None of the isolates of this second group that came from *Citrus* spp. originated from fruits with classical black spot lesions and pycnidia; several had been obtained from leaves. When isolated from citrus fruits, these had been either completely free of symptoms, or had carried tiny (<2 mm, mostly <1 mm), somewhat atypical spots of uncertain origin, never associated with pycnidia. Two isolates were from healthy looking parts of grapefruits that had carried such atypical symptoms. ITS sequences of group II isolates were identical to those of *P. capitalensis* (Figs. 1 and 2). ITS1 sequences did not resolve their relationships to other species (Fig. 1). ITS2 sequences suggested that *P. capitalensis* and group II isolates are the sister group of *G. philoprina* (Fig. 2). Both ITS1 and ITS2 sequences placed groups I and II distant from each other, with 62 and 16 bp differences, respectively.

Group III consisted of a single isolate (isolate 52), received as *G. citricarpa* from *Telopea speciosissima*. Isolate 52 was reclassified as *P. telopeae* on basis of its large conidia (14.0 to 16.0 × 8.9 to 10.6 µm), corresponding to the dimensions reported for *P. telopeae* (14 to 16.5 × 8 to 9.5 µm) (43) and well out of the range of those of *G. citricarpa* (6 to [9 to 10] to 13 × 5 to [6 to 7] to 9 µm) (36). ITS1 sequences placed isolate 52 close to *G. aesculi*, *G. philoprina*, and *P. pyrolae* (Fig. 1).

Group IV consisted of a single isolate (isolate 38) from an unknown host from Japan that had been received as *G. citricarpa*. Isolate 38 was reclassified as *P. spinarum* on basis of its ITS sequence.

ITS1 sequences grouped *G. bidwellii* and *P. eugeniae* together (Fig. 1). ITS1 and ITS2 sequences grouped *P. hypoglossi* and *P. spinarum* together (Figs. 1 and 2), and also *P. owaniana* and *P. podocarpi*. The reference strain of *P. abietis* clustered consistently at the root of the tree. Most other *Guignardia* and *Phyllosticta* spp. clustered inconsistently in both trees. The single isolate of *Phoma epicoccina* (= *Epicoccum nigrum*) clustered at the root of the tree.

AFLP grouping of isolates. AFLP patterns grouped a subset of 38 isolates in two main groups, corresponding with groups I and II resolved by ITS sequence analysis (Fig. 3). AFLP analysis supported the exclusion of isolates 38 (*P. spinarum*) and 52 (*P. telopeae*).

Growth rates of cultures. Growth rates of cultures of *G. citricarpa* sensu lato formed a continuum (Table 1; Figs. 4 and 5). The average colony diameter of group I isolates on CHA after 7 days growth at 22°C in darkness (25 to 30 mm) was lower than that of group II isolates (≥40 mm), both in the first experiment (Table 1; Fig. 4), and in the second, which comprised a subset of

TABLE 2. Investigated isolates of reference species of *Guignardia*, *Phyllosticta*, and *Phoma* used in this study

Species	Isolate ^a	Host	Source	Origin	Accession no.
<i>G. aesculi</i>	CBS 756.70	<i>Aesculus hippocastanum</i>	Leaf spot	Germany	AY042933, AY042934
<i>G. bidwellii</i>	CBS 237.48	<i>Parthenocissus tricuspidatus</i>	Unknown	Unknown	AY042931, AY042932
<i>G. philoprina</i>	CBS 447.68	<i>Taxus baccata</i>	Needle	The Netherlands	AF312014
<i>Phoma epicoccina</i> (= <i>Epicoccum nigrum</i>)	CBS 873.72 ^b	<i>Artocarpus heterophylla</i>	Leaf	India	AY042929, AY042930
<i>Phyllosticta abietis</i>	GCC ABCO 202	<i>Abies concolor</i>	Symptomless leaf	California	AF312012
<i>Phyllosticta beaumarisii</i>	CBS 535.87 (T)	<i>Muehlenbeckia adpressa</i>	Leaf	Australia	AY042927, AY042928
<i>Phyllosticta capitalensis</i>	CBS 226.77	<i>Paphiopedilum callosum</i>	Spotted leaves	Germany	AB041240
	CBS 398.80	Orchid	Unknown	New Zealand (ex Singapore)	AB041241
<i>Phyllosticta eugeniae</i>	CBS 445.82	<i>Eugenia aromatica</i>	Leaf	Indonesia	AY042925, AY042926
<i>Phyllosticta hypoglossi</i>	CBS 434.92	<i>Ruscus aculeatus</i>	Dead cladodes	Italy	AY042923, AY042924
<i>Phyllosticta owaniana</i>	GCC s.n.	<i>Brabejum stellatifolium</i>	Leaf spot	South Africa	AF312011
<i>Phyllosticta podocarpi</i>	GCC POLA 3-6	<i>Podocarpus lanceolata</i>	Symptomless leaf	South Africa	AF312013
<i>Phyllosticta pyrolae</i>	GCC s.n.	<i>Pyrola asarifolia</i>	Symptomless leaf	Oregon	AF312010
<i>Phyllosticta spinarum</i>	CBS 292.90	<i>Chamaecyparis pisifera</i>	Unknown	France	AF312009
	CBS 937.70 ^c	<i>Hedera helix</i>	Leaf litter	Italy	AF312008

^a CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; GCC, collection of G. C. Carroll.

^b Received as *Phyllosticta artocarpina*.

^c Received as *G. philoprina*.

the isolate collection (Table 3; Fig. 5). However, the range of growth rates of group I isolates (16- to 33-mm diameter in 7 days) fell within that of group II (16 to 59 mm). Growth rate differences were not or at best weakly reflected on OA and MEA (Fig. 5), although a tendency similar to that on cherry agar was observed on PDA (Table 3).

Pycnidia and conidia. All isolates formed conidiomata, which began to develop after 8 days, and produced conidia. The single exception was isolate 1 (an isolate from 1924), which formed pycnidia but failed to produce conidia any longer (it still had done so in 1973) (34). Pycnidia were produced faster by isolates belonging to group I than by those of group II. Isolates belonging to groups I and II had similarly sized pycnidia and conidia (Table 3). Conidia of isolates of group I were 9.4 to (10 to 12) to 12.7 × 5.0 to (6.0 to 7.5) to 8.5 µm, whereas those of group II isolates were 9.7 to (11 to 12) to 13.4 × 6.1 to (6.5 to 7.5) to 7.5 µm. The thickness of the mucoid sheath surrounding the conidium wall differed independently of the medium. Group I isolates mainly formed conidia with barely visible sheaths, or sheaths no thicker than 1.5 µm, whereas those of group II formed conidia with very

thick (1.5 to 2.5 (to 3) µm) mucoid sheaths (Fig. 6). Group I isolates also tended to form longer apical appendages, but this feature was more variable among strains. Moreover, appendages that adhere to the surface of the glass or other material in water mounts may be stretched and their actual length overestimated.

The morphology of pycnidia, conidia, and spermatia of group II isolates (those not associated with black spot symptoms) was the following: conidiomata were globose, occasionally pyriform, 150 to 300 × 100 to 250 µm, with a single papillate ostiole, glabrous or with short irregular hyphal outgrowths on the surface, walls similar to those of the perithecia. Conidiogenous cells were discrete, rarely integrated, determinate, holoblastic, short-cylindrical to ampulliform, 8 to 15 × 3.5 to 5.5 µm. Conidia were ovoid to almost pyriform, more rarely ellipsoid, hyaline, whitish in mass, multi-guttulate, 9 to 13.5 × 6 to 7.5 µm, truncate-rounded or truncate at the base, broadly rounded at the apex, with a flexible, 4 to 6 (to 10) µm long mucoid apical appendage, and the entire conidium wall surrounded by a stout, rigid 1.5 to 2.5 (to 3) µm thick mucoid sheath. The spermatial state belonged to the form genus *Leptodothiorella*. Spermatogenous cells were formed in the same or in

Fig. 1. Phylogram based on the internal transcribed spacer 1 region of the ribosomal DNA of 10 isolates of *Guignardia citricarpa* sensu lato group I (= *G. citricarpa* proper), 20 isolates of group II (= *G. mangiferae*), an isolate (38) later identified as *Phyllosticta spinarum*, an isolate (52) later identified as *P. telopeae*, and 15 isolates of reference species. Bootstrap values (1,000 replicates) are given above the nodes.

separate conidiomata, discrete or integrated, cylindrical, 4.5 to 9.5 × 1.0 to 2.5 µm. Spermatia were dumbbell-shaped, mostly 7 to 10 × 1.8 to 2.5 µm, containing a few minute guttules.

Perithecia and ascospores. Cultures of isolates belonging to group I produced rare infertile perithecia and no ascospores. Fertile perithecia and ascospores were formed in most isolates of group II. On CHA, numerous perithecia started to develop after 2 weeks and sporulated within 4 weeks. Fewer fertile perithecia were formed on OA. On MEA, perithecia were also occasionally formed, but mature asci were never observed. Perithecia mostly developed above the agar surface, within brittle, black, columnar or irregularly pustulate elevations, mainly composed of interwoven hyphae and fruiting bodies, that were in part also conidiomata that produced conidia as well as spermatia. Perithecia were globose, pyriform, or almost cylindrical, black, 250 to 400 × 175 to 250 µm, with a single papillate to rostrate ostiole, the surface often covered with irregular hyphal outgrowths, the outer wall layer composed of angular cells with brown thickened walls, the inner layer of angular to globose cells with thinner colorless walls. Asci were fasciculate, bitunicate, clavate, eight-spored, the apex

rounded, 65 to 100 × 10 to 14 µm shortly before rupture of the outer wall (at maximum turgor), then cylindrical-clavate and extending in length to 120 to 135 (to 150) µm prior to dehiscence. Ascospores were short-cylindrical and swollen in the middle, slightly curved, 15 to 17.5 × 6.5 to 7.5 µm, heteropolar with unequal obtuse ends, the smaller upper end provided with a 1.0 to 2.0 µm long truncate, noncellular, mucoid cap-like appendage, the lower end with a 3.0 to 6.0 µm long acute or ruffled appendage, multiguttulate. No differences in details of ascospore morphology were apparent when the ascospores produced in culture by group II isolates were compared with published illustrations of ascospores of *G. citricarpa* from perithecia formed naturally in fallen leaves of black spot-infected citrus trees that supposedly represent group I (16).

Cultural characteristics. Cultural characteristics were studied in detail for four isolates of ITS group I (isolates 35, 37, 46, and 70) and six isolates of ITS group II (isolates 12, 19, 36, 44, 48, and 73). Colonies of isolates belonging to ITS group I were less dark than those of group II, with a much wider translucent outer zone, and a distinctly lobate rather than entire margin. On OA, all group I isolates produced a distinct yellow pigment, with the

Fig. 2. Phylogram based on the internal transcribed spacer 2 region of the ribosomal DNA of 10 isolates of *Guignardia citricarpa* sensu lato group I (= *G. citricarpa* proper), 20 isolates of group II (= *G. mangiferae*), an isolate (38) later identified as *Phyllosticta spinarum*, an isolate (52) later identified as *P. telopeae*, and 15 isolates of reference species. Bootstrap values (1,000 replicates) are given above the nodes.

exception of isolate 40, which failed to do so despite repeated tests (Table 1; Fig. 7). Colonies of isolates belonging to ITS group II were darker, with only a narrow translucent outer zone and a more entire margin. On OA, none of the group II isolates produced the yellow pigment (Table 1).

Group I isolates produced immersed mycelium on OA that was always first pure yellow to straw before becoming dark herbage green. On CHA, they grew much slower than isolates belonging to group II, with a somewhat more irregular margin, lined by a much wider translucent zone of colorless immersed mycelium, which was largely covered by pure white, but later often more sulphur yellow or primrose, felty to woolly-floccose aerial mycelium that often reached the margin. Only the center of the colony was much darker, and here the aerial mycelium was gray or glaucous, often with numerous small tufts. The colony reverse was only very dark in the center, surrounded by areas of grayish sepia and buff. On MEA, group I isolates again had a much wider, brighter-colored zone that was largely hidden under a dense layer of white, later often faintly yellowish (straw), appressed woolly aerial mycelium. In the center of the colony, well-developed glaucous gray to greenish gray aerial mycelium was found. Stromatal development in the center was less pronounced than in group II. The colony reverse was also with a much wider, first buff, later often pale luteous marginal zone.

Colonies of group II isolates on OA had an almost even or more irregular glabrous colorless margin. The surface of the colony was smooth, plane, and the immersed mycelium dull green or gray olivaceous, almost olivaceous black in the center. Aerial myceli-

um formed only near the center, and was scanty, diffuse, woolly to woolly-floccose, grayish. The reverse was concolorous. Conidiomata began to develop after 8 days. On CHA, colonies had an even to slightly undulating, later mostly irregular, almost glabrous, colorless margin. The colony surface was iron gray or greenish gray after 8 days, to almost black after 14 days, except for a narrow translucent outer zone, plane, but already after 8 days with numerous black, semi-immersed globular conidiomata that were glabrous or bore gray aerial mycelial outgrowths. Soon afterwards, perithecia also started to develop and sporulate within 4 weeks. The aerial mycelium was well developed throughout, especially in the center of the colony, and was woolly-floccose, gray. The reverse was mostly dark slate blue. Colonies on MEA had a lobate or ruffled margin, appearing as a narrow, glabrous and colorless zone, or whitish due to diffuse, appressed, white aerial mycelium; colonies appearing restricted after 8 days, with an irregular, bumpy glaucous gray or greenish gray surface, the central part with numerous semi-immersed to almost superficial single globular, to complex, black stromatal masses bearing grayish white aerial hyphae; aerial mycelium well developed over most of the colony, felty to short-tufty, mostly glaucous gray, but locally white. The reverse was olivaceous black or dark slate blue, but with a narrow buff zone at the margin.

DISCUSSION

The molecular and morphological data presented in this study show that ITS groups I and II of *G. citricarpa* sensu lato are

Fig. 3. Unweighted pair group method dendrogram constructed from amplified fragment length polymorphism fingerprint patterns of 19 isolates of *Guignardia citricarpa* sensu lato group I (= *G. citricarpa* proper), 17 isolates of group II (= *G. mangiferae*), an isolate (38) later identified as *Phyllosticta spinarum*, and an isolate (52) later identified as *P. telopeae*.

distinct species. Further support for this assumption comes from variable intron sequences in genes for histone 4, translation elongation factor EF-1 α , chitin synthase, calmodulin, and spermidine synthase (G. C. Carroll, *unpublished data*), and from random amplified polymorphic DNA data (G. Glienke de Blanco, *unpublished data*). Similar conclusions were recently reached by Meyer et al. (24). Group I comprised all isolates from black spot-affected citrus fruit, and corresponded to McOnie's group of strains pathogenic to citrus (16) in their slow growth and lack of perithecia in pure culture. We therefore conclude that these strains correspond to *G. citricarpa* proper. Group II isolates generally grew rapidly, easily produced perithecia and ascospores, were never associated with black spot symptoms, and originated from a range of host species, all in line with McOnie's (16) unnamed *Guignardia* sp. from citrus and associated hosts. Groups III and IV consisted of single, apparently misidentified, strains of *P. telopeae* and *P. spinarum*, respectively.

ITS sequence analysis identified group II as possibly conspecific with *P. capitalensis*, a species described from orchids (36). *P. capitalensis* was recently found to grow endophytically in *Rhododendron* and *Enkianthus* in Japan, and to produce a hitherto unknown teleomorph newly described as *G. endophyllicola* (26). The morphological descriptions of the anamorphic and teleomorphic states of the strains of *P. capitalensis* from *Rhododendron*, *Enkianthus*, and orchids investigated in the latter study (including present reference strains CBS 226.77 and CBS 398.80 from orchids) confirm that the isolates from *Citrus* spp. and other hosts presently placed in ITS group II belong to *P. capitalensis*.

Our data show that *P. capitalensis* occurs endophytically on a wide range of hosts belonging to numerous families. An overview of families and genera of host plants of *P. capitalensis* (identified by ITS sequences and species-specific primer pairs) is given in Table 4. The recently reported endophytic occurrence of *P. capitalensis* in Ericaceae is therefore not surprising. The wide host range of *P. capitalensis* suggests that this fungus may have been described repeatedly from many different host species. Indeed, several older names in *Phyllosticta* and *Guignardia* probably are the same fungus. Based on the experience of one of the authors

that nearly all isolates from members of the Anacardiaceae and Theaceae give sequences typical of *P. capitalensis* (G. C. Carroll, *unpublished data*), and given that the morphology of these isolates is grossly the same as that of *P. capitalensis* (present study; 36), we conclude that *G. mangiferae* (anamorph: *P. anacardiacearum*) and *Guignardia* sp. (anamorph: *P. theacearum*) are the same species as *G. endophyllicola* (anamorph: *P. capitalensis*). Isolates from spotted fruits of *Psidium guajava* with the morphology of *G. citricarpa* also corresponded to *P. capitalensis* in a molecular analysis (C. Glienke de Blanco and W. Maccheroni Jr., *unpublished data*), indicating that *G. psidii* is likely a synonym as well. Many more synonyms are likely to exist. In North America alone, some 600 *Phyllosticta* spp. have been described, and sequences from about 150 isolates from various localities worldwide have so far revealed no more than 30 phylogenetic species; about half of these isolates showed the same ITS sequence as *P. capitalensis* (G. C. Carroll, *unpublished data*).

A survey of the taxonomic literature on *Phyllosticta* and *Guignardia* spp. shows that the oldest names associated with isolates belonging to group II probably are *G. mangiferae* and *P. capitalensis*. *P. capitalensis* P. Hennings was described in 1908 (7). *G. mangiferae* A.J. Roy was described in 1968 (29). An older possible name, *G. camelliae* (Cooke) E.J. Butler, was described in 1884 from *Camellia* as *Sphaerella* (*Laestadia*) *camilleae* Cooke and was transferred to the genus *Guignardia* Viala & Ravaz. after its establishment in 1892. However, the type material of *G. camelliae* proved to be *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (37), excluding the use of this name for the anamorph of *P. theacearum*. We, therefore, propose to designate isolates with the morphology and ITS sequence of group II as follows:

***Guignardia mangiferae* A.J. Roy**

Guignardia mangiferae A.J. Roy, Indian Phytopathol. 20(1968):340-341.—*G. endophyllicola* I. Okane, A. Nakagiri & T. Ito, Can. J. Bot. 79(2001):103.—*G. psidii* Ullasa & Rawal, Curr. Sci. 53(1984):436. Anamorphic state: *Phyllosticta capitalensis* P. Hennings, Hedwigia 48(1908):13.—*P. anacardiacearum* Van der Aa, Stud. Mycol. 5(1973):31.—*P. theacearum* Van der Aa, Stud. Mycol. 5(1973):97. Spermatial state: *Leptodothiorella* sp.

Fig. 4. Growth rate distribution of isolates of *Guignardia citricarpa* sensu lato group I (= *G. citricarpa* proper; black bars) and group II (= *G. mangiferae*; white bars) grown on cherry decoction agar (CHA). Colony diameters (x axis) were measured after 7 days growth in darkness at 22°C.

The present data confirm the reports by Chiu (4) and McOnie (16) that black spot is associated with slow growing *Phyllosticta* isolates, but not with the fast-growing ones that also form perithecia and ascospores in pure culture. Contrary to the main body of the black spot literature (11,32), followed in the European Union legislation (1), the nonpathogenic isolates are not strains or physiologically specialized forms of *G. citricarpa*, but a separate species, *G. mangiferae*. Despite this, unambiguous morphological distinction of *G. mangiferae* from *G. citricarpa* is notoriously difficult. None of the characteristics given by Chiu (4) or McOnie (16) was found to separate both species unambiguously. Growth rates formed a continuum, even on CHA. Pycnidia were formed by all isolates, although slower by *G. mangiferae* than by *G. citricarpa*. Not all isolates of *G. mangiferae* formed fertile perithecia on agar, whereas, incidentally, isolates of *G. citricarpa* formed infertile ones. The margin of cultures of isolates of *G. citricarpa* is more distinctly lobed than that of cultures of most of the isolates of *G. mangiferae*, but exceptions to this rule exist and therefore individual isolates cannot be reliably identified on this

criterion either. Altogether, the distinction made in quarantine legislation by the European Union and the United States, though scientifically justified, is nearly impossible to make in practice without molecular tools. The present study shows that the thickness of the mucoid sheath distinguishes *G. mangiferae* from *G. citricarpa*; however, the long period of time required for pycnidium formation makes this method unsuited for quarantine testing. The yellow pigment produced by *G. citricarpa* on OA is more useful, but even so sporulation is required because other fungi may resemble *G. citricarpa* while still sterile. The ITS sequences from this study have allowed us to produce a PCR method to test for the black spot fungus, which will be published in the near future.

The present study confirms previous reports that *G. citricarpa*, but not *G. mangiferae*, is associated with black spot symptoms. McOnie (16,19,21) and Lee (13; and literature citation 39) have shown experimentally, through inoculation trials, that the so-called nonpathogenic strains (= *G. mangiferae*) do not cause black spot. This justifies the exclusion of such "nonpathogenic strains of

Fig. 5. Growth of 17 isolates of *Guignardia citricarpa* sensu lato group I (= *G. citricarpa* proper) (▲) and nine isolates of group II (= *G. mangiferae*) (○) on cherry decoction agar (CHA), oatmeal agar (OA), and malt extract agar (MEA). Colony diameters were measured after 7 days growth in darkness at 22°C.

G. citricarpa” from quarantine legislation. Our data show that such strains belong to a cosmopolitan *Guignardia (Phyllosticta)* species that occurs on a wide range of woody plants, including *Citrus* spp., on all continents. The fungus is commonly present in Florida and also occurs in Europe, both on citrus and on orchids (and probably on more host species). Efforts to eradicate cosmopolitan *G. mangiferae* from citrus groves in regions in the European Union (Italy, Spain) and the United States (Florida) where this fungus occurs, but not *G. citricarpa*, will not serve any reasonable purpose. Similarly, preventing the introduction and spread of such strains when found on citrus fruit because of the

precautionary principle is not scientifically justifiable nor is it necessary from a technical point of view because the two fungi can be identified unambiguously and rapidly through PCR.

The biology and ecology of *G. mangiferae* differ considerably from that of *G. citricarpa*. The latter species does not produce fertile perithecia on agar media although it does produce them on fallen leaves in the field (9), possibly indicative of heterothallism, whereas *G. mangiferae* is evidently homothallic. *G. citricarpa* readily produces spermatia, while *G. mangiferae* produces them only erratically (C. Glienke de Blanco and W. Maccheroni Jr., unpublished data). *G. mangiferae* is a ubiquitous, cosmopolitan

TABLE 3. Average colony diameter and standard deviation after 7 days growth on cherry decoction agar (CHA), oatmeal agar (OA), malt extract agar (MEA), and potato dextrose agar (PDA), average length and width of conidia (in micrometers), and average pycnidium diameter (in millimeters) of representative isolates of internal transcribed spacer (ITS) groups I to IV of *Guignardia citricarpa* sensu lato

ITS group and isolate no.	Colony diameter (mm)				Conidial dimensions		Pycnidium diameter
	CHA	OA	MA	PDA	Length	Width	
ITS group I (=G. citricarpa)							
1	15 ± 1	20 ± 1	28 ± 4	8 ± 4	9–10 ^a	6–7 ^a	0.45 ± 0.09
35	25 ± 1	17 ± 1	23 ± 2	22 ± 4	10.6 ± 0.8	7.5 ± 0.6	0.28 ± 0.06
37	26 ± 3	14 ± 1	14 ± 0	28 ± 5	12.7 ± 0.9	8.5 ± 0.8	0.31 ± 0.03
46	20 ± 2	19 ± 1	24 ± 2	13 ± 4	11.5 ± 1.0	8.2 ± 0.6	0.11 ± 0.04
70	26 ± 0	22 ± 1	24 ± 1	25 ± 0	9.9 ± 0.7	6.9 ± 0.5	0.26 ± 0.04
ITS group II (=G. mangiferae)							
12	52 ± 2	27 ± 3	17 ± 0	32 ± 4	11.1 ± 0.9	7.0 ± 0.7	0.25 ± 0.03
19	45 ± 0	29 ± 2	23 ± 3	35 ± 3	11.0 ± 0.8	7.4 ± 0.5	0.36 ± 0.06
36	56 ± 2	17 ± 1	30 ± 5	35 ± 4	11.1 ± 0.7	7.4 ± 0.5	0.40 ± 0.06
44	40 ± 3	31 ± 1	22 ± 5	30 ± 1	11.5 ± 1.1	7.5 ± 0.7	0.24 ± 0.06
48	59 ± 1	23 ± 4	26 ± 0	44 ± 1	11.1 ± 0.8	6.5 ± 0.5	0.43 ± 0.07
73	65 ± 3	30 ± 2	25 ± 1	45 ± 3	11.2 ± 0.8	7.1 ± 0.6	0.57 ± 0.09
ITS group III (=G. telopeae)							
52	50 ± 4	28 ± 4	34 ± 2	31 ± 8	15.2 ± 1.1	10.6 ± 0.8	0.50 ± 0.7
ITS group IV (=P. spinarum)							
38	16 ± 4	8 ± 2	10 ± 1	13 ± 1	10.9 ± 2.2	8.2 ± 0.8	NA ^b

^a Isolate 1 (=CBS 111.20) failed to produce conidia. Values for this isolate have been taken from a previous study (36).
^b Not available: a few tiny pycnidia were produced in a single small dense cluster, which did not allow for measurement of individual pycnidial size.

Fig. 6. Comparison of the mucoid conidial sheath of *Guignardia citricarpa* sensu lato group I (= *G. citricarpa* proper) and group II (= *G. mangiferae*). A, Camera lucida drawings of conidia of group I (isolate 70). B, Camera lucida drawings of conidia of group II (isolate 48). C, Photograph of conidia of group II (isolate 36). Scale bar = 10 µm.

Fig. 7. Seven-day-old cultures of group I (= *Guignardia citricarpa* proper) isolates 1 and 37 (rows 1 and 2) and group II (= *G. mangiferae*) isolates 44 and 48 (rows 3 and 4). Left, oatmeal agar cultures; middle, malt extract agar cultures; and right, cherry decoction agar cultures.

TABLE 4. Hosts infected endophytically with *Guignardia mangiferae* (*Phyllosticta capitalensis*), identified as such by molecular analysis

Plant family	Host genus	Location	Source	Plant family	Host genus	Location	Source
Acanthaceae	<i>Mackaya</i>	South Africa	G. C. Carroll ^a	Oleaceae	<i>Schrebera</i>	South Africa	G. C. Carroll ^b
Anacardiaceae	<i>Comocladia</i>	Puerto Rico	G. C. Carroll ^a	Ophioglossaceae	<i>Botrychium</i>	Louisiana	G. C. Carroll ^a
	<i>Loxostylis</i>	South Africa	G. C. Carroll ^a	Orchidaceae	<i>Coelogyne</i>	Thailand	G. C. Carroll ^a
	<i>Mangifera</i>	Brazil	G. C. Carroll ^a		Orchid	New Zealand	Okane et al. (26)
		Ghana	Present study		<i>Paphiopedilum</i>	Germany	Okane et al. (26)
	<i>Rhus</i>	South Africa	Present study	Pittosporaceae	<i>Pittosporum</i>	Hawaii	Present study
	<i>Sclerocarya</i>	South Africa	G. C. Carroll ^a	Podocarpaceae	<i>Podocarpus</i>	South Africa	G. C. Carroll ^a
	<i>Spondias</i>	Brazil	G. C. Carroll ^a	Proteaceae	<i>Leucospermum</i>	Hawaii	G. C. Carroll ^a
Annonaceae	<i>Monanthes</i>	South Africa	G. C. Carroll ^a		<i>Protea</i>	Hawaii	G. C. Carroll ^a
Apocynaceae	<i>Secamone</i>	South Africa	G. C. Carroll ^a		<i>Telopea</i>	Australia	G. C. Carroll ^a
Aquifoliaceae	<i>Ilex</i>	Louisiana	G. C. Carroll ^a	Rhamnaceae	<i>Scutia</i>	South Africa	G. C. Carroll ^a
Araliaceae	<i>Cussonia</i>	South Africa	G. C. Carroll ^a		<i>Zizyphus</i>	South Africa	G. C. Carroll ^a
	<i>Hedera</i>	South Africa	G. C. Carroll ^a	Rosaceae	<i>Cliffortia</i>	South Africa	G. C. Carroll ^a
	<i>Polyscias</i>	Puerto Rico	G. C. Carroll ^a	Rubiaceae	<i>Canthium</i>	South Africa	G. C. Carroll ^b
	<i>Schefflera</i>	Costa Rica	Present study		<i>Coprosma</i>	Hawaii	Present study
Boraginaceae	<i>Cordia</i>	South Africa	G. C. Carroll ^b		<i>Gardenia</i>	South Africa	G. C. Carroll ^b
Capparaceae	<i>Maerua</i>	South Africa	G. C. Carroll ^b		<i>Pavetta</i>	South Africa	G. C. Carroll ^b
Celastraceae	<i>Putterlickia</i>	South Africa	Present study		<i>Rauvolfia</i>	South Africa	G. C. Carroll ^a
Chrysobalanaceae	<i>Parinari</i>	South Africa	G. C. Carroll ^b		<i>Rothmannia</i>	South Africa	G. C. Carroll ^b
Combretaceae	<i>Combretum</i>	South Africa	G. C. Carroll ^a	Rutaceae	<i>Citrus</i>	Argentina, Brazil, Australia, South Africa, Taiwan, Hong Kong, Mexico, Florida	G. C. Carroll ^a and present study
Cornaceae	<i>Curtisia</i>	South Africa	Present study		<i>Fortunella</i>	Louisiana	G. C. Carroll ^a
Ebenaceae	<i>Diospyros</i> (3 species)	South Africa	G. C. Carroll ^a		<i>Vitex</i>	South Africa	G. C. Carroll ^b
	<i>Euclea</i> (2 species)	South Africa	G. C. Carroll ^a		<i>Zanthoxylum</i>	Puerto Rico	Present study
Ericaceae	<i>Enkianthus</i>	Japan	Okane et al. (26)	Sapindaceae	<i>Allophylus</i>	South Africa	G. C. Carroll ^a
	<i>Rhododendron</i>	Japan	Okane et al. (26)		<i>Dodonaea</i>	Hawaii	G. C. Carroll ^a
Euphorbiaceae	<i>Clutia</i>	South Africa	Present study		<i>Litchi</i>	South Africa	G. C. Carroll ^b
	<i>Croton</i>	South Africa	G. C. Carroll ^b		<i>Paullinia cupana</i>	Brazil	C. Glienke de Blanco ^c
	<i>Ctenomeria</i>	South Africa	G. C. Carroll ^b	Smilacaceae	<i>Smilax</i>	South Africa	Present study
Flacourtiaceae	<i>Dovyalis</i>	South Africa	G. C. Carroll ^b	Stangeriaceae	<i>Stangeria</i>	South Africa	Present study
Iteaceae	<i>Itea</i>	Louisiana	G. C. Carroll ^a	Sterculiaceae	<i>Sterculia</i>	Puerto Rico	G. C. Carroll ^a
Lauraceae	<i>Ocotea</i>	South Africa	G. C. Carroll ^a	Theaceae	<i>Camellia</i>	Louisiana	Present study
Lecythidaceae	<i>Barringtonia</i>	South Africa	Present study	Tiliaceae	<i>Grewia</i>	South Africa	G. C. Carroll ^b
Loganiaceae	<i>Strychnos</i>	South Africa	G. C. Carroll ^a	Trimeniaceae	<i>Xymalos</i>	South Africa	G. C. Carroll ^b
	<i>Anthocleista</i>	South Africa	G. C. Carroll ^b	Ulmaceae	<i>Trema</i>	South Africa	G. C. Carroll ^b
Magnoliaceae	<i>Magnolia</i>	Louisiana	G. C. Carroll ^a	Veronicaceae	<i>Hebe (Veronica)</i>	South Africa	G. C. Carroll ^b
Menispermaceae	<i>Cocculus</i>	Louisiana	G. C. Carroll ^a	Viscaceae	<i>Viscum obscurum</i>	South Africa	Present study
Meliaceae	<i>Ekebergia</i>	South Africa	G. C. Carroll ^b	Vitaceae	<i>Rhoicissus</i>	South Africa	Present study
	<i>Trichilia</i>	South Africa	Present study		<i>Ampelopsis</i>	Louisiana	G. C. Carroll ^a
Moraceae	<i>Artocarpus</i>	Thailand	Present study		<i>Cyphostemma</i>	South Africa	G. C. Carroll ^b
Musaceae	<i>Musa</i>	Australia	Present study	Zamiaceae	<i>Encephalartos</i>	South Africa	Present study
Myrtaceae	<i>Eucalyptus</i>	South Africa, Brazil	G. C. Carroll ^a ; C. Glienke de Blanco ^c		<i>Zamia</i>	Florida	Present study
	<i>Psidium</i>	Brazil	C. Glienke de Blanco ^c				

^a Internal transcribed spacer sequence data.^b Data obtained by using species-specific primer pairs.^c Unpublished random amplified polymorphism DNA data.

endophyte of woody plants, most of which seem to harbor the fungus without any visual symptoms. On mango (15,27) and guava (C. Glienke de Blanco, *unpublished data*), however, the fungus has been associated with leaf spot and minor fruit spot, respectively. Under the name *P. capitalensis* it is also known as a pathogen of orchids (36). Our experience is that minute spots on grapefruit, lemon, and lime fruits may sometimes indeed produce cultures of *G. mangiferae*. However, in such cases, the fungus may have been present endophytically in the peel prior to lesion formation by pathogenic *Colletotrichum* spp., which are nearly always present in the peel. When selecting fruits with symptoms, it is therefore inevitable that endophytic fungi that do not cause these symptoms are also isolated. Altogether, *G. mangiferae* proves to be a ubiquitous endophyte that has been associated with leaf spots in few host species, not including *Citrus*. Co-occurrence of *G. citricarpa* and *G. mangiferae* on the same host (*Citrus* spp.) does not indicate evolutionary convergence in host specialization in two *Guignardia* spp. but clearly is a matter of coincidence. Many other host species of *G. mangiferae* also carry other *Guignardia* and *Phyllosticta* spp. (36).

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