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Comparison of Molecular Procedures for Detection and Identification of *Guignardia citricarpa* and *G. mangiferae*

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ABSTRACT

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Citrus black spot, caused by *Guignardia citricarpa*, is a serious fruit spot disease and is widely distributed in Asia, southern Africa, and South America, but does not occur in North America or the Mediterranean region. A nonpathogenic species, *G. mangiferae*, is cosmopolitan with a wide host range and can colonize citrus fruit and leaves saprophytically. Detection and identification of *Guignardia* spp. on citrus fruit is necessary for epidemiological, management, and regulatory purposes. In this study, we compared published and unpublished polymerase chain reaction primer sets for their specificity and sensitivity in the detection and differentiation of the two *Guignardia* spp. All primers evaluated successfully identified the two species using purified DNA from fungal cultures or mycelia as source materials. However, some primer sets were not highly effective in detecting *G. citricarpa* when DNA was extracted directly from single characteristic black spot lesions on fruit. Thus, new primer pairs for both species were designed from the internal transcribed spacer region that were highly sensitive and specific for detection of *G. citricarpa* using DNA recovered from single lesions on fruit by a rapid DNA extraction procedure.

Citrus black spot, caused by *Guignardia citricarpa* Kiely, has become a serious problem for citrus production in Asia and in many areas of the southern hemisphere (8,17,18,28). The pathogen produces airborne ascospores in decomposing leaf litter on the grove floor that form quiescent infections on fruit and leaves (12,13,17,18,20,22,31). Conidia produced by pycnidia of the anamorph of the fungus, *Phyllosticta citricarpa* (McAlpine) van der Aa, also may infect fruit and leaves. Symptoms on fruit include hard spot lesions (raised black edges and tan centers that often bear pycnidia), false melanose (small, raised, dark lesions that often coalesce and have been attributed to conidial infection), freckle spots (sunken reddish lesions that may coalesce to form large areas called virulent spot), and cracked spots (raised dark lesions with irregular margins) (10,14,17,18). Infections usually occur early in the season, from petal fall until about midsummer, but symptoms

usually do not develop until the fruit begin to mature (29). If symptoms develop early, fruit may abscise prematurely, reducing crop yield. Fruit with symptoms are not suitable for fresh market use; however, fruit that are asymptomatic at harvest may still develop symptoms during transport or in the market. Quiescent infections occur on leaves, but leaf spot symptoms are uncommon and occur mostly on highly susceptible species such as lemon.

Black spot has not been reported in the United States or in Mediterranean citrus-producing countries. Regulatory measures restrict market access for countries with black spot and reduce the availability of citrus fruit for consumers in the northern hemisphere (26) but potentially protect against the dissemination of the pathogen to other citrus-producing areas. The European Community accepts fruit from citrus areas with black spot, but fruit must be free of symptoms (4,23). The United States does not currently allow importation of fresh citrus fruit from areas with black spot. Because symptoms may develop during transport, entire loads of fruit may be rejected at the port of entry. Effective fungicide programs during the season may substantially reduce the number of infections in the field and quiescent infections that develop on fruit after harvest (1). Fungicide applications immediately before harvest or in the packinghouse, however, have been minimally effective or ineffec-

tive in preventing symptom development (1-3,25,30,36). Cold storage or storage in the dark delays but does not prevent symptom development (1,6,16,32).

For many years, pathogenic and nonpathogenic isolates of *G. citricarpa* have been recognized (7,13,19,21). The nonpathogen was distinguished by faster growth, colonies with entire rather than lobed margins, and production of pycnidia and ascogmata in culture. The nonpathogen occurs widely on many tropical and subtropical noncultivated and crop plants, including citrus (11), and its geographic distribution on citrus is much wider than that of the pathogenic strain (21). Meyer et al. (24) considered the pathogenic and nonpathogenic strains to be different species. Baayen et al. (4) described the nonpathogenic strain as *G. mangiferae* A. J. Roy and differentiated it from the pathogenic *G. citricarpa* based on the above morphological and cultural characteristics and the presence of mucoid sheaths on the conidia of *G. mangiferae*. The two species also were separated based on DNA sequences of the internal transcribed spacer (ITS) region and amplified fragment length polymorphisms.

Diagnosis of black spot is difficult unless typical hard-spot symptoms containing pycnidia of the fungus are present on fruit. The false melanose, virulent, freckle, and cracked spot symptoms can be confused with other diseases and disorders of citrus. *Guignardia* spp. are not easily isolated from fruit or leaves because the fungus is rather slow-growing and often overgrown by other fungi. No selective medium has been developed for these species. The regulatory protocol for the European Union calls for plating of lesion pieces onto agar and incubation for 14 days for formation of typical colonies of *G. citricarpa* (5). Lesions that were colonized by *G. mangiferae* have been misdiagnosed initially as black spot using this protocol on fruit in shipments from Florida. However, inoculations of wounded or unwounded fruit with this species did not produce lesions (*unpublished*). Although *G. mangiferae* generally is considered an endophyte or saprophyte, in Argentina this species has been associated with a symptom on lemon fruit referred to as "moteado" or speckled blotch (*G. Fogliata, personal communication*). Thus, once

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isolated, the fungus must be identified as *G. citricarpa* or *G. mangiferae*. The two species can be differentiated by colony morphology and other traits as described above; however, about 10 to 14 days are required for cultures to produce enough growth to be identified (4). Because fruit infections by *G. citricarpa* typically are quiescent for long periods, pathogenicity tests on fruit are difficult and require extended periods. Thus, molecular diagnostic tests are important to be able to identify the species associated with specific types of lesions for etiological studies.

Glienke-Blanco et al. (9) developed a polymerase chain reaction (PCR) technique using specific primers for *G. citricarpa* with sequences derived from random amplified polymorphic DNA (RAPD) markers. Bonants et al. (5) developed a PCR test using primers from the ITS region to detect *G. citricarpa* directly from fruit lesions. The test was effective in differentiation of *G. citricarpa* from *G. mangiferae* and other common fungi occurring on citrus fruit. However, a five-lesion minimum sample was required to attain a high level of accuracy and the DNA extraction procedure was lengthy. This technique was patented and was available commercially (MGT, Inc., Eugene, OR), but sale of this product has been discontinued. Subsequently, Meyer et al. (23) developed a 1-day PCR method to detect and distinguish *G. citricarpa* and *G. mangiferae* using primers from the ITS region. This method uses a commercially available and relatively expensive kit for rapid DNA extraction from fruit lesions that may not be available in some situations. In many cases, the number of lesions present in fruit shipments is low and lesions are not necessarily identical. Pooling of such lesions for analysis may result in dilution of DNA of the pathogen with that from saprophytes in wounds.

The objectives of this study were to compare the molecular methods available to determine the most effective means of rapid detection and differentiation of *G. citricarpa* and *G. mangiferae* using small amounts of tissue. In addition, new primers and a rapid, less-expensive protocol for detection and differentiation of these two species were developed.

MATERIALS AND METHODS

Fungal isolates and culture. Thirty isolates of *G. citricarpa* were obtained from small sections from the edge of typical black spot symptoms such as hard spot, virulent spot, and false melanose from fruit of sweet orange (*Citrus sinensis*) in the state of São Paulo, Brazil. Seven isolates of *G. mangiferae* were recovered in Florida from injuries on fruit peel of sweet orange or grapefruit (*C. paradisi*) that sometimes resembled hard spot symptoms but had no pycnidia. Another six isolates each of *G. citricarpa* and *G. mangiferae*

from Brazil were supplied by C. Aguilar (Centro de Citricultura, Cordeiropolis, São Paulo, Brazil). For isolations, fruit pieces were dipped in 70% ethanol for 30 s, surface disinfested in 1% NaOCl for 2 min, rinsed twice in sterilized distilled water, and blotted dry. Tissue pieces were placed on carrot dextrose agar (200 g of macerated carrot, 68.5 g of dextrose, and 20 g of agar per 1 liter of distilled water) and incubated at 24°C with a 12-h photoperiod for 10 to 12 days. Isolates were maintained as dry cultures on sterile filter paper in sealed plastic containers with silica gel at -20°C (27).

DNA extraction from fungal isolates. For DNA extraction from fungal cultures, isolates were grown for 7 days in potato-dextrose broth (Difco Laboratories, Detroit) on a rotary shaker and mycelia were recovered and ground to a fine powder using liquid nitrogen. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. In other cases, small amounts of mycelia from the surface of the colonies produced on agar plates were collected with a sterile pipette tip and were used directly as DNA templates.

DNA extraction from fruit lesions. For DNA extraction from single, very small fruit lesions (approximately 2 to 4 mm in diameter), the diseased tissue was dissected out, removing and discarding as much as possible of the surrounding healthy flavedo, the pigmented outer tissue of the fruit rind. Different methods of tissue disruption, such as grinding with liquid nitrogen or sand, use of an electric rotary tool (Dremel, Racine, WI), or exposure to ultrasound or heat, were tested with the following DNA extraction techniques: (i) the DNeasy Plant Mini Kit according to the manufacturer's instructions, (ii) a Chelex DNA extraction protocol (34) that was modified by centrifugation for 5 min at 12,000 × g instead of for 10 to 20 s, (iii) a protocol using polyvinylpyrrolidone (PVPP) spin columns (Sigma-Aldrich Inc., St. Louis) according to the manufacturer's instructions, (iv) a combination of the Chelex procedure followed by purification with the PVPP spin columns, (v) a procedure using the DNAzol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, (vi) the Ultra Clean Plant DNA isolation and Ultra Clean Soil DNA kits (MO Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions, (vii) a single-step protocol for preparation of plant tissue for PCR (33), (viii) an alkaline lysis DNA extraction protocol (15), (ix) the alkaline lysis DNA extraction protocol followed by purification with PVPP spin columns, and (x) the alkaline lysis DNA extraction protocol followed by purification using a dipstick method. With the dipstick method, 150 µl of 100% ethanol and a small piece

of cellulose thin-layer chromatography plate (dipstick) were added to the 2-ml tube after alkaline lysis. Tubes were placed on their sides on ice and shaken for 30 min. Liquid was aspirated off and 500 µl of wash buffer (10× [Tris, Na₂EDTA, and NaCl, pH 7.0], and 95% ethanol) diluted to 25% was added and tubes were inverted to mix the contents. Washing was repeated twice. The dipsticks were placed in a new tube and dried under vacuum. Tubes were placed on their sides and 50 µl of Tris-EDTA buffer was added. After incubation for 5 min, tubes were spun for 10 s, the dipstick was removed and discarded, and the DNA was recovered.

DNA amplification. Amplifications were carried out in total reaction volume of 20 µl. PCR mixtures contained 8 µl of 2.5× Eppendorf MasterMix (Taq DNA polymerase at 0.06 U/µl), 2.5× Taq reaction buffer (with 125 mM KCl, 75 mM Tris-HCl, pH 8.4, 4 mM Mg²⁺, and 0.25% Nonidet-P40, 500 µM each of dNTP, and stabilizers), 0.8 µl of each primer at 10 µM, and 2 µl of template DNA or mycelia. Amplifications were performed in either a PTC100 or PTC200 thermocycler (MJ Research, Watertown, MA) programmed for 94°C for 2 min; followed by 39 cycles at 94°C for 30 s, annealing for 30 s, and 72°C for 1 min; and a final extension for 10 min at 72°C. Annealing temperatures are given in Table 1. PCR products were separated by electrophoresis in 1% agarose gels in 1× Tris-borate EDTA buffer.

Comparison of species-specific primers. Seven primer sequences for *G. citricarpa* and five primer sequences for *G. mangiferae* were used (Tables 1 and 2). The specificity and the sensitivity of all primers were evaluated using purified DNA extracts and mycelia from *G. citricarpa* and *G. mangiferae* cultures.

Evaluation of species-specific primer pairs using DNA extracted from single black spot fruit lesions. The detection of *G. citricarpa* using DNA from the different extraction techniques from a single black spot lesion was done initially with primer pairs LM-SA-ITS-Gc and CB-BR-RP-Gc, and general primers ITS1 and ITS4 (35). Mycelia from pure fungal cultures were used as positive controls. Fungal mycelia also were mixed with DNA extracted from single black spot lesions to determine the effect of potential inhibitors in the PCR reactions. All experiments were repeated at least once and most many times with consistent results, except as otherwise noted.

Development of new *Guignardia* sp.-specific primers from rDNA ITS sequence data. Primers GCN and GCMR (NP-Br-ITS-Gc) for *G. citricarpa* and GMN and GCMR (NP-Br-ITS-Gm) for *G. mangiferae* were designed by the authors based on DNA sequences from the ITS region of rDNA deposited in the GenBank

(accessions AY042911, AY042913, AY042915, AY042917, AY042919, and AY042921). Primers were constructed from the part of the ITS region that showed the greatest differences between *G. citricarpa* and *G. mangiferae*. Annealing temperatures and formation of secondary structure for the primers were evaluated using the computer program DNA Calculator from Sigma-Genosys. Primers were tested under different annealing temperatures using DNA extracts or using mycelia directly as template DNA.

Sensitivity of *Guignardia* sp.-specific primers. The detection limits of selected primer sets NP-Br-ITS-Gc, PB-N-ITS-Gc, and LM-SA-ITS-Gc were evaluated by performing PCR using dilutions of known amounts of purified DNA. DNA concentration was determined by UV spectrophotometry. PCR conditions were the same as described above for each primer set. The sensitivity of primers also was evaluated using known amounts of fungal DNA mixed with citrus fruit DNA. For this evaluation, mycelia from pure fungal cultures were added to dilutions of citrus tissue extract (1 g of fruit peel in 9 ml of distilled water) or known amounts of purified fungal DNA was mixed with citrus fruit DNA. The specificity of primer pairs

NP-Br-ITS-Gc, PB-N-ITS-Gc, and LM-SA-ITS-Gc also was tested using DNA of some common citrus pathogens, including *Alternaria alternata*, *Colletotrichum acutatum*, *C. gloeosporioides*, *Diaporthe citri*, *Mycosphaerella citri*, and *Penicillium digitatum*. All experiments were repeated at least once and most many times with consistent results, except where otherwise noted.

Evaluation of primers for detection of *Guignardia* spp. from black spot-like symptoms in Florida. The NP-Br-ITS-Gc and NP-Br-ITS-Gm primer pairs were evaluated for detection of *Guignardia* spp. in Florida. Necrotic lesions on grapefruit and orange fruit collected from groves near Lake Alfred, FL were dissected and half of each lesion was used for isolations on culture media and the other half for PCR. In addition, decaying leaves collected from grapefruit groves near Lake Alfred with fungal structures fitting the description of *G. mangiferae* were used to evaluate the detection of this species with primer pair NP-Br-ITS-Gm. Leaves without those fungal structures were used for comparisons. All experiments were repeated at least once and most many times with consistent results, except where otherwise noted.

RESULTS

Evaluation of species-specific primers using purified DNA from fungal isolates or mycelium. Primer pairs listed in Tables 1 and 2 were evaluated for their ability to detect and differentiate *G. citricarpa* and *G. mangiferae* using purified DNA extracted from cultures of these species. All pairs produced amplicons specifically of *G. citricarpa* or *G. mangiferae* with the respective primer pairs (Table 3). However, primer pairs GC-OR-ETS-1-Gc, GC-OR-ETS-2-Gc, GC-OR-ETS1-Gm, GC-OR-ETS2-Gm, and GC-OR-CS-Gm were not as consistent as some of the other primer pairs.

All primer pairs were also effective in specifically detecting *G. citricarpa* and *G. mangiferae* using mycelium directly in PCR. However, primer pairs GC-OR-ITS-Gc, CB-Br-RP-Gc, LM-SA-ITS-Gc, PB-N-ITS-Gc, and NP-Br-ITS-Gc were more consistent in showing an intense amplicon of expected size (Table 3).

For example, primer pair LM-SA-ITS-Gc, developed in South Africa, produced specific bands with mycelia of 20 different isolates of *G. citricarpa* from Brazil, but did not produce bands with the 12 different isolates of *G. mangiferae* (Fig. 1A). With specific primers for *G. mangiferae*, primer

Table 1. Primer pairs used in polymerase chain reaction (PCR) to detect and identify *Guignardia citricarpa*

Designation	Primer pair (reference)	Primer sequence	Origin ^a	Annealing temperature (°C)	PCR product size (bp)
GC-OR-ITS-Gc	ITS 1F	TAAAAAAGCCGCCGACCTAC	ITS	55	300
	5.8S 2R	TGCAATTCACATTACTTATCGC
GC-OR-ETS1-Gc	FRAG 1-4F	TCAGACACTCGGGGGTAAG	ETS	53	280
	1468 VI-1R	TGCTGGCACTCGTGAGAG
GC-OR-ETS2-Gc	1468 VI-1F	TCTCACGAGTGCCAGCAG	ETS	53	380
	SSU 9R	CTAGAATTACCACGGTTATCC
GC-OR-CS-Gc	CIT 2-1 CHS-2F	TATGTCTCCCCTTTCCTCG	Chitin synthase	53	180
	CHS 4R	TGATTCCTTTCTTCAAGCAG
CB-Br-RP-Gc	GCP1 (9)	AAGTGTGAGTGTCGAAGGTGG	RAPD	67	370
	GCP2 (9)	GACGACTCGCTTTTCTACGGC
LM-SA-ITS-Gc	CITR11 (23)	GAAAGGTGATGGAAGGGAG	ITS	55	580
	ITS4 (35)	TCCTCCGCTTATTGATATGC
PB-N-ITS-Gc	GCF3 (5)	AAAAAGCCGCCGACCTACCT	ITS	65	490
	GCR7 (5)	TGTCCGGCGCCAG
NP-Br-ITS-Gc	GCN	CTGAAAGGTGATGGAAGGGAGG	ITS	64	300
	GCMR	CATTACTTATCGCATTTTCGCTGC

^a ITS = internal transcribed spacer, ETS = external transcribed spacer, and RAPD = random amplified polymorphic DNA.

Table 2. Primer pairs used in polymerase chain reaction (PCR) to detect and identify *Guignardia mangiferae*

Designation	Primer pair (reference)	Primer sequence	Origin ^a	Annealing temperature (°C)	PCR product size (bp)
GC-OR-ETS1-Gm	CITR 3-1 VI-1F	CTCGCAAAGTAACCCCTTC	ETS	53	300
	SSU 11R	TCTAATAAATACACCCCTTCC
GC-OR-ETS2-Gm	CITR 3-1 VI-3F	TAATACTCTGCCCTCAC	ETS	53	320
	SSU 11R	TCTAATAAATACACCCCTTCC
GC-OR-CS-Gm	CHS 1F	CAGTTTGAAGCCTTTCGGG	Chitin synthase	53	180
	CHS 1R	AGAGTTGATTTTCTTTTGATTC
LM-SA-ITS-Gm	CAMEL2 (23)	AGTATACAAAACCTCAAGAATTC	ITS	55	430
	ITS4 (35)	TCCTCCGCTTATTGATATGC
PB-N-ITS-Gm	TAECTTCTATTGAAAGGTTCCAGAGT	TAECTTCTATTGAAAGGTTCCAGAGT	ITS	65	210
	GCR4 (5)	TCAGGACTTCACAAAATGAATTCTT
NP-Br-ITS-Gm	GMN	CGTACAACGCCGAAATGA	ITS	64	290
	GCMR	CATTACTTATCGCATTTTCGCTGC

^a ETS and ITS = external and internal transcribed spacer, respectively.

pair GC-OR-ETS2-Gm produced bands of the expected size with all 13 different isolates of *G. mangiferae*, but did not yield any products with 26 isolates of *G. citricarpa* (Fig. 1B). Primer pair LM-SA-ITS-Gm did not amplify any products with mycelia of 20 different isolates of *G. citricarpa*, but amplified specific products with all 11 isolates of *G. mangiferae* tested (Fig. 1C).

Evaluation of species-specific primers using DNA extracted from single black spot lesions. The 10 DNA extraction procedures described in Materials and Methods were evaluated by performing PCR using DNA from single fruit lesions to determine the most effective extraction procedures. However, *G. citricarpa* was not detected using the primer pair LM-SA-ITS-Gc with any of the extraction procedures tested and bands were produced only with the positive controls where mycelia from fungal colonies were used as DNA templates (Table 3). When positive samples were added to the DNA extracted from single fruit lesions, products were amplified effectively and, thus, there was no evidence for the presence of inhibitors. Primer pairs GC-OR-ITS-Gc, GC-OR-ETS2-Gc, GC-OR-CS-Gc, and LM-SA-ITS-Gc all amplified products with mycelia, but only primer pair GC-OR-ITS-Gc proved to be more sensitive than the others when purified DNA from a single fruit lesion was used (Fig. 2). In general, products were amplified much more effectively when using mycelia rather than DNA from single fruit lesions (Table 3).

Evaluation of new primers developed from ITS sequence data. Because existing primer pairs showed low sensitivity for detecting *Guignardia* spp. when using DNA extracted from single fruit lesions using a quick extraction method, the new primer pairs NP-Br-ITS-Gc and NP-Br-ITS-Gm (Tables 1 and 2) were designed from the ITS region using data available in

GenBank. Primer pair NP-Br-ITS-Gc was compared with the previously published primer pairs (5,23), designated herein as PB-N-ITS-Gc and LM-SA-ITS-Gc, respectively, for sensitivity in detection of *G. citricarpa* using purified DNA extracted

from fungal cultures, mycelia, and DNA from single fruit lesions as source materials. For the latter samples, the alkaline hydrolysis procedure followed by the dipstick method was chosen. When DNA from pure fungal cultures was used, tem-

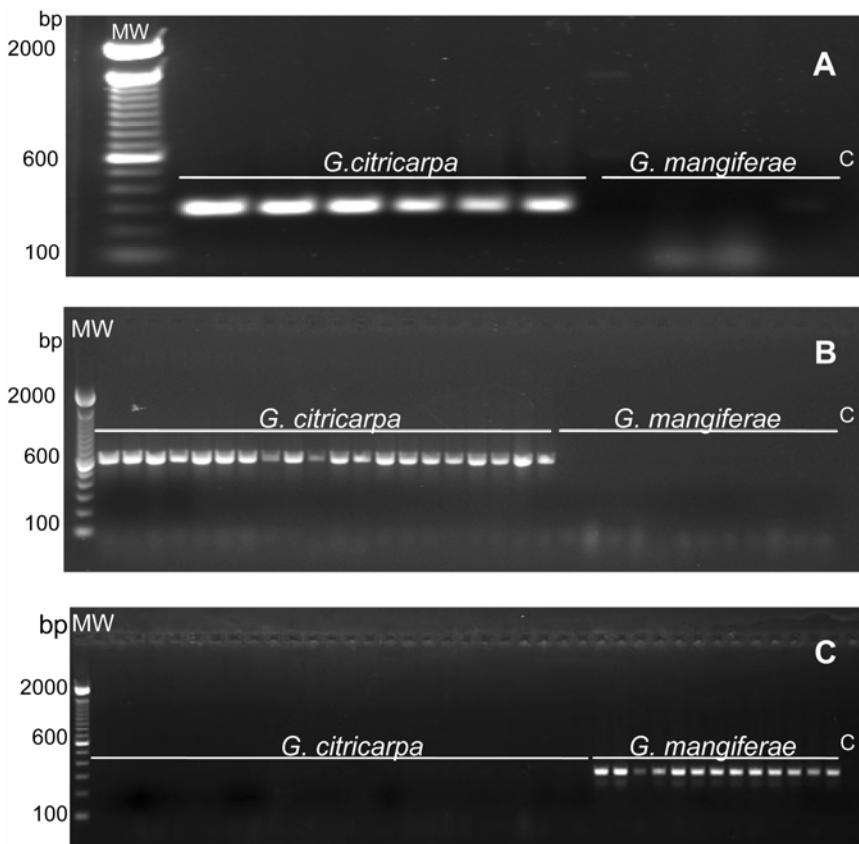


Fig. 1. Detection and identification of *Guignardia citricarpa* using DNA extracts from fungal cultures or mycelia directly for polymerase chain reaction. **A**, Primer pair CB-Br-RP-Gc: lanes 2–7, DNA extracts of different isolates of *G. citricarpa*; lanes 8–11, DNA extracts of different isolates of *G. mangiferae*; lane 12, no DNA control. **B**, Primer pair LM-SA-ITS-Gc: lanes 2–21, mycelia of different isolates of *G. citricarpa*; lanes 22–33, mycelia of different isolates of *G. mangiferae*; lane 33, no DNA control. **C**, Primer pair GC-OR-ETS2-Gm: lanes 2–26, mycelia of different isolates of *G. citricarpa*; lanes 27–39, mycelia of different isolates of *G. mangiferae*; lane 40, no DNA. The molecular weight (MW) standard is a 100-bp DNA ladder.

Table 3. Comparison of species-specific primers for *Guignardia citricarpa* and *G. mangiferae* to detect and identify each fungus using DNA extracts from fungal cultures, mycelia directly, or extracts from single black spot lesions as templates in polymerase chain reaction (PCR) reactions

Species ^b	Primer pair	Identification in DNA extracts from fungal cultures		Detection using mycelia directly in PCR		Detection from lesion ^a
		<i>G. citricarpa</i>	<i>G. mangiferae</i>	<i>G. citricarpa</i>	<i>G. mangiferae</i>	<i>G. citricarpa</i>
<i>G. citricarpa</i>	GC-OR-ITS-Gc	++ ^c	–	++	–	±
<i>G. citricarpa</i>	GC-OR-ETS1-Gc	±	–	+	–	–
<i>G. citricarpa</i>	GC-OR-ETS2-Gc	±	–	+	–	–
<i>G. citricarpa</i>	GC-OR-CS-Gc	++	–	+	–	–
<i>G. citricarpa</i>	CB-Br-RP-Gc	++	–	++	–	–
<i>G. citricarpa</i>	LM-SA-ITS-Gc	++	–	++	–	–
<i>G. citricarpa</i>	PB-N-ITS-Gc	++	–	++	–	–
<i>G. citricarpa</i>	NP-Br-ITS-Gc	++	–	++	–	+
<i>G. mangiferae</i>	GC-OR-ETS1-Gm	–	±	–	+	n/t
<i>G. mangiferae</i>	GC-OR-ETS2-Gm	–	±	–	+	n/t
<i>G. mangiferae</i>	GC-OR-CS-Gm	–	±	–	+	n/t
<i>G. mangiferae</i>	LM-SA-ITS-Gm	–	+	–	+	n/t
<i>G. mangiferae</i>	PB-N-ITS-Gm	–	+	–	+	n/t
<i>G. mangiferae</i>	NP-Br-ITS-Gm	–	+	–	+	n/t

^a Detection in extract from a single black spot lesion.

^b Specificity of designed primer pair.

^c Symbols: ++ = intense amplicon of expected size produced, + = amplicon of expected size produced, – = no amplicon produced, ± = inconsistent reactions, and n/t = not tested.

plate thresholds for the detection of *G. citricarpa* were concentrations of 0.001 ng/μl for NP-Br-ITS-Gc, 0.01 ng/μl for PB-N-ITS-Gc, and 0.1 ng/μl for LM-SA-ITS-Gc (Fig. 3). When primer pairs LM-SA-ITS-Gc were tested using an annealing temperature of 64°C instead of the recommended 56°C (23), detection of *G. citricarpa* was achieved with a template concentration of 0.001 ng/μl (data not shown).

The NP-Br-ITS-Gc primer pair was evaluated for the ability to detect *G. citricarpa* using DNA from single fruit lesions from different symptoms on fruit. In this case, the alkaline hydrolysis procedure followed by the dipstick method also was used. This primer pair consistently amplified products of expected size from hard spot, virulent spot, and cracked spot lesions. In the example in Figure 4, specific products for *G. citricarpa* were amplified from 15 of 16 lesions. When fruit extracts or fruit DNA were added to purified DNA of *G. citricarpa*, no inhibitory effects on the level of detection of *G. citricarpa* were found (Fig. 5). Primer pair NP-Br-ITS-Gc was specific for *G. citricarpa* and did not produce products using DNA or mycelia from other citrus pathogens such as *A. alternata*, *C. acutatum*, *C. gloeosporioides*, *D. citri*, *M. citri*, or *P. digitatum* (data not shown).

Evaluation of primers for detection of *Guignardia* spp. from black-spot-like symptoms in Florida. When the NP-Br-ITS-Gc and NP-Br-ITS-Gm primer pairs were evaluated for detection of *Guignardia* spp. on fruit lesions in Florida, neither primer pair detected the presence of these fungi. However, *Guignardia* spp. were not isolated from the other half of the lesions either. Addition of DNA from *G. mangiferae* to DNA from negative samples from fruit lesions showed that inhibitors probably did not play a role in the failure to detect *Guignardia* spp. from fruit lesions in Florida (Fig. 5). When the NP-Br-ITS-Gc and NP-Br-ITS-Gm primer pairs were evaluated for detection of *Guignardia* spp. on decaying leaves bearing fungal struc-

tures, primer pair NP-Br-ITS-Gm was able to detect *G. mangiferae* from one of nine leaf samples (data not shown).

DISCUSSION

Molecular procedures for detection and differentiation of *G. citricarpa* and *G. mangiferae* have been developed and improved in recent years (5,9,23,24). Species-specific primers were developed for *Guignardia* spp. from the ITS region (5,23,24) and from RAPD bands (9). Bonants et al. (5) developed a PCR detection method using primers from the ITS region which was highly effective and specific for detecting *G. citricarpa*. However, the DNA extraction procedure required was lengthy and a five-lesion sample was required for extraction. Meyer et al. (23) were able to successfully detect and differentiate the two species using different primers from the ITS region and a rapid DNA extraction technique. In our studies, we were not able to detect *G. citricarpa* from one single black spot lesion when using their protocol. In addition, the DNA extraction procedure used a commercial kit that may not be available or affordable in some areas. However, that procedure (23) proved to be more effective when the annealing temperature was increased.

The original purpose of our study was to compare the various primers and procedures available and to evaluate their effectiveness. Nearly all of the primer sets developed previously from RAPD bands (9) and from the ITS region (5,23,24), as well as previously unpublished primer sequences from the external transcribed spacer and chitin synthase regions, were effective for detecting and differentiating the two species when purified DNA extracted from fungal cultures or mycelia were used as DNA templates. Thus, it appeared that any of the primer sets would be useful for identification of these species in culture and for detection in fruit if sufficient tissue were available.

Because *Guignardia* spp. are slow-growing fungi and sometimes difficult to

isolate, a rapid detection direct from fruit lesions would be ideal. However, problems were encountered in the detection using DNA from a single fruit lesion. Thus, primer sets LM-SA-ITS-Gc and CB-BR-IP-Gc were selected to evaluate a number of DNA extraction procedures. This choice was unfortunate because it subsequently was found that other primer sets, such as GC-OR-ITS-Gc, proved more sensitive for detection from fruit lesions using quick DNA extraction protocols (Fig. 3). The main difficulty encountered for DNA extraction from single lesions from fruit tissue was that the small size of the tissue piece made it very difficult to grind or macerate. Thus, the alkaline lysis protocol

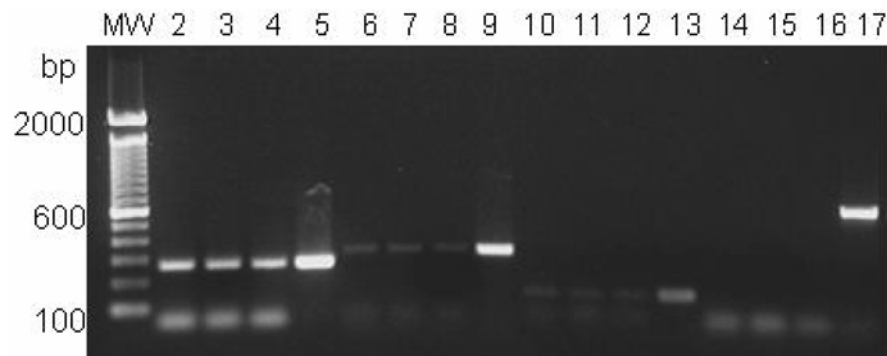


Fig. 2. Sensitivity of selected primer pairs (as indicated) for detection of *Guignardia citricarpa* using DNA from symptomatic orange peel lesions that was isolated by a quick extraction protocol adapted from Klimyuk et al. (15). The fourth lane in each set is a positive control using mycelia from isolate BS-CCSM-1; whereas, in the first three lanes in each set, DNA extracts from fruit tissue were used as DNA templates. The molecular weight (MW) standard is a 100-bp DNA ladder.

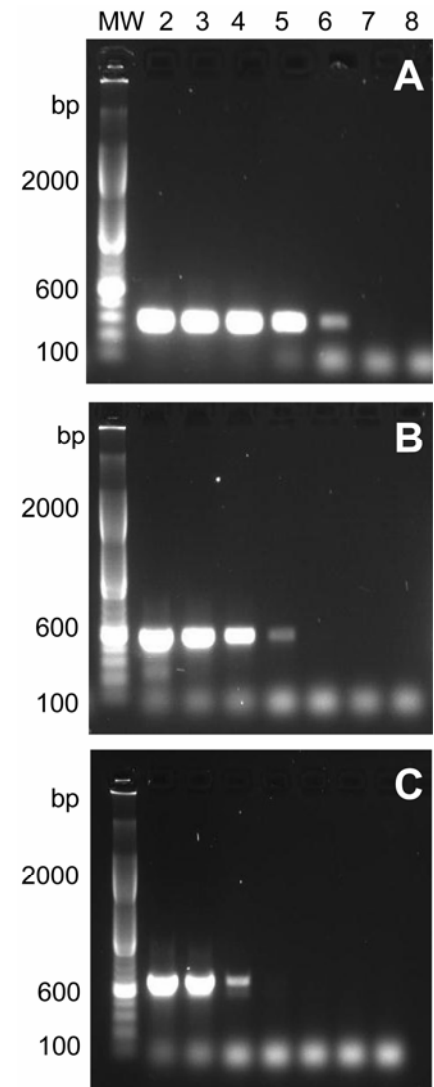


Fig. 3. Comparison of the sensitivity of primers designed in this study with other previously published primers. DNA was extracted from cultures of *Guignardia citricarpa* and polymerase chain reaction were carried out with **A**, primer pairs NP-Br-ITS-Gc; **B**, primer pairs PB-N-ITS-Gc; and **C**, primer pairs LM-SA-ITS-Gc. Lane 2, 5 ng/μl; lane 3, 1 ng/μl; lane 4, 0.1 ng/μl; lane 5, 0.01 ng/μl; lane 6, 1 pg/μl; lane 7, 0.1 pg/μl; lane 8, no DNA control. The molecular weight (MW) standard is a 100-bp DNA ladder.

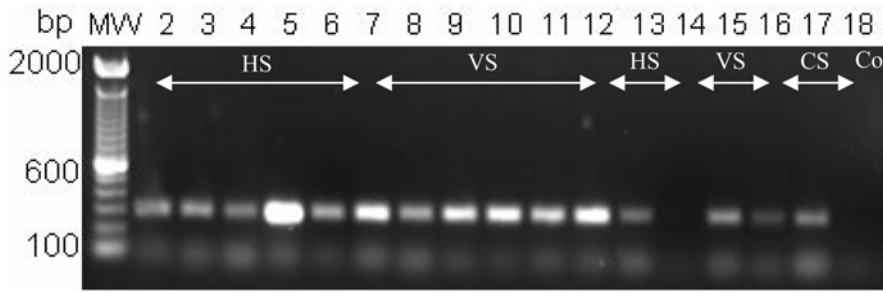


Fig. 4. Detection of *Guignardia citricarpa* in single hard spot (HS), virulent spot (VS), or cracked spot (CS) fruit lesions using primer pair NP-Br-ITS-Gc. The last lane is a no-DNA control. The molecular weight (MW) standard is a 100-bp DNA ladder.

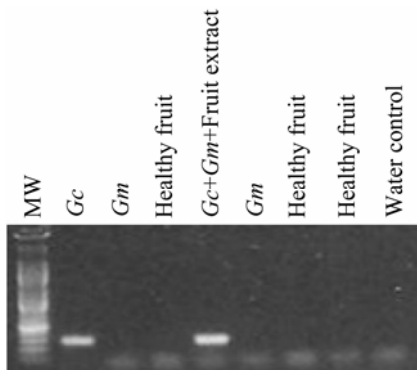


Fig. 5. Effect of flavedo extracts on polymerase chain reaction detection of DNA of *Guignardia citricarpa* (*Gc*) and *G. mangiferae* (*Gm*) using primer pair NP-Br-ITS-Gc. *G. citricarpa* was detected in the *Gc+Gm+fruit extract* mixture. Controls consisted of water or extracts from healthy fruit. The molecular weight (MW) standard is a 100-bp DNA ladder.

followed by the dipstick, where boiling was used to break down the tissue, was chosen and proved to be very effective.

Subsequently, we developed another primer set from the ITS region that proved to be highly effective for the detection of *G. citricarpa* from single fruit lesions using the alkaline lysis rapid DNA extraction procedure. This primer set NP-Br-ITS-Gc was able to detect *G. citricarpa* in various types of black spot lesions and was very sensitive, and flavedo extracts did not interfere with the detection. In limited tests, the *G. mangiferae* primer set NP-Br-ITS-Gm was able to detect this fungus in decaying citrus leaves in Florida. However, we detected *G. mangiferae* in only one of the nine samples; thus, it appears that the fungal structures observed on the other leaves were of an unrelated fungal species. It did not detect *G. mangiferae* in fruit lesions, but neither were *Guignardia* spp. isolated from any of these lesions.

We have developed a highly specific, rapid, and inexpensive assay for the detection and differentiation of *G. citricarpa* and *G. mangiferae*. Most of the other primer sets evaluated were species specific and useful for detection. However, in our work, they often were less sensitive than the new primers when quick DNA extraction protocols from single fruit lesions

were used. Several molecular systems are now available and can be used for detection on commercial fruit and in studies of the etiology of this disease on citrus.

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