SPECIES-SPECIFIC PCR PRIMERS FOR *GUIGNARDIA CITRICARPA* AND *GUIGNARDIA MANGIFERAE*

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ABSTRACT

The plant pathogen *Guignardia citricarpa* causes citrus black spot and is not considered to be present in New Zealand. Species-specific polymerase chain reaction (PCR) primers were designed to identify *G. citricarpa* and *G. mangiferae*, a closely related saprotroph that is present in New Zealand. These PCR primers were tested against a range of other saprotrophic and pathogenic fungi viz. *Botrytis cinerea*, *Botryosphaeria dothidea*, *B. parva*, *Cladosporium* sp., *Colletotrichum acutatum*, *C. gloeosporioides*, *Cryptosporiopsis* sp., *Epicoccum* sp., *Nigrospora* sp., *Penicillium* sp., *Pestalotia* sp., *Phialophora* sp., *Phlyctema* sp., *Phoma* sp., *Phomopsis* sp., *Stemphylium* sp. and *Venturia inaequalis*. The primers JRGGc were specific to *G. citricarpa*, and JRGGm to *G. mangiferae*. A 226 bp product was amplified from *G. mangiferae* DNA using JRGGm primers, and a 501 bp product was amplified from *G. citricarpa* DNA using JRGGc primers. These primers thus distinguished *G. citricarpa* from *G. mangiferae*, and can be used to rapidly identify incursions by citrus black spot.

Keywords: citrus black spot, saprotrophic strain, PCR, species-specific primers, ITS.

INTRODUCTION

Citrus black spot is a fungal disease caused by *Guignardia citricarpa* Kiely, first reported in Australia by Benson (1895) and later named and described by Kiely (1948). Citrus black spot can cause significant economic losses from disfigurement of fruit, rejection for export, and, in severe outbreaks, fruit drop in the orchard. All varieties of citrus apart from sour orange (*Citrus aurantium*) are affected. Symptoms of the disease are round, dark, depressed spots of increasing size and severity described as occurring in three stages: hard spot, freckle spot and virulent spot (Kiely 1948).

Citrus black spot has been recorded in Russia, Asia (Bhutan, China, Indonesia and Philippines), Africa (Kenya, Mozambique, South Africa, Zambia and Zimbabwe), South America (Argentina and Brazil) and Oceania (Australia, New Zealand and Vanuatu) (CAB International 2000). Citrus black spot was recorded as present in New Zealand on the basis of one isolate (ICMP 8336) from Seminole tangelo that was identified by morphological features as *Guignardia citricarpa*. Sequence analysis of the internal transcribed spacer (ITS) region (ITS1, 5.8S and ITS2) showed that this isolate was 99.7% identical to the ITS region of *G. mangiferae*, a closely related saprotroph (Everett & Rees-George 2006). Citrus black spot symptoms have not been seen on fruit grown in New Zealand (MAF 1992; Everett & Rees-George 2006). On this basis the record of citrus black spot occurring in New Zealand was re-examined and is no longer considered present.

Meyer et al. (2001) and Baayen et al. (2002) showed overlap in morphological features that were used to distinguish *G. mangiferae* from *G. citricarpa* and that DNA sequence analysis of ITS regions reliably separated the two strains. Species-specific polymerase chain reaction (PCR) primers can utilise the polymorphisms in ITS sequence, and PCR is less time-consuming than ITS sequencing. Primers utilising ITS sequence were

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designed by other workers, but were patented and were not available for general use (Bonants et al. 2003). Recently Meyer et al. (2006) have also designed and published primers for detection of this pathogen. Everett & Rees-George (2006) identified a region of polymorphisms in ITS sequence that was suggested as being suitable for primer design. Primers complementary to this region were designed and results are presented here.

METHODS

Fungi used in PCR tests

Four cultures deposited as *G. citricarpa* were obtained from the International Collection of Micro-organisms from Plants (ICMP; Manaaki Whenua, Landcare Research, Lincoln, New Zealand) (Table 1). A number of isolates of commonly occurring saprophytic and pathogenic fungi from kiwifruit, avocado, apple and squash identified by culture and spore morphology were deposited in ICMP (Table 1). These isolates were used for cross-genera tests of PCR primer specificity.

TABLE 1: ICMP number and plant species from which fungi used in cross-genera PCR tests were isolated.

Fungus	ICMP number	Plant of origin
1. *Botryosphaeria dothidea*	15694	Avocado
2. *Botryosphaeria parva*	15695	Avocado
3. *Botrytis cinerea*	15696	Squash
4. *Cladosporium* sp.	15697	Avocado
5. *Colletotrichum acutatum*	15698	Avocado
6. *C. acutatum*	13949	Apple
7. *C. gloeosporioides*	12071	Apple
8. *Cryptosporiopsis* sp.	15699	Kiwifruit
9. *Epicoccum* sp.	15700	Squash
10. *Guignardia citricarpa*	7825	Citrus
11. *G. citricarpa*	7874	Citrus
12. *G. mangiferae*	7872	Citrus
13. *G. mangiferae*	8336	Citrus
14. *Nigrospora* sp.	15701	Avocado
15. *Penicillium* sp.	15702	Avocado
16. *Pestalotia* sp.	15703	Avocado
17. *Phialophora* sp.	15704	Kiwifruit
18. *Phoma* sp.	23[1]	Avocado
19. *Phomopsis* sp.	12[1]	Avocado
20. *Phlyctema* sp.	15708	Kiwifruit
21. *Stemphylium* sp.	15707	Avocado
22. *Venturia inaequalis*	13258	Apple

[1]Isolate numbers are laboratory numbers and these isolates were not deposited in ICMP because of mortality in storage. Similar isolates ICMP 15705 (*Phoma* sp.) and ICMP 15706 (*Phomopsis* sp.) were deposited instead.

Fungal DNA extraction

Fungal spores and mycelia were harvested from 12-week cultures grown on Difco® Potato Dextrose Agar and DNA extracted using the methods described in Everett & Rees-George (2006).

Species-specific PCR primers

Species-specific primers to distinguish *G. citricarpa* and *G. mangiferae* were designed from ITS and ribosomal DNA regions of rDNA (Table 2). The PCR reaction mixture

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(25 µl final volume) included dNTP (0.2 mM of each), Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) (0.75 U), *Taq* DNA polymerase buffer (supplied with the enzyme), $MgCl_2$ (1.5 mM), 0.5 µM of each primer, and approximately 5 ng of template DNA. Thermocycling conditions were an initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 30 s (denaturation), 62.5°C for 30 s (annealing) and 72°C for 60 s (elongation), and a final elongation of 72°C for 7 min. The quality and sizes of PCR products were determined on an agarose gel (10 g/litre) stained with ethidium bromide and visualised under UV illumination.

TABLE 2: Sequences of two pairs of species-specific primers derived from the ITS region of rDNA designed to distinguish *G. mangiferae* (JRGGm) and *G. citricarpa* (JRGGc).

Primer pair	Sense	Sequence (5'-3')	Product size	T_m[1]
JRGGm	Forward	CGCTACAACGCCGAAATG	226 bp	52.6
	Reverse	CGATGCCAGAACCAAGAGAT		51.6
JRGGc	Forward	CGTAATCCTGAAAGGTGATGG	501 bp	51.3
	Reverse	CCTCCTCCAAAGCGAGATATT		51.9

[1]Thermal melting point (ºC) calculated using the oligonucleotide analysis function of Vector NTI 8.

Sequence analysis

The BLAST sequence analysis programme available at the National Centre of Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) was used to compare primer sequences to sequence deposited in GenBank.

RESULTS

Primers JRGGcF and JRGGcR produced a 501 bp amplicon when used in PCR reactions with DNA from ICMP 7825 and 7874 (*G. citricarpa*), but no product when used in PCR reactions with DNA from ICMP 8336 and 7872 (*G. mangiferae*). Primers JRGGmF and JRGGmR produced a 226 bp amplicon when used in PCR reactions with DNA from ICMP 8336 and 7872 (*G. mangiferae*), but no product when used in PCR reactions with DNA from ICMP 7825 and 7874 (*G. citricarpa*) (Fig. 1). Neither primer set produced products when used to amplify DNA from a range of other pathogenic and saprophytic fungi listed in Table 1 (Fig. 1).

BLAST sequence analysis resulted in 19 out of a possible 22 hits for JRGGcF to deposited *G. citricarpa* sequence, and primer sequence JRGGmF to all available ITS1 *G. mangiferae* sequence. JRGGcF was complementary to position 73-95 (see Fig. 1; Everett & Rees-George 2006), and JRGGmF to position 38-63, both in ITS1. The reverse primers were not specific and were complementary sequence of the 5.8S subunit, position 308-289, for JRGGmR, and ITS2, position 548-527, for JRGGcR.

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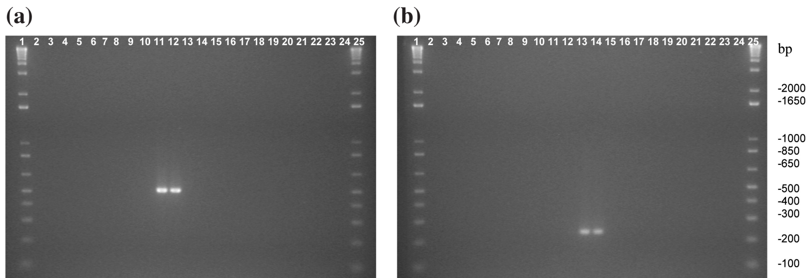

FIGURE 1: PCR products produced with (a) JRGGc or (b) JRGGm primers using DNA from 2. *Botryosphaeria parva*, 3. *Botryosphaeria dothidea*, 4. *Botrytis cinerea*, 5. *Cladosporium* sp., 6. *Colletotrichum acutatum* Ca6, 7. *C. acutatum* 13949, 8. *C. gloeosporioides*, 9. *Cryptosporiopsis* sp., 10. *Epicoccum* sp., 11. *Guignardia citricarpa* 7825, 12. *G. citricarpa* 7874, 13. *G. mangiferae* 7872, 14. *G. mangiferae* 8336, 15. *Nigrospora* sp., 16. *Penicillium* sp., 17. *Pestalotia* sp., 18. *Phialophora* sp., 19. *Phlyctema* sp., 20. *Phoma* sp., 21. *Phomopsis* sp., 22. *Stemphylium* sp., 23. *Venturia inaequalis* and 24. water control. Lanes 1 and 25 are 1 kb plus DNA marker (Invitrogen®).

DISCUSSION

The species-specific PCR primers designed and tested in this work were highly specific to *G. mangiferae* and *G. citricarpa* by PCR reactions, and by comparing primers with sequence deposited in the NCBI database using the programme BLAST.

These primers have also been tested against closely related fungi as well as fungi that are generally present in New Zealand orchards as saprotrophs.

Although 19/22 ITS1 sequences of *G. citricarpa* deposited in NCBI were detected by PCR primer JRGGcF following BLAST analysis, three were not. The reason BLAST analysis showed lack of homology to these sequences is due to an extra Adenine base at position 55 (see Fig. 1 in Everett & Rees-George 2006). Sequence AY042919 also had an additional Cytosine base at position 39. In PCR reactions these small differences may not affect specificity, therefore these isolates also need to be tested by PCR.

There are two sequences of *G. citricarpa* isolate CBS111.20 deposited in GenBank, AF346772.1 and AY042921. AF345772.1 is 100% homologous to primer JRGGcF, but AY042921 (one of the three non-homologous sequences by BLAST) has the extra Adenine base at position 55. Because of this difference for the same isolate, these sequences need to be investigated further.

Geographically related differences in ITS sequence have been found in fungal populations, for example, PCR primers to *Verticillium fungicola* failed to detect European isolates (Romaine et al. 2002). The isolates tested in this study were from Taiwan, South Africa and New Zealand (Everett & Rees-George 2006). Isolates from other countries with citrus black spot that are likely to export citrus to New Zealand, such as Australia, have not been tested. Therefore the primers presented here also require testing against isolates of *G. citricarpa* and *G. mangiferae* from more geographic locations.

A region of ITS1 (from bases 37-103) was identified by Everett & Rees-George (2006) as being suitable for specific primer design. The forward primers tested here were both designed to this region (JRGGmF bases 73-95, JRGGcF bases 38-63). The specificity of these primers to their respective fungi demonstrates the usefulness of this region of polymorphisms for primer design.

Further work is required to compare and validate PCR protocols as they become available in the public domain for use in New Zealand. For instance, although the primers

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developed by Bonants et al. (2003) and Meyer et al. (2006) were highly specific for *G. citricarpa*, they need to be tested against saprotrophs and closely related pathogens common in orchards in New Zealand before their use can be recommended.

Once a PCR protocol has been validated for use in New Zealand, diagnostic plant pathologists can use this test to rapidly and reliably identify suspected incursions of citrus black spot disease. Methods have been developed to use citrus tissue directly in PCR tests (Taylor et al. 2002), thus removing the time-consuming DNA extraction step. Using these methods, the causal organism of a lesion from a citrus fruit could be identified in 3-4 hours compared with 1 week for ITS sequencing or 6 weeks by morphology. If the fungus was not *G. citricarpa* this would enable the rest of the consignment to be sold before spoiling.

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