Two distinct *Guignardia* species associated with citrus in South Africa

L. Meyer*†, B. Slippers*, L. Korsten*, J.M. Kotzé* and M.J. Wingfield*

The fungus *Guignardia citricarpa* causes a phytosanitary restrictive disease called citrus black spot (CBS). Some researchers have suggested the existence of two strains, but these cannot be distinguished by mere microscopy. South African *Guignardia* isolates from different lesion types, as well as from symptomless fruit, were compared by means of ribosomal DNA internal transcribed spacer sequence analysis, proving the existence of two *Guignardia* species on citrus. Restriction enzyme (*Cfo*I) digestion fingerprints of the PCR products clearly distinguished the two species, providing a quick yet reliable identification tool. Growth rate in culture also corresponded with the two species. The first species, *G. citricarpa*, is confirmed as the causal organism of CBS and is restricted to citrus. This fungus occurs in all major citrus-producing areas of South Africa, except the Western Cape. The pathogen can be isolated from hard spots, virulent spots, freckled spots and false melanose lesions from sweet orange, grapefruit, lemon and tangerine. The second species, which is harmless to fruit, can be isolated from symptomless citrus products, but also from avocado, mango, banana, cabbage tree and kumquat, which occur in various geographical areas. Phytosanitary measures may be used against the export of citrus fruit suspected of being infected with CBS. The DNA tests we have devised are able, for the first time, to distinguish the pathogenic from the harmless endophyte of citrus and other plants.

Background

South Africa is a relatively small producer of *Citrus* L. in global terms, yet it exports on average 56% of its crop.[1] This makes the country the third biggest exporter of fresh citrus fruit in the world. This important source of foreign exchange is seriously threatened by restrictive quarantine regulations linked to a fruit disease known as citrus black spot (CBS), caused by the fungus *Guignardia citricarpa* Kiely (asexual state: *Phyllosticta citricarpa* (McAlpine) Aa).[2-4]

Preharvest CBS lesions resulting from field infections spoil the appearance of the fruit and thus significantly reduce sales. In addition, latent infections that are not associated with symptoms at the time of harvesting can also develop on fruit in transit.[3,5] CBS is most prevalent in South Africa, Argentina, Australia and Brazil, and has not been reported in Europe or the U.S.A. (on citrus).[4,5] Since South Africa exports the bulk of its fruit to the countries of the European Union and is currently negotiating entry into the United States market, phytosanitary measures might restrict future exports.

The typical symptom of CBS-infected fruit, hard spot, is a circular lesion on the rind. Although the rind may become extensively necrotic, it seldom causes post-harvest decay.[5] Fruit symptoms can be classified into five categories: hard spot/shot-hole, freckle spot, virulent spot, false melanose (in South Africa) and cracked spot (in Argentina).[6-8] Except for Seville orange (*Citrus aurantium* L.) and its hybrids, all commercially grown *Citrus* spp. are susceptible to the CBS pathogen. Lemons (*C. limonia* Osbeck) are particularly susceptible, and heavy losses may occur on late Valencia and navel oranges [*C. sinensis* (L.) Osbeck] and grapefruit (*C. paradisi* Macfady).[3,4]

Some researchers have suggested that there are two different, but morphologically indistinguishable, forms of the CBS pathogen.[5,9-11] One of these is pathogenic, host specific and the causal agent of the disease, while the other is believed to be non-pathogenic, occurs on numerous hosts and is relatively unimportant.

The aim of this investigation was to compare *Guignardia* isolates from the rind of apparently healthy fruit with those from CBS-spotted citrus rind and different alternative hosts based on nuclear ribosomal DNA internal transcribed spacer (ITS) sequences and certain morphological features. Nucleic acid characters, and especially the internal transcribed spacers ITS1 and ITS2, have been successfully used in resolving intra- and interspecific relationships in numerous fungi.[12-16]

Materials and methods

Fungal isolates: a set of 35 *Guignardia* isolates obtained from the major citrus-producing areas in South Africa, from different cultivars [lemons, sweet oranges, grapefruit, tangerine (*C. reticulata* Blanco) and kumquat (*Fortunella* Swingle)] and different lesion types from citrus fruit, twigs and leaves, as well as from avocado (*Persea americana* Mill.), mango (*Mangifera indica* L.), banana (*Musa acuminata* Colla) and cabbage tree (*Cussonia* Thunb.) were included in the study (Table 1). The fungal samples were obtained by direct isolation from host tissue onto potato-dextrose agar (Biolab Diagnostics, Midrand), supplemented with 250 mg chloramphenicol (Premier Pharmaceutical, Bryanston). An ex-holotype culture of the species, *Phyllosticta citricarpa* (CBS111.20), was used as comparative standard in all tests.

DNA amplification and sequence analysis: DNA was extracted from freeze-dried mycelium using the technique described by Raeder and Broda,[17] and a portion of the ITS region of the ribosomal DNA operon amplified using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG GG-3') and ITS4 (5'-GCT GCG TTC TTC ATC GAT GC-3').[18] The amplified DNA fragments were visualized on a 1.0% (w/v) agarose gel to assess the amplification and purified using a Wizard Preps DNA purification system kit (Promega Corp., Madison, WI). Sequencing reactions were carried out using standard protocols recommended by the manufacturers. The sequence data for the *Guignardia* isolates were processed using Sequence Navigator version 1.0.1 (Perkin-Elmer). These DNA sequences were aligned with each other, as well as with ITS sequences of *Guignardia philoprina* (Berkeley & Curtis) van der Aa (GenBank accession number AF312014).

Analysis: sequences were studied using Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1.[19] A heuristic search with tree-bisection-reconnection (TBR) was carried out and trees were rooted to the sequence of *G. philoprina*. The confidence limits were determined from a bootstrap analysis with 1000 replications.

RFLP analysis: amplified DNA was digested with *Cfo*I (Roche Diagnostics, Mannheim). Digestions were prepared using 2 units of enzyme, 2.5 μl of the accompanying enzyme buffer (Buffer L), 2.1 μl H_2O and 20 μl of each PCR reaction sample. The mixtures were incubated at 37°C for 1 h. The fragments were separated on a 2% agarose gel stained with ethidium bromide.

*Department of Microbiology and Plant Pathology, and Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002 South Africa.
†Author for correspondence. E-mail: lindam@postino.up.ac.za

Table 1. Isolates of *Guignardia* and *Phyllosticta* species compared by means of ITS sequences data.

Isolate code	Host cultivar and tissue type	Lesion type on citrus	Origin	GenBank accession number
GC3	*Citrus sinensis* (cv. Valencia) fruit	Hard spot (HS)	Melmoth, KZN*	AF374369
GC30	*C. sinensis* (cv. Valencia) fruit	Hard spot	Kaapmuiden, MP*	AF374364
GC46	*C. sinensis* (cv. Valencia) fruit	False melanose	Letaba, MP	AF374368
GC62	*C. sinensis* (cv. Valencia) fruit	Red margin	Letsitele, MP	AF346779
GC63	*C. sinensis* (cv. Valencia) fruit	Freckled spot	Letsitele, MP	AF346780
GC68	*C. sinensis* (cv. Valencia) fruit	Freckled spot	Croc Valley, MP	AF346781
GC87	*C. sinensis* (cv. Valencia) fruit	False melanose	Nelspruit, MP	AF374371
GC95	*C. sinensis* (cv. Valencia) fruit	Hard spot	Lisbon, MP	AF346777
GC130	*C. sinensis* (cv. Valencia) fruit	Hard spot	Komatipoort, MP	AF374357
GC134	*C. sinensis* (cv. Valencia) fruit	Virulent spot	Komatipoort, MP	AF374358
GC152	*C. sinensis* (cv. Valencia) fruit	Virulent spot	Komatipoort, MP	AF374361
GC153	*C. sinensis* (cv. Valencia) fruit	Hard spot	Komatipoort, MP	AF374360
GC25	*C. sinensis* (cv. Valencia) leaf	Hard spot	Hoedspruit , MP	AF374365
GC31	*C. paradisi* (cv. Marsh) fruit	Hard spot	Swaziland	AF374366
GC117	*C. paradisi* (cv. Marsh) fruit	—	Nkwaleni, KZN	AF346766
GC119	*C. paradisi* (cv. Marsh) fruit	Red margin HS	Nkwaleni, KZN	AF374356
GC146	*C. paradisi* (cv. Star Ruby) fruit	—	Malelane, MP	AF346767
GC147	*C. paradisi* (cv. Star Ruby) fruit	—	Malelane, MP	AF346770
GC149	*C. paradisi* (cv. Star Ruby) fruit	Hard spot	Malelane, MP	AF346774
GC151	*C. paradisi* (cv. Star Ruby) fruit	Hard spot	Malelane, MP	AF374359
GC154	*C. paradisi* (cv. Marsh) fruit	Red margin HS	Nkwaleni, KZN	AF346775
GC4	*C. limon* (cv. Eureka) fruit	Hard spot	Zebediela, MP	AF346778
GC41	*C. limon* (cv. Eureka) fruit	—	Patensie, S. Cape*	AF346764
GC51	*C. limon* (cv. Eureka) fruit	Hard spot	Croc Valley, MP	AF346773
GC66	*C. limon* (cv. Eureka) fruit	Hard spot	Karino, MP	AF374367
GC75	*C. limon* (cv. Eureka) twig	Hard spot	Croc Valley, MP	AF346782
GC80	*C. limon* (cv. Eureka) fruit	Hard spot	Croc Valley, MP	AF374370
GC45	*C. reticulata* (cv. Nova) fruit	Hard spot	Alkmaar, MP	AF346776
GC10	*Fortunella* sp. (Kumquat) fruit	—	Letaba, MP	AF374362
GC6	*Cussonia* sp.	—	Pretoria, GP*	AF346768
GC172	*Mangifera indica*	—	Letaba, MP	AF346769
GC5	*Musa acumunata*	—	Nelspruit, MP	AF346771
GC14	*M. acumunata*	—	Port Edward, KZN	AF374363
GC18	*Persea americana*	—	Letaba, MP	AF346765
CBS111.20 (ex-holotype)	*Citrus* sp. (cv. unknown)	—	NSW, Australia	AF346772

*Provinces in South Africa: KZN = KwaZulu-Natal; MP = Mpumalanga; S. Cape = Southern Cape part of the Western Cape Province; GP = Gauteng.

Culture and conidial characteristics: to measure growth rate, 5-mm-diameter mycelial plugs were transferred from the margin of a single, morphologically uniform colony on PDA to a 9-cm-diameter plastic Petri dish containing PDA. Three plates of each strain were incubated at 22°C under continual, wide spectrum fluorescent light. Colony diameter was measured at 5, 10 and 20 days. Colour and morphology of fungal growth were noted in the above treatments after 15 days. After sporulation was induced on PDA, the lengths and widths of 50 conidia mounted in water were measured for each isolate using bright field microscopy.

Results

DNA amplification: A Single DNA fragment of approximately 660 base pairs was obtained for all PCR amplification reactions.

Sequence analysis: sequences of approximately 610 bp were attained from the ITS region of the rDNA operon and manually aligned for all the isolates. Of the aligned data set of 610 bp, 80 characters were parsimony informative. Four most parsimonious trees of 133 steps were obtained (CI = 1.000, RI = 1.000). The topography of these trees was the same, with rearrangements occurring only within the main groups. Isolates were grouped into two main clades, which were supported by 100% bootstrap values at the branching points (Fig. 1). All isolates from CBS lesions grouped with the pathogen *P. citricarpa* (CBS111.20) (group B), whereas all the endophytic isolates grouped separately from this species (group A).

RFLP analysis: *Cfo*I digestion of the ITS PCR products resulted in fragment patterns that clearly distinguished the two groups (Fig. 2). The sizes of fragments for group B, were similar to that of the ex-holotype of *P. citricarpa* (CBS111.20).

Cultural and conidial characteristics: two distinct colony types were produced on PDA at 22°C.

Guignardia citricarpa *isolates*: colony on PDA black, sometimes with a thin yellow margin, deeply lobed, mycelium dense, felty, appressed, reverse black. Conidiomata pycnidial in culture, globose, grey to black, hairy, in dense clusters embedded in the mycelium and formed over the whole colony, conidial mass white to cream. Conidia 8–11 × 6.5–8 μm. Spermatia present.

Endophytic isolates: colony on PDA black to olive green, margin less lobed than *G. citricarpa* isolates, never yellow. Mycelium less dense and woolier. Otherwise, cultural and conidial characteristics were the same as for the *G. citricarpa* isolate group.

Growth rate: *G. citricarpa* isolates (including CBS111.20) grew at 2.7–3.2 mm/day,whereas isolates from the endophytic group grew at 4.5–5.3 mm/day on PDA (22°C).

Discussion

This study confirms the existence of two *Guignardia* taxa on citrus, and suggests that they are two distinct species. Growth rate and colony morphology correspond with the two main clades of the ITS phylogeny, but the species could not be

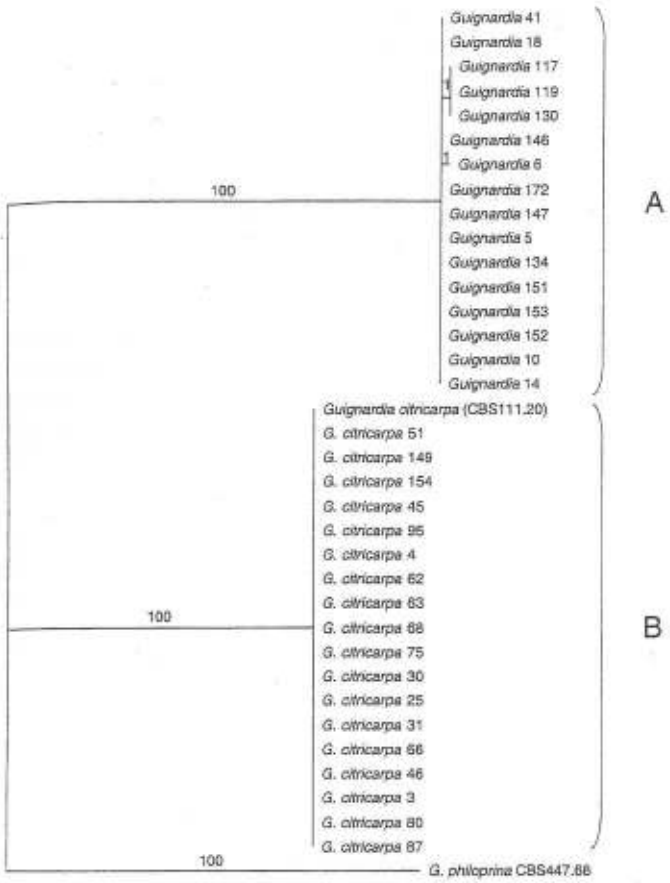

Fig. 1. Dendogram with bootstrap values (1000 replicates) derived from DNA sequence data of the ITS region of the rRNA operon (ITS1, 5.8S rDNA, ITS2) for 35 *Guignardia* strains examined. Group A represents the endophytic isolates and group B represents the pathogen *Guignardia citricarpa*. The GenBank sequence of *G. philoprina* (AF312014) was included as the outgroup.

separated based on other morphological characteristics. No differentiation of pathogenic isolates from different cultivars or different fruit symptoms or the various geographical areas was evident from the ITS sequence analysis. Group B represents the true *G. citricarpa* isolates as it also includes the ex-holotype, *P. citricarpa* (CBS111.20). All these isolates were taken from either hard spots, virulent spots, freckled spots or false melanose lesions from sweet orange, grapefruit, lemon and tangerine. The pathogen is thus restricted to citrus, but occurs in all the main citrus-producing areas of South Africa except the Western Cape.

The second fungus (group A) represents another species of *Guignardia*, which is apparently not pathogenic and was isolated from the above cultivars, as well as from avocado, banana, cabbage tree, mango and kumquat. Isolates were obtained from plant material from various geographic areas.

Kiely[6] first suggested that CBS, latent infection of citrus fruit, and infection of other semi-tropical non-citrus plants, might all be caused by *G. citricarpa*. The CBS pathogen, *G. citricarpa*, was subsequently described on 21 plant families worldwide.[5,11,20] The latent nature of the pathogen in citrus[6,9,10,21] contributed to even greater uncertainty.

Van der Aa[22] studied selected species of *Phyllosticta* Pers. and stated that *P. citricarpa* could not be distinguished on morphological grounds alone, as morphologically similar isolates were obtained from different substrates or hosts. He recommended that host specificity be used to delimit species. Various new names have therefore been given to species of *Guignardia* and *Phyllosticta* occurring on hosts where none has been identified previously. Sometimes species described from closely related host species or genera have been disregarded. Since morphologically, physiologically and genetically identical isolates from citrus, mango, avocado, banana and cabbage tree were retrieved in this study, the practice of applying new names to these fungi from new hosts is clearly not advisable. A similar conclusion was reached by Okane *et al.*,[16] who studied isolates of *Guignardia* from various ericaceous hosts. It is clear, therefore, that a detailed molecular taxonomic study of this genus is required, as this would eliminate the establishment of unnecessary names resulting from the practice of naming fungal isolates solely according to host.

Conclusion

The fungus causing CBS is *Guignardia citricarpa* (anamorph *Phyllosticta citricarpa*); another, morphological similar but distinct species of *Guignardia*, which has not yet been named, is a harmless endophyte of citrus and several other plants. In the past, these two fungi were treated as a single entity responsible for CBS. There are, however, two distinct species, and therefore appropriate quarantine decisions, affecting exports for example, can only be made after careful study of isolates from the fruit. These studies must rely on DNA tests, which provide the only robust approach to distinguish the two species. Failure to discriminate correctly between these fungi may severely threaten future international trade in citrus fruit from South Africa.

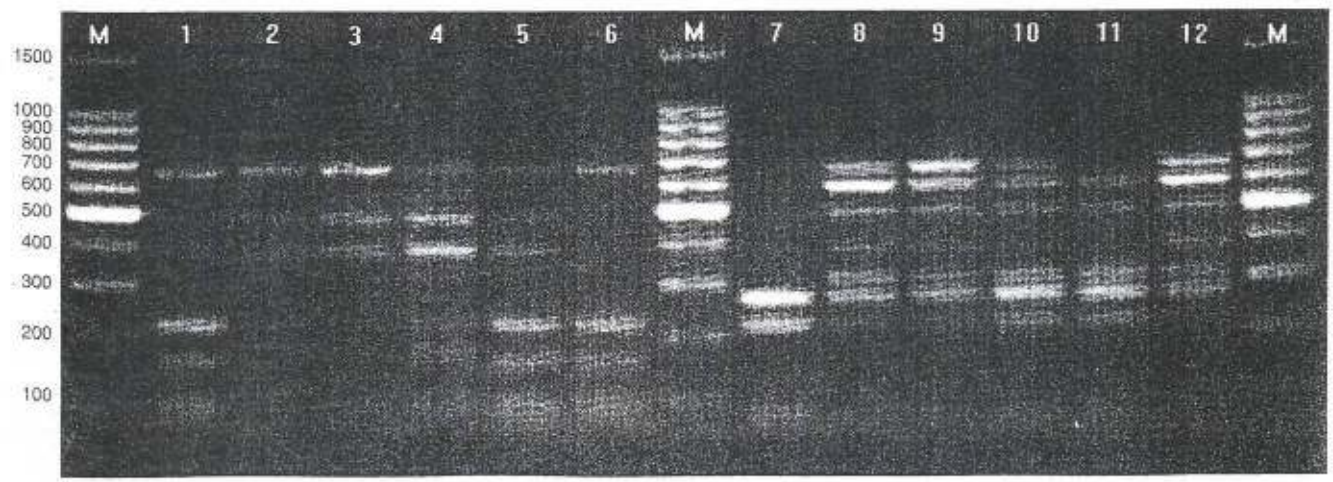

Fig. 2. Restriction enzyme (*Cfol*) digestion patterns of *Guignardia* ITS PCR products. M = 100 bp DNA molecular weight marker. Lanes 1–6 represent *Guignardia citricarpa* isolates (CBS111.20, GC25, GC63, GC95, GC149, GC154). Lanes 7–12 represent the endophytic isolates (GC5, GC10, GC119, GC134, GC146, GC151).

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