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FULL RESEARCH PAPER

Pathogenicity, colony morphology and diversity of isolates of *Guignardia citricarpa* and *G. mangiferae* isolated from *Citrus* spp.

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Abstract In the present study, the pathogenicity of 36 isolates of *Guignardia* species isolated from asymptomatic 'Tahiti' acid lime fruit peels and leaves, 'Pêra-Rio' sweet orange leaves and fruit peel lesions, and a banana leaf were characterized. For pathogenicity testing, discs of citrus leaves colonized by *Phyllosticta citricarpa* under controlled laboratory conditions were kept in contact with the peels of fruit that were in susceptible states. In addition, pathogenicity was related to morphological characteristics of colonies on oatmeal (OA) and potato dextrose agar (PDA). This allowed the morphological differentiation between *G. citricarpa* and *G. mangiferae*. Polymerase chain reactions (PCRs) were also used to identify non-pathogenic isolates based on primers specific to *G. citricarpa*. A total of 14 pathogenic isolates were detected during pathogenicity tests. Five of these were obtained from leaf and fruit tissues of the 'Tahiti', which until this time had been considered resistant to the pathogen. Given that the *G. citricarpa* obtained from this host was pathogenic, it would be more appropriate to use the term insensitive rather than resistant to categorize *G. citricarpa*. A non-pathogenic isolate was obtained from lesions characteristic of citrus black spot (CBS), indicating that isolation of *Guignardia* spp. under these conditions does not necessarily imply isolation of pathogenic strains. This also applied to *Guignardia* spp. isolates from asymptomatic citrus tissues. Using fluorescent amplified fragment length polymorphism (fAFLP) markers, typically pathogenic isolates were shown to be more closely related to one another than to the non-pathogenic forms, indicating that the non-pathogenic isolates display higher levels of genetic diversity.

Keywords *Citrus latifolia* · *Citrus sinensis* · fAFLP markers · *Phyllosticta capitalensis* · *Phyllosticta citricarpa*

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Introduction

Brazilian citriculture has experienced severe phytosanitary problems, including citrus black spot (CBS). This disease is caused by *Guignardia citricarpa* (anamorph: *Phyllosticta citricarpa*, which depreciates the commercial value of fruit intended for the fresh fruit market, reduces crop productivity due to premature fruit falls, and increases considerably the costs of production. In

addition, *G. citricarpa* is classified as an A1 quarantine disease by the European Union and the USA, causing restrictions on the importation of Brazilian citrus products into these countries.

There are two forms of *Guignardia* spp. associated with citrus plants: *G. citricarpa* and *G. mangiferae* (Baayen et al. 2002). However, even though *G. citricarpa* is responsible for the expression of the symptoms of the disease, *Guignardia* spp. can be isolated from leaves, branches, and fruit of asymptomatic citrus plants (McOnie 1964). The endophytic form is distinct from the genus *Guignardia* (McOnie 1964), it as a saprophyte (Stone 1988), or an avirulent form (Kotzé 1988). Both forms of *Guignardia* belong to distinct species, with the endophytic asymptomatic form being classified as *G. mangiferae* (*Phyllosticta capitalensis*; Baayen et al. 2002). According to Kotzé (1988) and Baayen et al. (2002), the colonies of *G. citricarpa* and *G. mangiferae* have different morphologies when grown on potato dextrose agar (PDA) According to these authors, colonies of *G. mangiferae* display a more 'spongy' shape, with invaginations causing them to be irregular. On the other hand, colonies of *G. citricarpa* are more compact and lack invaginations.

Except for sour orange (*Citrus aurantium*) and its hybrids, nearly all remaining species and varieties of citrus plants are susceptible to the pathogen (Kotzé 1981). Varieties of sweet orange (*C. sinensis*) and lemon (*C. limon*) are usually very susceptible to the fungus. However, 'Tahiti' acid lime (*C. latifolia*), does not exhibit disease symptoms under field conditions, even in areas of high inoculum pressure. The reasons for this resistance are still unknown.

Although *G. citricarpa* does not induce disease symptoms on 'Tahiti' acid lime, *Guignardia* species have been isolated from leaves and fruit of this host. However, these isolates have not been characterized taxonomically. There are no data on the structures of populations of *Guignardia* species associated with citrus in Brazil. Therefore, the objectives of this study were to comparatively (1) analyze the pathogenicity of *Guignardia* isolates from 'Tahiti' acid lime and 'Pêra-Rio' sweet orange, and an isolate from 'Prata' banana (*Musa* species); (2) describe the colony characteristics of these isolates on culture media; and (3) determine the relationships among these isolates by the polymerase chain reaction (PCR) and fluorescent amplified fragment length polymorphism (fAFLP) markers.

Materials and methods

Isolate collection

Asymptomatic 'Tahiti' acid lime leaves and fruit and 'Pêra-Rio' sweet orange fruit showing symptoms of CBS were collected from a site with high inoculum pressure. Fragments of leaf and tissues, of approximately 5 mm^2 were immersed in 70% ethanol for 1 min, followed by immersion in a solution of sodium hypochlorite: sterile water (1:3 *v*/*v*) for 3 min. The fragments were then rinsed in sterile water for 30 sec and dried on sterile filter paper. The fragments were then plated on the surface of complete medium (CM) as described by Blanco (1999), and incubated under ambient laboratory conditions at approximately 25°C for 7 days. Isolates from monosporic culture were obtained from ascospores captured in a wind tunnel as described by Whiteside (1973). All the isolates used in this study are listed in Table 1.

Production and collection of ascospores

In order to obtain ascospores, samples of approximately 1 kg of physiologically mature leaves from the 'Tahiti' acid lime and 'Pêra-Rio' sweet orange trees were collected, stored in mesh bags (1 mm mesh size) and maintained under ambient conditions for 30 days. After this period, samples of approximately 200 g were soaked in water for 20 min and later maintained in ascospores collectors in a wind tunnel, consistent with the method developed by Whiteside (1973), except for the modification of placing a glass slide with water agar at the air flow exit to trap particles. The presence of *Guignardia* species ascospores was checked every 45 min. In order to capture the ascospores attached to the agar-water slides, an adapted optical microscope (400× magnification) was used. This consisted of an electromechanical device that enabled micrometrical movements of a micropin attached to its extremity. Ascospores, therefore, that were present on the surface of the slide could be captured individually and later transferred to Petri dishes with PDA for incubation at 25°C.

Pathogenic characterization

Pathogenicity tests

For pathogenicity tests, 'Pêra-Rio' sweet orange fruit were used in an orchard located at the UNESP–

Table 1 Isolates of *Guignardia* species used for pathogenicity tests, colony characterization, PCR and fAFLP

Isolates	Material of origin	Location	*G. citricarpa*	*G. mangiferae*
1	Peel of fruit of Tahiti acid lime	Conchal SP		X
2	Peel of fruit of Tahiti acid lime	Conchal SP[a]		X
3 A	Peel of fruit of Tahiti acid lime	Conchal SP[b]		X
3 B	Peel of fruit of Tahiti acid lime	Conchal SP[b]		X
3 C	Peel of fruit of Tahiti acid lime	Conchal SP[b]		X
4	Ascospores of leaves of Pêra-Rio[a]	Conchal SP		X
5	Peel of fruit of Pêra-Rio–FS[d]	Conchal SP[a]	X	
6	Leaves of Tahiti acid lime	Conchal SP[a]		X
7	Leaves of Tahiti acid lime	Conchal SP[b]	X	
8	Ascospores of leaves of Pêra-Rio[a]	Conchal SP[b]	X	
9	Leaves of Tahiti acid lime	Conchal SP[b]		X
10	Peel of fruit of Pêra-Rio–FM[e]	Itacotiara/AM	X	
11	Peel of fruit of Pêra-Rio–FS[d]	Conchal SP[b]	X	
12	Peel of fruit of Tahiti acid lime	Conchal SP[b]		X
13	Peel of fruit of Tahiti acid lime	Conchal SP[a]	X	
14	Peel of fruit of Tahiti acid lime	Conchal SP[a]		X
15	Peel of fruit of Pêra-Rio–FM[e]	Conchal SP[a]	X	
16	Leaves of Tahiti acid lime	Conchal SP[a]		X
17	Leaves of Tahiti acid lime	Conchal SP[b]	X	
18	Leaves of Tahiti acid lime	Conchal SP[a]		X
19	Leaves of Tahiti acid lime	Conchal SP[a]		X
20	Peel of fruit of Tahiti acid lime	Conchal SP[b]		X
21	Peel of fruit of Pêra-Rio–FM[e]	Conchal SP[b]	X	
22	Peel of fruit of Tahiti acid lime	Conchal SP[b]		X
23	Peel of fruit of Pêra-Rio–HS[f]	Conchal SP[a]	X	
24	Ascospores of leaves of Tahiti acid lime	Conchal SP[a]		X
25	Leaves of Tahiti acid lime	Conchal SP[a]		X
26	Peel of fruit of Tahiti acid lime	Conchal SP[a]		X
27	Peel of fruit of Pêra-Rio–FS[d]	Itacotiara AM		X
28	Ascospores of leaves of Tahiti acid lime[a]	Conchal SP[b]	X	
29	Peel of fruit of Pêra-Rio–HS[f]	Itacotiara AM	X	
30	Ascospores of leaves of Tahiti acid lime[c]	Conchal SP[b]		X
31	Ascospores of leaves of Tahiti acid lime[c]	Conchal SP[b]		X
32	Peel of fruit of Tahiti acid lime	Conchal SP[a]	X	
33	Peel of fruit of Pêra-Rio–HS[f]	Conchal SP[b]	X	
35	Leaves of banana tree	Silva Jardim RJ		X

[a] Farm Bela Vista

[b] Farm São Paulo

[c] Ascospores obtained from wind tunnel

[d] Freckle spot

[e] False melanose

[f] Hard spot

Jaboticabal in State of São Paulo. 'Valência' sweet orange fruit were used in an orchard near Araraquara in São Paulo State. Neither orchard had any record of a history of CBS in previous years. Fruit were bagged in 18×15.5 cm paper bags when the plants had shed approximately 75% of their leaves to prevent any possibility of fungal infection. The inoculum was produced from 'Valência' sweet orange leave discs (12 mm diam) colonized by *P. citricarpa*. These discs, previously autoclaved, were placed on the surface of

water agar together with 5 mm-diam discs of *P. citricarpa* obtained from the periphery of the colony of each isolate (Table 1) and incubated for 30 days at 25°C and a 12 h photoperiod to promote the colonization. Colonized leaf discs contained pycnidia, conidia and immature pseudothecia of *P. citricarpa*, in >50% of the disc area, and were used for inoculation on fruit surfaces.

The pathogenicity of *G. citricarpa* (isolates 4, 7, 13, 17, 23, 32, 33) was tested using the variety 'Pêra-Rio' sweet orange fruit. The pathogenicity of all other isolates was evaluated using 'Valência' sweet orange. Each isolate was tested on 15 previously bagged fruit of either variety, with each fruit measuring 20 and 30 mm diam. Six discs with pathogen structures were placed on each fruit surface. A very thin meshed fabric was used to facilitate the contact between the leaf disc and the fruit. The bags were moistened daily for 30 days and the temperature average was 25°C to ensure conditions conductive for infection. The fruit remained in the bags until the ripening stage, when they were macroscopically evaluated for the presence or absence of CBS symptoms. If positive, the fungus was reisolated from the tissues showing lesions typical of CBS, and this isolate was considered to be pathogenic; the absence of symptoms on the fruit was used to classify an isolate as non-pathogenic.

Culture characterization of colonies of the isolates of Guignardia spp. in culture media

The characterization of *Guignardia* species colonies was made using oatmeal (OA) as described by Baayen et al. (2002). A 5 mm-diam disc obtained from the periphery of the colony, in areas of active growth, was placed at the centre of Petri dishes and maintained on PDA. After incubating at 25°C and 12 h photoperiod for 7 days, the dishes were evaluated for the presence of a yellow halo around the colonies characteristic of *G. citricarpa* and indicative of pathogenicity (Baayen et al. 2002; EPPO 2003). Four plates were used for every isolate. Each Petri dish was considered as a sampling unit, and the isolate was only considered pathogenic if the typical yellow colouration was present in all four replications.

Evaluation of isolates based on molecular studies

DNA from the *Guignardia* species isolates was extracted using the protocol of Shillito and Saul (1988). DNA quantification was determined spectrophotometrically by measuring the absorbance of each sample in relation to DNA-free, distilled water at 260 and 280 nm wavelengths (Sambrook et al. 1989). DNA preparations with A260/A280=1.8–2.0 were considered to be of sufficient quality for PCR and fAFLP.

PCRs using specific primers

Specific primers (GCP1 and GCP2) were designed by Blanco (1999) for the amplification of a 373-bp diagnostic fragment specific for *G. citricarpa* isolates. PCR amplifications were carried out using 2 μl of 1X buffer solution (50 mM KCl, 200 mM TRIS–HCl, pH 8.4), 0.8 μl of 5 mM $MgCl_2$; 0.4 μl of each dNTP (10 mM); 0.2 μl of Taq DNA polymerase, 5 pmol of each primer, 50 ng of genomic DNA and 20 μl of nanopure water. Reactions were carried out using a PTC-100 programmable thermal controller (MJ Research, Inc.) using the following settings: 1 cycle at 94°C for 2 min, 39 cycles of 94°C for 1 min, 67°C for 1 min and 72°C for 1.5 min, and 1 final cycle at 72°C for 5 min. Amplified samples were electrophoresed in 1.5% agarose gels with 0.5 μg ml^{-1} of ethidium bromide, and visualized under UV light in photodocumentation equipment (GEL DOC 1000, BioRad).

Development of fAFLP markers and analysis

fAFLP markers were developed using the 'AFLP microbial fingerprinting kit' (Applied Biosystems do Brazil LTDA) following the manufacturer's instructions. The digestion of the DNA extracts was carried out using the EcoRI and MseI restriction enzymes, which were pre-amplified and ligated to the adaptors. Selective amplification was conducted using the FAM *Eco*RI AC–*Mse*I CA, NED *Eco* RI C–*Mse*I A and JOE *Eco*RI G–*Mse*I CG primer pairs. Samples were loaded on a 5% denaturating Long Ranger gel using 1× TEB as the running buffer. A 36 cm sequencing plate was used in 2.5 h runs at 2.500 V (ccd 4) in an automated DNA sequencer (ABI Prism 377), along with a GeneScan-500 (ROX) molecular weight standard. Markers between 50 and 500 bp were analyzed using GeneScan (ABI Prism version 1.0) and Genotyper (ABI Prism version 1.03) software for data collection and their transformation into a binary matrix, which was analyzed using the PAUP (phylogenetic analysis using parsimony, version 3.01;

Felsenstein 1985) software to generate a distance matrix. Finally, the distance matrix was analyzed using the MEGA (version 2.1; Kumar et al. 2005) software using a distance method through the neighbour-joining algorithm (Sambrook et al. 1989) to generate a phylogram with the phylogenetic relationships between the isolates (Saitou and Nei 1987).

Results

Production and collection of ascospores

Ascospores were produced, release, and collected from both 'Tahiti' acid lime leaves and 'Pêra-Rio' sweet orange by the ascospore collector. Individual ascospores were collected from the agar-water slides, generating four monosporic cultures from 'Pera-Rio' sweet orange leaves and two monosporic cultures from 'Tahiti' lime leaves (Table 1). The first release of the ascospores occurred 53 and 48 days respectively, from leaves of 'Pêra-Rio' and 'Tahiti'.

Pathogenicity tests and culture characterization of colonies of the isolates of *Guignardia* species

According to the pathogenicity tests, all isolates that had been classified as *G. citricarpa* were shown to be pathogenic and caused the typical symptoms of CBS (Table 1). These typical symptoms of CBS were clearly observed on mature fruit. The use of the OA culture medium allowed the efficient differentiation between pathogenic and non-pathogenic isolates because of the manifestation of the characteristic yellow halo in colonies of the former (Fig. 1). The isolates 5, 7, 8, 10, 11, 13, 15, 17, 21, 23, 28, 29, 32, 33 had colonies with a yellow halo (pathogenic) and the remaining did not have a yellow halo (non-pathogenic). Moreover, the results were consistent with those obtained in the pathogenicity tests.

Results of the characterization of isolates on the PDA medium indicated morphological differences between *G. citricarpa* and *G mangiferae*. Morphological differences in colony morphological patterns were previously shown by Kotzé (1988) and thus confirmed in the present study. Among the 36 isolates grown on PDA, *G. citricarpa* were more compact, with differentiated regions of dark pigmentation. The colony edges of *G. citricarpa* did not show invaginations. On the other hand, colonies of *G. mangiferae* had a spongy appearance, with homogeneous mycelial growth and invaginating round edges (Fig. 1).

PCRs and fAFLP analysis

Guignardia citricarpa and *G. mangiferae* isolates were also differentiated by PCR using primers GCP1/GCP2 (Blanco 1999) to amplify a 373 bp DNA fragment specific to pathogenic isolates of *G. citricarpa*. Isolates 13, 32, 7, and 17, obtained from leaves and asymptomatic fruit of 'Tahiti' acid lime were classified as pathogenic, whereas isolates 3A, 3B, 3C, 12, 20, 22, 1, 2, 14, 26, 9, 6, 16, 19, 25, 18, 30, and 31, also obtained from leaves and asymptomatic fruit of 'Tahiti' acid lime, were classified as non-pathogenic.

A phylogram based on the genetic polymorphism of these 36 isolates using fAFLP markers to differentiate pathogenic and non-pathogenic forms is presented in Fig. 2. Pathogenic isolates were more closely related to one another than to other isolates, forming a subgroup in the phylogenetic tree. Endophytic isolates, in comparison, showed more ramifications and were more genetically distant from one another. This can be seen in isolates 3C, 11 and 31, which formed a group completely distinct from the remaining isolates.

Discussion

Guignardia citricarpa caused infection and fruit damage with the ascospores being the primary agent of inoculum. In South Africa under natural conditions, the formation and release of ascospores occurs between 40 and 180 days after leaf fall (Kotzé 1981). The inoculation method used was shown to be very efficient, underscoring its applicability to studies of this nature. This novel approach is particularly useful because the origin of the isolate under evaluation can be determined. There are other useful methods to conduct pathogenicity tests of *Guignardia* species; however, the random collection of leaves in the orchard does not allow determination of the origin of an isolate. Lemir et al. (2000) reported positive pathogenicity tests through the in vitro production of great quantities of ascospores. However, our experience using this method has not been successful.

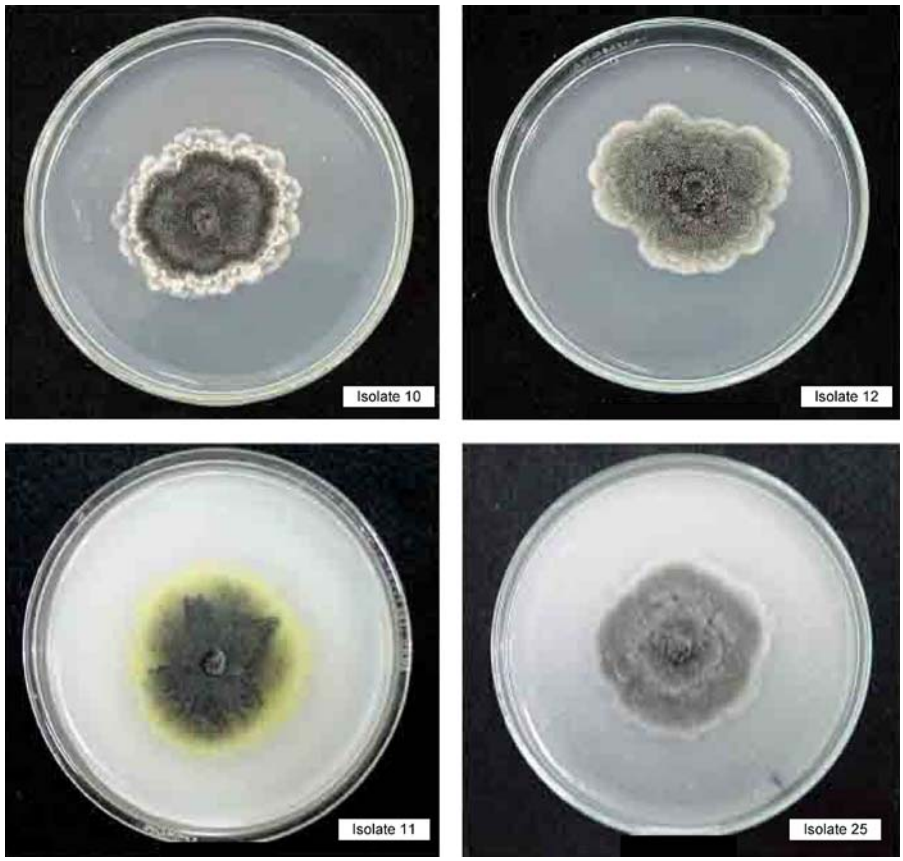

Fig. 1 Seven-day-old cultures of *Guignardia* species pathogenic (*row 1*) and non-pathogenic (*row 2*) cultures. Isolates 10 and 12: growth on potato-dextrose-agar; isolates 11 and 25 on oatmeal agar

The results from the analysis of morphological characteristics of cultures grown on PDA were consistent with those obtained by Baayen et al. (2002), corroborating our approach as a simple, efficient, and relatively rapid alternative to differentiate isolates of *G. citricarpa* and *G. mangiferae*. The differential morphological characteristics of these *Guignardia* species on OA were also consistent with differential pathogenicities of these isolates. Thus, *Guignardia* species that produced a yellow pigment at the edges of colonies were consistently pathogenic as reported previously (Baayen et al. 2002). PCR using GCP1 and GCP@ was also useful for rapid, specific differentiation of *G. citricarpa* and *G. mangiferae*.

Although 'Tahiti' acid lime does not show foliar or fruit symptoms of CBS, both *G. citricarpa* and *G. mangiferae* can coexist on this host. In addition, both *G. citricarpa* and *G. mangiferae* are capable of producing viable ascospores on decomposing 'Tahiti' acid lime leaves. Therefore, this host must have a significant role in the epidemiology of *G. citricarpa* and CBS. However, the extent of this is currently unknown. In the case of *G. citricarpa* and 'Tahiti' acid lime interactions, the terms 'resistant' (Agrios 1995), 'immune' (Camargo 1995) and 'tolerant' (Camargo 1995) host do not apply, as the pathogen infects the host, yet does not elicit disease symptoms. We propose that an appropriate term for this host-pathogen relationship may be 'insensitive' host.

The isolate 27, despite being obtained from lesions on 'Pêra-Rio' sweet orange fruit in Itacotiara, State of Amazonas, was shown to be non-pathogenic and distinguishable from pathogenic isolates by colony characteristics and molecular analyses. However, the isolates 10 and 29, which were obtained from the same fruit and different lesions, were shown to be pathogenic. These results indicate, therefore, that simply obtaining isolates from lesions does not guarantee pathogenicity. Likewise, obtaining isolates from asymptomatic tissues does not ensure that they are endophytic. Thus, both *G. citricarpa* and *G. mangiferae* can coexist on the same lesion. Such occurrence can be coincidental, although additional studies are necessary to determine the reasons for the presence of both forms on the same tissue.

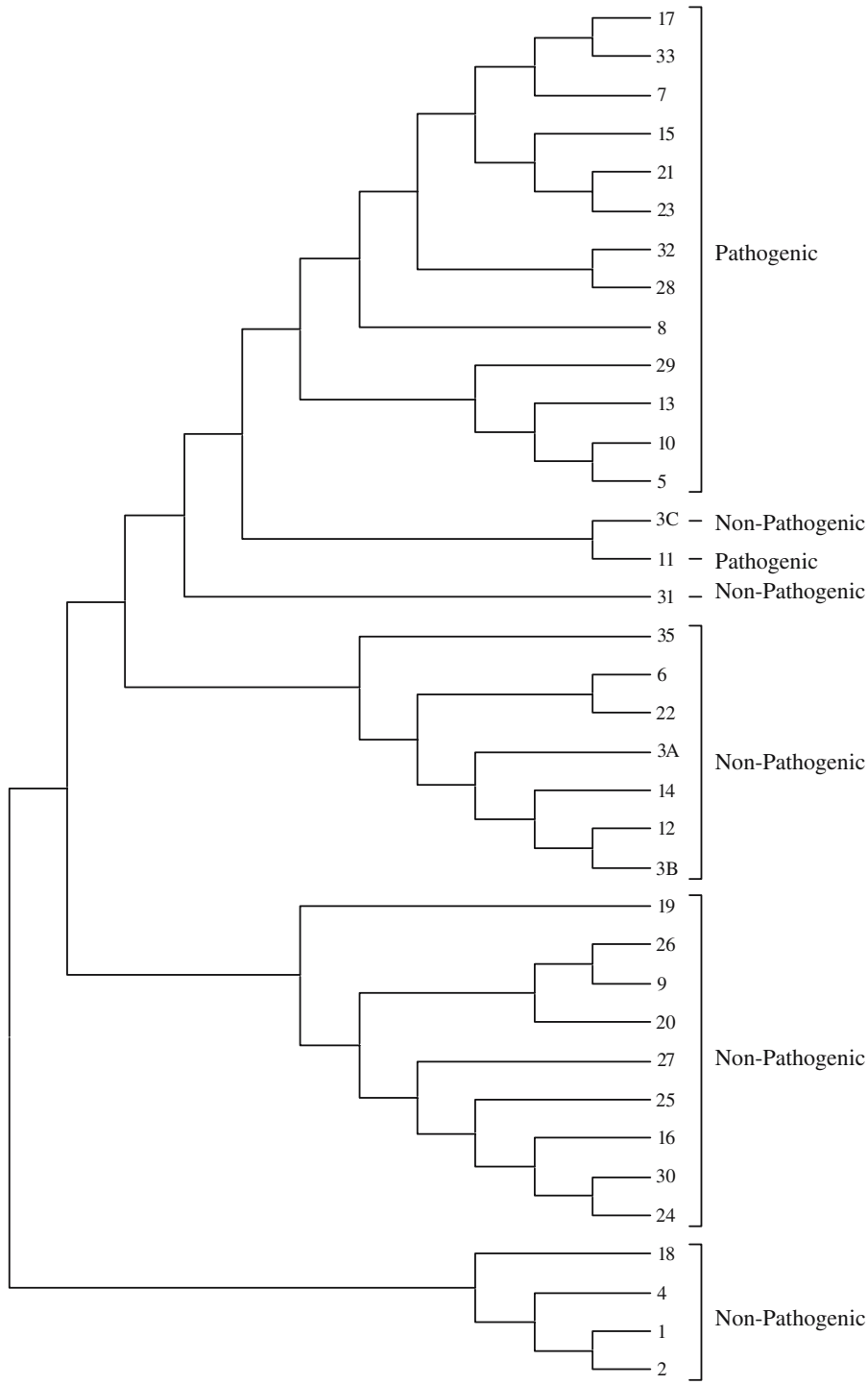

Fig. 2 Phylogram build for method of distance and algorithm of Neighbour Joining showing the phylogenetic relations of 36 isolates of *Guignardia* species. The isolates are clustered with characteristics of pathogenicity and non-pathogenicity. Major variability was shown by non-pathogenic isolates, in three different groups (1, 2, and 3)

From an evolutionary perspective, it was thought that non-pathogenic individuals represent more ancient lineages than pathogenic ones. Theoretically, that would entail an even greater genetic divergence among these individuals, given that neutral mutation could accumulate over time, generating increased gene diversity. If the hypothesis that pathogenic and non-pathogenic individuals share common ancestors is true, genetic factors such as mutations and drift, or environmental factors, contributed to the appearance

and maintenance of changes, which allowed the currently observed evolutionary divergence. Given that *Citrus* spp. are cultivated species of great economic interest, and therefore subject to massive interference from cultural traits, the hypothesis that the appearance of *G. citricarpa* is due to selection pressure by some cultural trait or set of traits which would have altered the genetic and morphological structure of the population of *G. mangiferae* cannot be dismissed.

The results obtained in the present study confirm existing information present in the literature on the coexistence of *G. citricarpa* and *G. mangiferae* in tissues of *C. sinensis* (Baayen et al. 2002; Bonants et al. 2003; Blanco 1999; Glienke 1995). However, the present study is the first record of the coexistence of the two forms of *Guignardia* in 'Tahiti' acid lime.

Contrary to what has been suggested by Baayen et al. (2002), there is no substantial difference between the ecologies of *G. citricarpa* and *G. mangiferae*, because of the coexistence of both forms on the same host, as in *C. sinensis* and *C. latifolia*. This is probably not coincidental, indicating a possible evolutionary interaction which is still not understood. Isolate 11, classified as pathogenic using conventional, molecular and morphological methods, had an intermediate position between the pathogenic and endophytic subgroups. Likewise, a similar intermediate position was found for isolates 3C and 31, which were shown to be endophytic. A better understanding of these interactions, as well as knowledge about the existence of non-pathogenic forms, will improve methods to control *G. citricarpa* that are more rational and ecologically safe.

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