# High Molecular Diversity of the Fungus *Guignardia citricarpa* and *Guignardia mangiferae* and New Primers for the Diagnosis of the Citrus Black Spot

**Danyelle Stringari[1], Chirlei Glienke[1*], Daniel de Christo[1], Walter Maccheroni Jr.[2] and João Lucio de Azevedo[2]**

[1]*Departamento de Genética; Universidade Federal do Paraná; C. P.: 19071; 81531-990; Curitiba -PR - Brasil.*
[2]*Departamento de Genética; Universidade de São Paulo; C. P.: 9; 13418-900; Piracicaba - SP - Brasil*

## ABSTRACT

*RAPD markers were used to investigate the distribution of genetic variability among a group of* Guignardia citricarpa*,* G. mangiferae*, and* Phyllosticta spinarum *isolates obtained from several hosts in Brazil, Argentina, Mexico, Costa Rica, Thailand, Japan, United States and South Africa. Pathogenic isolates* G. citricarpa *Kiely (anamorph form* P. citricarpa *McAlp Van Der Aa) are the etiological agent of the Citrus Black Spot (CBS), a disease that affects several citric plants and causes substantial injuries to the appearance of their fruits, thus preventing their export. Several previous studies have demonstrated the existence of an endophytic species with high morphological similarity to the causal agent of CBS that could remain latent in the same hosts. Consequently, the identification of the plants and fruits free from the causal agent of the disease is severely hampered. The RAPD analysis showed a clear discrimination among the pathogenic isolates of* G. citricarpa *and endophytic isolates (*G. mangiferae *and* P. spinarum*). In addition, a Principal Coordinate Analysis (PCO) based on a matrix of genetic similarity estimated by the RAPD markers showed four clusters, irrespective of their host or geographical origin. An Analysis of Molecular Variance (AMOVA) indicated that 62.8% of the genetic variation was found between the populations (*G. citricarpa*,* G. mangiferae*,* P. spinarum *and* Phyllosticta *sp.). Substantial variation was found in the populations (37.2%). Exclusive RAPD markers of isolates of* G. citricarpa *were cloned, sequenced and used to obtain SCARS (Sequence Characterized Amplified Regions), which allowed the development of new specific primers for the identification of* G. citricarpa *PCR (Polymerase Chain Reaction) analysis using a pair of primers specific to pathogenic isolates corroborating the groupings obtained by the RAPD markers, underscoring its efficiency in the identification of the causal agent of CBS.*

**Key words:** *Guignardia*, SCARS, Citrus Black Spot, Citrus, Diagnosis, RAPD

## INTRODUCTION

The orange fruit is currently the most cultivated plant in the world, with an annual production exceeding 60 million tones. Brazil is responsible for nearly a third of the world production of orange, as well as 85% of the control of the international orange juice market. Therefore, the cultivation of citric fruits is among the most important activities in Brazilian agrobusiness (Agrianual, 2005).
However, citriculture has several phytosanitary problems, particularly the Citrus Black Spot (CBS), which has caused substantial losses due to the need for pulverization with fungicides for their

* Author for correspondence: cglienke@ufpr.br

control and the ensuing increases in the costs of production (Agrianual, 2005). There is considerable depreciation in the value of fruits with CBS in the *in natura* market, with strong opposition to its import by the European Union, which is currently Brazil's most important consumer. Therefore, the diagnosis of the fruits destined for export is of fundamental importance. The CBS is caused by the fungus *Guignardia citricarpa* Kiely (anamorphic form is *Phyllosticta citricarpa* Van Der Aa) and was first described in 1895 in Australia in orange fruits of the 'Valência' variety, causing considerable losses. This disease is currently found in several countries, with the maximum impact in South Africa, Japan, Argentina, and particularly in Brazil, where the disease has been reported most frequently after 1995 and is strongly affecting the local citriculture (Feichtenberger, 1996).

Although the identification of the causal agent of CBS can be obtained by the conventional techniques such as culturing and microscopy, these procedures require weeks to be completed. This delay is impractical in the *in natura* business of the fruit. In addition, there have been reports of the existence of an endophytic species (*G. mangiferae*) that is very similar morphologically with the causal agent of CBS, inhabiting latently the same hosts (Glienke-Blanco et al., 2002, Baayen et al., 2002). Therefore, the identification of the plants and fruits free from the causal agent of CBS is made even more difficult. Although these two fungus species are morphologically nearly identical, their physiological differences are clear. Baayen et al. (2002) showed that the most significant morphological difference among *G. citricarpa* and *G. mangiferae* is the thickness of the mucoid conidial sheath, which is smaller than 1.5 μm in the former but ranges between 1.5 and 3.0 μm in the latter. However, the long time required for the isolation, culturing and pycnidium formation makes this method unsuitable for the quarantine purposes.

Baayen et al. (2002) investigated ITS sequences and showed that the endophytic species isolated in *Citrus* spp. was the anamorphic state of *P. capitalensis*. Okane *et al.* (2001) reported the endophytic occurrence of *P. capitalensis* inhabitanting Ericaceae plants, describing the teleomorphic state as *G. endophyllicola*. A survey of the taxonomic literature showed that the oldest name associated with the teleomorphic state of

these isolates probably was *G. mangiferae* A. J. Roy, described in 1968. In fact, the endophytic *G. mangiferae* is found inhabiting a wide range of hosts, and probably has been repeatedly described from many different host species, causing extensive several cases of synonymy.

The present work investigated the Brazilian isolates of *Guignardia* spp. and *Phyllosticta* spp. with the following objectives: 1) to verify the endophytic occurrence of *G. citricarpa* and *G. mangiferae* in *Citrus* spp and *llex paraguariensis*; 2) to assess the genetic variability and population structure of endophytic isolates, as well as those obtained from CBS lesions; 3) to differentiate the isolates of *G. mangiferae* and *G. citricarpa* based on RAPD markers and to develop SCARS specific to the causal agent of CBS. The development and validation of an identification method for *G. citricarpa* using PCR could expedite the identification process of contaminated fruits and orchards.

## MATERIALS AND METHODS

### Fungal Material

The isolates investigated in this work were obtained from monosporic cultures. The strains of *G. citricarpa* were isolated directly from CBS injuries. The endophytic strains of *G. mangiferae* and *Phyllosticta* spp. were isolated from healthy host plants without any apparent lesions (Pimentel et al., 2006). The geographical origin, host plant and nature (endophytic or pathogenic) of all the isolates are listed in Tables 1 and 2.

Three strains of *G. citricarpa* and three strains of *G. mangiferae* that were tested positive and negative for pathogenicity, respectively (Baldassari, 2005) were used in the validation of the PCR diagnostic (Table1).

### DNA Extraction

The fungi were grown in the complete agar medium (Azevedo and Costa, 1973) at 28°C for three days. The mycelium was then harvested, frozen at –20°C and freeze–dried. The dried mycelium was placed in a mortar and ground with liquid nitrogen and a pestle into a fine powder. The genomic DNA was isolated according to the protocol of Raeder and Broda (1985). The DNA was precipitated with ethanol, washed in ethanol (70%), dried and dissolved in milli-Q sterilized

water. The DNA solution was treated with RNAse (50μg/mL) and the concentration of DNA was measured by agarose gel electrophoresis, followed by staining with ethidium bromide.

**Table 1** - Isolates of the citrus pathogen *G. citricarpa*, the citrus endophyte *G. mangiferae*, and *Phyllosticta* species from other host species used in the RAPD analyses and PCR tests as reference isolates (Baayen et al., 2002; Baldassari, 2005).

| Isolates | Nature / Host | Origin |
|---|---|---|
| *G. citricarpa (P. citricarpa)* | | |
| PC3C, PC6C, PC8C, PC9C, PC10C | Pathogen/*Citrus* sp | South Africa [1] |
| PC12C | Pathogen/*Citrus* sp | Brazil [1] |
| PC15C | Pathogen/*Citrus* sp | Japan [1] |
| PC18C | Pathogen/*Citrus* sp | Mexico [1] |
| PC28/05, PC32/05, PC33/05 | Pathogens/*Citrus* sp [P] | Brazil [2] |
| *G. mangiferae (P. capitalensis)* | | |
| EC1C | Endophyte/Pittosporaceae | EUA [1] |
| EC2C | Endophyte/Zamiaceae (**cycad palm family**) | South Africa [1] |
| EC4C | Endophyte/Zamiaceae (**cycad palm family**) | EUA [1] |
| EC5C | Endophyte/Moraceae (**mulberry family**) | Thailand [1] |
| EC7C, EC17C | Endophytes/Citrus sp | EUA [1] |
| EC11C | Endophyte/Sapotaceae | EUA [1] |
| EC13C | Endophyte/Lauraceae | South Africa [1] |
| EC14C | Endophyte/Araliaceae | South Africa [1] |
| EC16C | Endophyte/*Fortunella margarita* | EUA [1] |
| EC19C | Endophyte/Araliaceae (**ginseng family**) | Costa Rica [1] |
| EC20C | Endophyte/Rubiaceae | EUA [1] |
| EC21C | Endophyte/*Camellia japonica* | EUA [1] |
| EC 01/05[N], EC19/05[N], EC22/05[N] | Endophytes/Citrus sp | Brazil [2] |

[1] Strains kindly provided by Dr. George Carroll / University of Oregon-USA.
[2] Dr. Antonio de Goes, UNESP/Jaboticabal – SP/Brazil
[P] Positive strains in pathogenicity tests for CBS (Baldassari, 2005).
[N] Negative strains in pathogenicity tests for CBS (Baldassari, 2005).

**RAPD**

Random Amplified Polymorphic DNA (RAPD) analysis was carried out using 50 ng of genomic DNA. The amplification reaction was done in a final volume of 25 µl containing 1.5 U of *Taq* DNA polymerase (*Invitrogen*, Carlsbad, CA, USA), 0.4 µM of primer, 0.2 mM of dNTPs (*Pharmacia*, Freiburg, Germany), 3.0 mM of $MgCl_2$ and 1 X PCR Buffer (50 mM of KCl, 200 mM of Tris-HCl, pH 8.4). Negative controls containing all the components, except genomic DNA were included in all the experiments. The primers selected were obtained from Operon Technologies (Alameda, CA, USA): OPX08, OPX12, OPX13, OPX14, OPX17, OPX19.

The amplification was performed in a MJ Research[®] thermocycler (Watertown, MA, USA) according to the following steps: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation for 1 min. at 94°C / annealing for 1.5 min at 37°C / extension for 2 min. at 72°C, and a final extension for 3 min at 72°C.

**Data Analysis**

RAPD markers were interpreted as binary data based on the presence or absence of bands. A matrix of Jaccard genetic similarities (Sneath and Sokal, 1973) was generated using NTSYS 2.1 (Rohlf, 1997). A significance test of the correlation between the genetic similarity matrix and a cophenetic matrix of the dendrogram was carried out with the Mantel matrix correlation test (Mantel, 1967). From the genetic similarity matrix, a Principal Coordinate Analysis (PCO) was performed to verify possible clustering of the isolates. Based on the similarity values, an UPGMA cluster was generated proceeding an artificial dendrogram. The robustness of each node in the dendrogram was evaluated by the bootstrap analysis (Felsenstein, 1985) using the software Bood v.3.03 (Coelho, 2005). An analysis of molecular variance (AMOVA) was conducted using the software *Arlequin 3.0* (Excoffier et al., 2005).

**Table 2 -** Isolates of the citrus pathogen *G. citricarpa*, the citrus endophyte *G. mangiferae*, *Phyllosticta spinarum* and *Phyllosticta* species from other host species used in the RAPD analyses and PCR tests.

| Isolates | Nature / Host | Origin |
|---|---|---|
| ***G. citricarpa (P. citricarpa)*** | | |
| PC7LI3, PC7LA2, PC7LD6, PC7LB6, PC7LB8, PC7LE10 | Pathogens/*Citrus* sp | SP - Brazil [1] |
| PC13/96, PC13, PC19, PCP6 | Pathogens/*Citrus* sp | SP - Brazil [2] |
| ***G. mangiferae (P. capitalensis)*** | | |
| ECOL23, EC145, EC131, EC01/96, EC16/96 | Endophytes/*Citrus* spp | SP - Brazil [3] |
| ECMi1, ECMi4, ECMi5, ECMi6, ECMi7, ECML2, ECML7, | Endophytes/*Citrus* spp | PR - Brazil [4] |
| EEM09, EEM39, EEM75, EM153, EEM163, EEM176, EEM192, EEM202, EEM375, EEM371 | Endophytes/*Ilex paraguariensis* | PR - Brazil [5] |
| PGB1515 | Pathogen/*Psidium guajavae* | Brazil [6] |
| EG3Z | Endophyte/*Paullinia cupana* | Manaus - Brazil [7] |
| ECF75LP | Endophyte/*Citrus* sp | Tucumám - Argentina [8] |
| ***Phyllosticta* sp** | | |
| ECPR5, ECPR6, ECPR8 | Endophyte/*Citrus limon* | PR - Brazil [9] |

[1] Dr. Antonio de Goes, UNESP/Jaboticabal – SP/Brazil
[2] Centro de Citricultura Sylvio Moreira – IAC - SP/Brazil
[3] Strains isolated by Blanco (1999).
[4] Strains isolated in the metropolitan region of Curitiba – PR (Stringari, 2004).
[5] Strains isolated by Penna (2000).
[6] Strain kindly provided by Dr. Luimar José Tozetto / UnB
[7] Strain isolated and kindly provided by Dr. J. O. Pereira – UFAM
[8] Strain kindly provided by Dr. Estela Duran, Tucumám - Argentina.
[9.] Strains isolated in the region of Rio Negro - PR, by Blanco (1999).

**Development of Specific PCR Primers**

Following the detection of RAPD bands that were specific to strains of *G. citricarpa*, a new RAPD reaction was carried out using the DNA from isolate PC13/96 and the OPX14 primer. The DNA from the purified band (p373) was used, together with the OPX14 primer, in a reamplification reaction using the same conditions as before. This 373 bp fragment was purified, cloned in a *E. coli* DH5αF using the pGEM-T Easy Vector Systems kit (*Promega*®, Madison, WI, USA), and sequenced using the T7 Sequencing kit (*USB*, Cleveland, Ohio, USA) and the sequencing method of double-stranded DNA. The resulting sequence was used to design the following primer pair for *G. citricarpa:* GCP1 5' AAGTGTGAGTGTCGAAGGTGG 3' and GCP2 5' GACGACTCGCTTTTCTACGGC 3', resulting in a 340 bp amplicon (Blanco, 1999). Reaction conditions were: 1 X PCR buffer (50 mM of KCl, 200 mM of Tris-HCl, pH 8.4), 1.5 U of *Taq* DNA polymerase (*Invitrogen*, Carlsbad, CA, USA), 0.25 μM of each primer, 0.2 mM of each dNTP (*Pharmacia*, Freiburg, Germany), 25 ng of DNA and 1.5 mM of $MgCl_2$ in a total volume of 25 μl.

Amplification was carried out in a MJ Research ® thermocycler (Watertown, MA, USA) using the following conditions: initial denaturation at 94°C for 2 min; 35 cycles of 1 min at 94°C / 1 min at 69°C / 1 min at 72°C; and a final extension of 3 min at 72°C.

A positive control for the amplification was conducted in a reaction with the primers ITS1/ITS4 and the conditions described in White et al. (1990). Reaction conditions were: 50 ng of DNA, 1 X PCR buffer (50 mM of KCl, 200 mM of Tris-HCl, pH 8.4), 1.5 U of *Taq* polymerase (*Invitrogen*, Carlsbad, CA, USA), 0.25 μM of each primer, 0.2 mM of each dNTP (*Pharmacia*, Freiburg, Germany), and 1.5 mM of $MgCl_2$ in a total volume of 25 μl. Amplification was carried out in a MJ Research® thermocycler (Watertown, MA, USA) using the following conditions: initial denaturation at 94°C for 2 min; 35 cycles of 1 min at 94°C / 1 min at 50°C / 1 min at 72°C; and a final extension of 3 min at 72°C.

The robustness and the specificity of the primer pair were assessed using DNA from the reference isolates of *G. citricarpa* and *G. mangiferae* (Table 1) that had been validated using the pathogenicity

tests (Baldassari, 2005). Specificity was also tested against the cultures of the common citrus endophyte, *Colletotrichum gloeosporioides*, and against leaves of orange and lemon trees.

## RESULTS AND DISCUSSION

RAPD markers were used in the present study to investigate the distribution of genetic variability among the isolates of *G. citricarpa*, *G. mangiferae* and *Phyllosticta* spp. (Tables 1 and 2), resulting in 141 polymorphic markers. Species-specific bands were found both for *G. citricarpa* and *G. mangiferae*.

The analysis using RAPD markers allowed for the discrimination of the isolates into four main groups with 5% genetic similarity. The high bootstrap values (*P*>90%; Fig. 1) confirmed the robustness of the groupings. A Principal Coordinate Analysis (PCO) based on the genetic similarities estimated with the RAPD markers (Fig. 2) showed high diversity of the isolates of groups I and II, with the clear formation of subgroups that corroborated those observed in Fig. 1.

These four groups (I - IV in Figs. 1 and 2) were considered as distinct populations according to the AMOVA, which indicated that 62.8% of the genetic variation was between the populations, although substantial variation was found in populations (37.2% *P*<0.0001) (Table 3). The high genetic variability observed in and among the groups was surprising, given that all the studied strains belonged to the genus *Guignardia* and its anamorph *Phyllosticta*. Using the same method, several studies have demonstrated that such low levels of similarity were mainly found among the genera, including the phylogenetically distant ones (Motta et al., 2002; Azevedo et al., 2000; Mehta, 2001).

**Table 3** - Analysis of molecular variance (AMOVA).

| Source of variation | DF | SS | Variation components | % of variation |
|---|---|---|---|---|
| Among populations | 3 | 599.430 | 18.11 | 62.80 |
| Within populations | 55 | 590.028 | 10.73 | 37.20* |
| Total | 58 | 1189.458 | 28.84 | |

*(*P* < 0.01)

The low genetic similarity among these groups has also been observed using data from AFLP and ITS sequences. Baayen et al. (2002) suggested the existence of at least three distinct species of *Guignardia* coexisting in Brazilian citrus plants: *G. citricarpa* (causing CBS) and two endophytic species: *G. mangiferae* (Baayen et al., 2002), and *P. spinarum*.

Group I included the *G. citricarpa* isolated from the CBS lesions (Fig. 1). The high variability observed in this group did not seem to be associated with the geographical origin of these strains, which were obtained in different continents. Two subgroups could be recognized in this group: Ia and Ib. Subgroup Ia included the strains isolated in Brazil and one strain isolated in South Africa.

Subgroup Ib was composed of strains obtained in Brazil, Japan, South Africa, and Mexico.

Group II included the isolates of considerable genetic diversity, with broad geographical distribution and considerable host diversity (Table 3). This was shown by the composition of the subgroup IIa, which included the strains of *G. mangiferae* from South Africa, Brazil, Costa Rica, United States, and Thailand obtained from different hosts (Tables 1 and 2). In addition, the fungus *P. capitalensis* has already been described as causing disease in orchids (Okane et al., 2001).
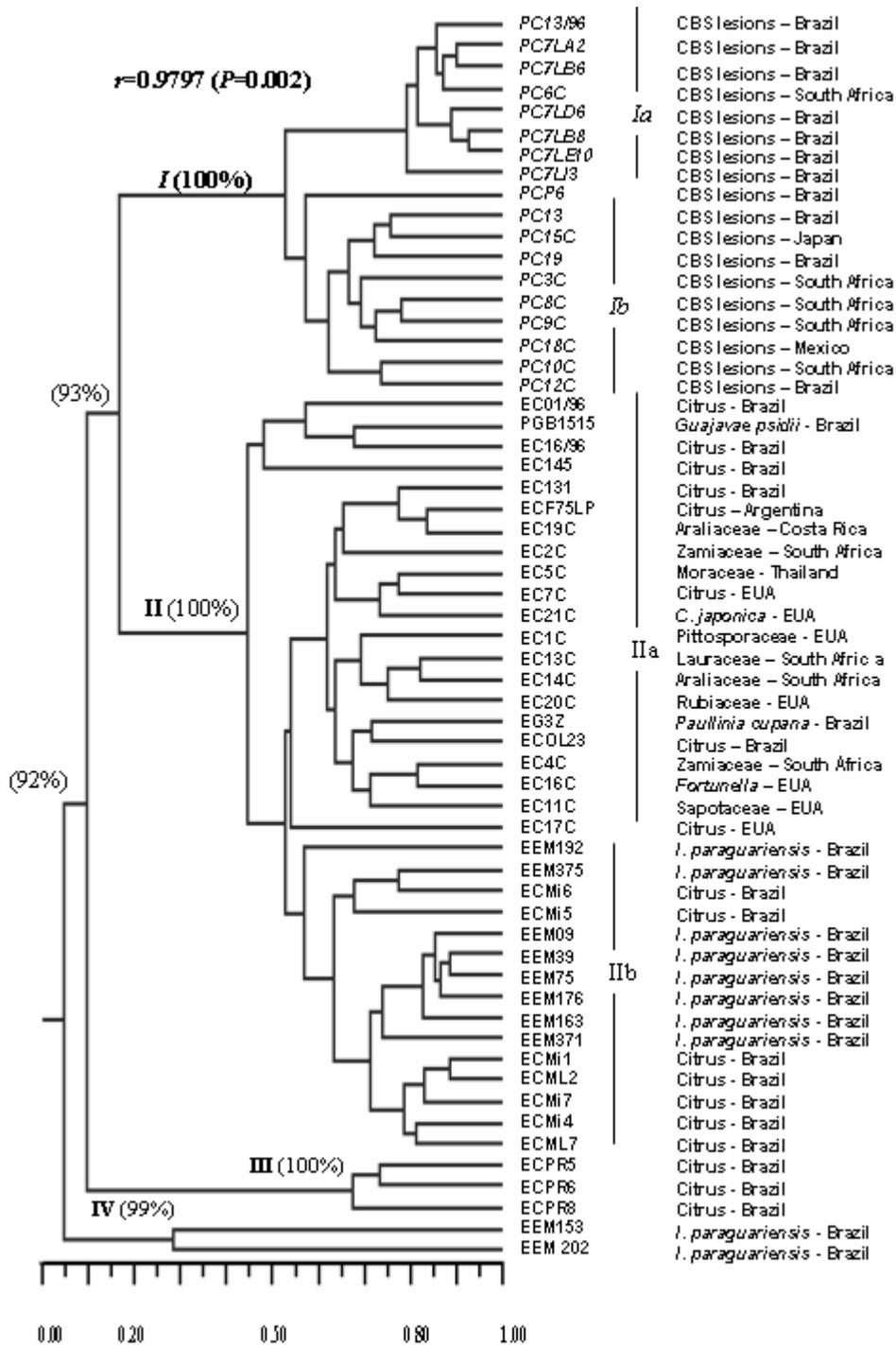
**Figure 1 -** Dendrogram generated from the genetic similarity matrix obtained using RAPD analysis of the isolates from Tables 1 and 2. ***Group I:*** *G. citricarpa*; **Group II:** *G. mangiferae* and *Phyllosticta* sp.; **Group III:** *Phyllosticta* sp, **Group IV:** *Phyllosticta* sp. Bootstrap values (%) of each group are shown in parentheses.
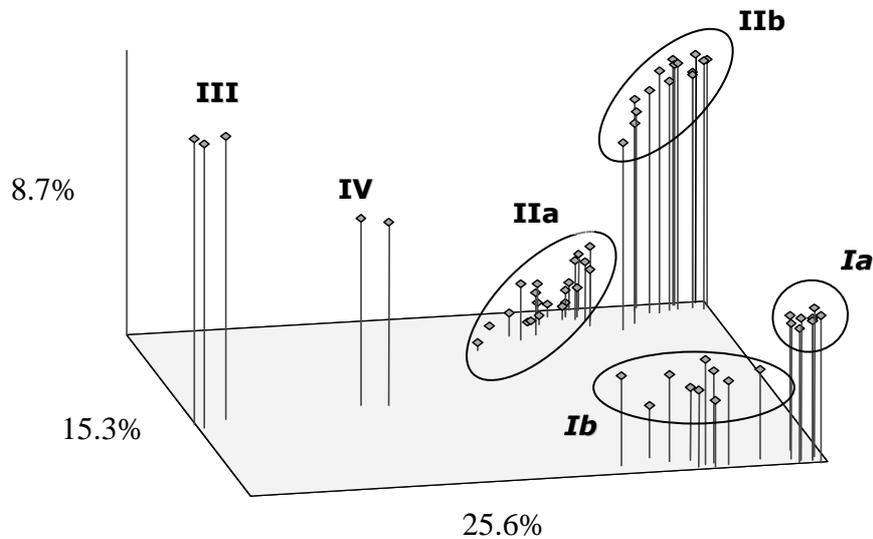
**Figure 2 -** Principal Coordinate Analysis (PCO) of endophytes based on genetic similarity. *Group I: G. citricarpa*; **Group II:** *G. mangiferae* and *Phyllosticta* sp.; **Group III:** *Phyllosticta* sp., **Group IV:** *Phyllosticta* sp. *Ia, Ib*, IIa e IIb indicate the subgroups identified in Figure 1.

Subgroup IIa included all the reference strains of *G. mangiferae*, six endophytic isolates from citrus, one endophytic isolate from *Paullinia cupana* (Guaraná), and the strain PGB1515 obtained from a lesion in *Guajavae psidii* (Table 1). The presence of the *Guignardia psidii*, a pathogen of the guava tree, in subgroup IIa indicated that this strain might belong to the *G. mangiferae* species and was classified as *G. psidii* due to its host plant in another case of synonymy. This reinforced the idea that the same microorganism could behave as an endophyte or as a pathogen depending on the host. This difficulty in establishing the limits between the endophytic and pathogenic species has been underscored by several authors (e.g. Azevedo J.L. et al., 2000; Araújo, et al., 2000). On the other hand, given the high genetic diversity in group II, it was possible that more than one species of *Guignardia* similar to *G. mangiferae* was endophytic in these plants.

Eight endophytic isolates obtained from *Ilex paraguariensis* (erva-mate) in 2000 were classified in subgroup IIb, as well as seven endophytes isolated from citric plant, all of which in the plants located in the State of Paraná, Brazil. It was noteworthy that although they were obtained from distantly related hosts,

the strains in this subgroup showed genetic similarity higher than those obtained from the same host plants, such as ECMi5 and ECOL23 (Fig. 1). The formation of the subgroup IIb indicated a tendency for grouping strains based on the geographical location of their host plants.

Despite the genetic diversity observed in group II, all the isolates were considered as belonging to the species *G. mangiferae* (*P. capitalensis*). Future studies including the sequencing of the ITS1-5.8S-ITS2 region of the rDNA might help elucidate this issue.

Group III included three isolates of *Phyllosticta* sp. endophytic of *C. limon* obtained in the municipality of Rio Negro, Paraná, Brazil in 1997. These isolates showed low genetic similarity (<10%) with the remaining isolates. These results not only corroborated the data obtained using other markers by Baayen et al. (2002), but also suggested that these strains could belong to another species of *Phyllosticta*. Sequencing data (not shown) suggested that these strains belonged to *P. spinarum*. The positioning of these lineages in relation to the remaining groups was clearly shown by the PCO in Fig. 2. Group IV was composed only of two endophytic isolates from *Ilex paraguariensis* (Figs. 1 and 2), which showed low genetic similarity with the remaining groups (Fig. 1). In addition, they were

intermediate between group III and the remaining isolates of *G. mangiferae* and *G. citricarpa* according to the PCO (Fig. 2). Given that it was not possible to identify the species based on the morphological characters alone, the strain was classified as *Phyllosticta* sp. Therefore, there were at least three species that are morphologically similar to *G. citricarpa* and were endophytic in *Citrus* spp. and *Ilex paraguariensis* in Brazil, namely *G. mangiferae*, *P. spinarum* and *Phyllosticta* sp.

It was clear that the isolation from *Citrus* spp. that were morphologically similar to *G. citricarpa* did not necessarily meant that the host was contaminated with the causal agent of CBS. The identification using the morphological and physiological characters was imprecise and time-consuming, being inadequate for the analysis of fruits to export (Baayen et al*.,* 2002). Thus, it was necessary to develop diagnostic methods that would be rapid and efficient (Bonants et al*.*, 2003).

Results also underscore the efficiency of RAPD markers, not only in the studies of population structure and in the assessment of genetic variability in fungus populations, but also to suggest the presence of distinct species of a given genus in the surveys of the endophytic community of the studied hosts.

**Development of Specific PCR Primers**
The strains in Tables 1 and 2 were tested using PCRs with the GCP1/GCP2 primer pair. Only strains in Group I (Fig. 1) showed the amplification of a band of approximately 350 bp (Fig. 3).

The primers ITS1 and ITS4 were used as positive controls of the amplification (White et al., 1990). These primers are universal for fungi and allow for the amplification of the ITS1 –5.8S – ITS2 region of the rDNA. All the strains amplified an approximately 650 bp fragment (Fig. 3), which was consistent with the expected fragment sizes ca. 700 bp for ascomycete (White et al., 1990).
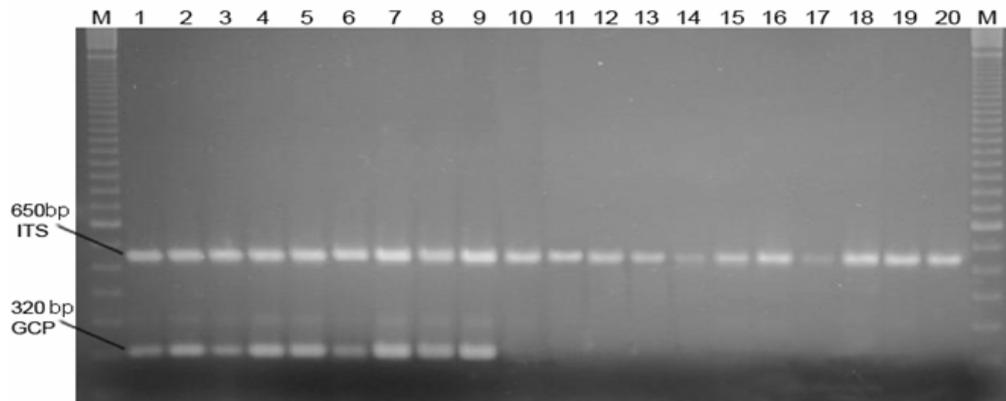


**Figure 3 -** Agarose gel electrophoresis of PCR using the primer pairs GCP1/GCP2 and ITS1/ITS4, and isolates of *G. citricarpa* and *G. mangiferae.* Legend: M: 100 bp. DNA Ladder. 1: Reference strain of *G. citricarpa* PC28/05 (Table 1); 2 - 9: *G. citricarpa* (Table 2); 10: Reference strain of *G. mangiferae* EC22/05 (Table 1); 11 - 20: *G. mangiferae* (Table 2).

Therefore, the isolates of the pathogenic fungus *G. citricarpa* obtained from several different countries could be discriminated from the endophytic strains of *Guignardia* spp. isolated from *Citrus* spp. using the GCP1 and GCP2 primers. The correct assignment of the strains of

*G. citricarpa* and *G. mangiferae* (Fig. 4) validated in the pathogenicity test for CBS (Baldassari, 2005) demonstrated the efficiency in obtaining the SCARS and developing new primers for the identification of the causal agent of CBS.
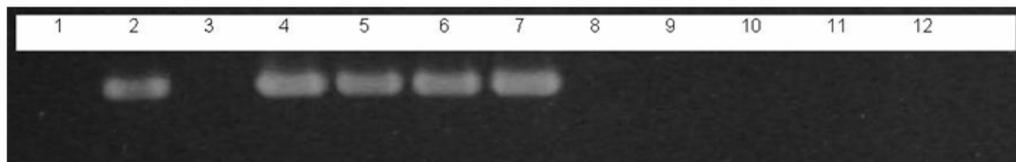
**Figure 4 -** Agarose gel electrophoresis of PCR using the primer pair GCP1/GCP2 and isolates of *G. citricarpa*, *G. mangiferae* and *Phyllosticta* sp. 1: Negative control without DNA; 2: pGEM-T: plasmid containing the 373bp cloned fragment; 3: *P. spinarum* (ECPR05); 4 - 6: Reference strains of *G. citricarpa* (PC28/05, PC32/05, PC33/05); 7: Strain PC13/96 of *G. citricarpa*; 8 - 10: Reference strains of *G. mangiferae* (EC 01/05, EC19/05, EC22/05); 11: Negative control: *Colletotrichum gloeosporioides*; 12: Negative control: DNA of citrus leaves.

## CONCLUSIONS

The high genetic variability observed among the groups obtained using RAPD markers corroborated previous studies (Baayen et al., 2002; Rodrigues et al., 2004) with respect to the existence of at least three distinct species of *Guignardia* and its anamorph *Phyllosticta* that were very similar morphologically to the pathogen *G. citricarpa*, coexisting in *Citrus* spp. and hampering the identification of the plants and fruits free from the causal agent of CBS.

The use of RAPD markers allowed for the classification of isolates into *G. citricarpa*, *G. mangiferae* and *Phyllosticta* sp.. The fact that most endophytic isolates from different hosts and regions could be grouped as belonging to *G. mangiferae* confirmed that a single fungus species could endophytically colonize several host species. In addition, the high genetic similarity between the strain of *G. psidii*, a guava pathogen, with the strains of *G. mangiferae* indicated that the same microorganism might be found acting as an endophyte in some plants and as a pathogen in others. In other words, *G. mangiferae* could colonize certain types of the plants without causing symptoms of CBS (citric plants and other hosts) and became pathogenic in other plant species (guava trees and orchids).

The GPC1/GCP2 was efficient in the identification of *G. citricarpa* as the causal agent of CBS through PCR.

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## RESUMO

Marcadores de RAPD foram utilizados para investigar a distribuição da variabilidade genética de linhagens de Guignardia citricarpa, G. mangiferae, e Phyllosticta spinarum isolados em diversos hospedeiros no Brasil, Argentina, México, Costa Rica, Tailândia, Japão, EUA e África do Sul. O fungo Guignardia citricarpa Kiely (Phyllosticta citricarpa McAlp Van Der Aa) é o agente causal da Mancha Preta dos Citros (CBS), uma doença que afeta diversas plantas cítricas, causando dano a aparência dos frutos, prejudicando a exportação. Diversos estudos têm demonstrado a existência de uma espécie endofítica muito semelhante morfologicamente a G. citricarpa, e que permanece de forma endofítica no mesmo hospedeiro. Dificultando assim, a identificação de plantas e frutos livres do agente causa da CBS. A análise do perfil de RAPD revelou uma clara discriminação entre isolados patogênicos de G. citricarpa e isolados endofíticos (G. mangiferae e P. spinarum). A Análise de Coordenadas Principais (PCO) baseada na matriz de similaridade genética dos marcadores RAPD, demonstrou a formação de quatro grupos, sem relação com origem geográfica ou com hospedeiros utilizados. A análise de Variância de Marcadores Moleculares (AMOVA) indicou que 62,8% da variação genética é encontrada entre as populações (G. citricarpa, G. mangiferae, P. spinarum and Phyllosticta sp.). Entretanto,

variação substancial foi encontrada dentro destas populações (37,2%). Bandas de RAPD exclusivas de isolados de G. citricarpa foram clonadas, sequenciadas e utilizadas na obtenção de SCARS (Sequence Characterized Amplified Regions), que permitiram o desenvolvimento de novos primers específicos para a identificação de G. citricarpa. Reações de PCR (Polymerase Chain Reaction) utilizando este par de primers corroboraram os agrupamentos obtidos pelos marcadores de RAPD, revelando sua eficiência na identificação do agente causal da CBS.

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