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Amplification of the *cap*20 Pathogenicity Gene and Genetic Characterization Using Different Markers Molecular in *Colletotrichum gloeosporioides* Isolates

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ABSTRACT

Studies were performed to analyze the genetic characterization using RFLP-ITS and Intron (primer EI1) markers and the amplification of the cap20 pathogenicity gene by PCR in Colletotrichum gloeosporioides *isolates of different hosts plant. The genetic variability was accessed using RFLP-ITS and Intron markers and grouping by UPGMA method. Primers to cap20 gene were constructed using selected sequences of the GenBank (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov) with the Primer 3 program. The dendrograms analysis showed that the RFLP-ITS marker was more informative to separate the Colletotrichum sp, and that primer EI1 demonstrated greater genetic diversity. The amplification of the DNA of the* Colletotrichum *isolates to the cap20 gene with primers P1 and P2 indicated that this gene could present variations into* C. gloeosporioides *related with the host, and also that it was present in other Colletotrichum sp.*

Key words: *cap20* gene, *Colletotrichum gloeosporioides*, pathogenicity

INTRODUCTION

Anthracnose caused by *Colletotrichum* species is the main disease in post-harvest fruit and is considered disease of high economic importance in the Northeast of Brazil (Serra and Silva, 2004). *Colletotrichum* is a filamentous fungus that affects fruit of many varieties of botanical species in pre and post-harvest, causing quiescent infections particularly in tropical and subtropical regions, resulting in losses of billions of dollars annually (Korsten and Jeffries, 2000; Kramer-Haimovich et al., 2006).

Several factors are responsible for the post-harvest losses, including post-harvest fungal diseases, which cause considerable losses on the products collected, fruits and vegetables. The losses range around 20 to 50%, which depending on the year and quality of storage, can be high in developing countries (Smith et al., 2003; Agrios, 2005).

The *Colletotrichum* genus displays two modes of nutrition, biotrophic and necrotrofic and develops a series of specialized infection structures, such as: germ tube, appressorium, peg penetration, vesicles infectives, primary and secondary hyphaes (O'Connell et al., 2000). The stages of formation of these structures are marked by the production of specific metabolites, which may act as pathogenicity factors. The penetration of the tissue is favored by the action of pectinolytic enzymes,

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excreted on the host tissue and by the mechanical force exerted by appressorium on the peg of penetration (Agrios, 2005).

Recently several genes of the phytopathogenics fungi have been identified, that after deletions, resulted in reduction or total loss of symptoms of the disease. Some of these genes are responsible for the degradation on the cell wall of the plant; others induce the formation of the specialized structures such as appressorium that penetrates into the epidermis and others produce toxins to overcome the defenses of the plants (Idnurm and Howlett, 2001). Oliver and Osboum (1995) sugested that the pathogenicity genes were those required for the development of the disease and have been identified, in recent years, to increase the knowledge of process and control of the disease (Idnurm and Howlett, 2001).

Differential screening of a cDNA library produced by a subtractive hybridization approach yielded the genes designated *cap* expressed uniquely during appressorium formation of *C. gloeosporioides* induced by the wax present on the surface of the fruit of avocado. The *cap*20 gene encodes a protein of 20 kDa which may have a role in the apressorium function. The CAP20 peptide showed homology with the proteins of the cell wall and could be part of the wall of the apressorium. Mutants with disruption of this gene was unable to express a protein of 20 kDa and showed drastic reduction in virulence in the fruits of avocado and tomato (Hwang et al., 1995).

Several methods can be used for inter and intraspecific characterization of *Colletotrichum* sp.. Currently, a promising tool for the study of population dynamic is the use of molecular markers that can infer the genetic variability (Katan, 2000). The existence of species-specific *primers* based on nucleotides sequences of the ITS1 rDNA region have made the Polymerase Chain Reaction (PCR) a powerful tool for the identification of *Colletotrichum* species (Peres et al., 2002; Afanador-Kafuri et al., 2003; Bueno, 2005; Tozze Junior et al, 2004; Andrade et al., 2007).

The aim of this study was to amplify the pathogenicity gene *cap*20 by PCR and intraspecific genetic characterization using molecular markers RFLP-ITS and ISSP in *C. gloeosporioides* isolates obtained from different hosts plant.

MATERIAL AND METHODS

Strains *Colletotrichum*

Twenty eight isolates of *Colletotrichum* sp of different hosts were obtained from the Department of Mycology, Federal University of Pernambuco (URM-UFPE) (Table 1). The isolates were maintained on potato dextrose agar (PDA) medium for seven days. Out of these, 17 (1-17) were used for genetic characterization with molecular markers CgInt-ITS4, RFLP-ITS and ISSP and three isolates of other species (20-21 and 25) were used as outgroup for comparative analysis.

Table 1 - *Colletotrichum gloeosporioides* isolates according from hosts.

Isolates	Access number	Geographic origin	Host
1	URM4626	Brejão/PE	Stalk of the onion
2	URM4627	Belém de S. Francisco/PE	Leaf of the onion
3	URM4628	Brejão/PE	Inflorescence of the onion
4	URM4629	Petrolina/PE	Leaf of the onion
5	URM4894	Brejão/PE	Leaf of the cashew
6	URM4896	Garanhuns/PE	Leaf of the cashew
7	URM4900	São João/PE	Leaf of the cashew
8	URM4905	Igarassu/PE	Leaf of the cashew
9	URM4908	Igarassu/PE	Inflorescence oh the cashew
10	URM4852	Garanhuns/PE	Leaf of the mango
11	URM4854	Itambé/PE	Leaf of the mango
12	URM4856	Igarassu/PE	Leaf of the Pink mango
13	URM4857	Igarassu/PE	Leaf of the mango
14	URM4858	Brejão/PE	Leaf of the pinkm mango
15	URM4859	Recife/PE	Leaf of the pink mango
16	URMCi	São Paulo/SP	Leaf of the ciclamen
17	URMPI 13	São Paulo/SP	Fruit of the chili
18	URM1633	Bragança Paulista/SP	Fruit of the chili
19	*C. gloeosporioides* "Esalq"	Bragança Paulista/SP	Fruit of the chili

Table 2 - *Colletotrichum* sp. isolates according from hosts.

Isolates	Fungi	Access number	Geographic origin	Host
20		URMPI15	São Paulo/SP	Fruit of the chili
21	*C acutatum*	URMAcu	São Paulo/SP	Fruit of the peach
22	*C. boninense*	*C. boninense* "Esalq"	Caxias do Sul/RS	Fruit of the chili
23	*C. coccodes*	*C. coccodes* "Esalq"	Caxias do Sul/RS	Fruit of the chili
24	*C. capsici*	URM1245	Mamanguape/PB	Fruit of the papaya
25	*C. sublineolum*	URMSub	São Paulo/SP	Leaf of the sorgum
26	*C. dematium*	URM 1023	Balsas/MA	Stem of the soy
27	*C. graminicola*	URM 1041	Recife/PE	Leaf of the mayze
28	*C. gossypii var. cephalosporioides*	URM 1147	Montevidéu/GO	Leaf of the cotton

For the amplification of the *cap20* gene were used them all the 28 isolates, of wich nine isolates of other species (20-28) were used as outgroup for comparative analysis.

DNA extraction

Flasks containing 100 ml liquid minimum medium were inoculated with 3 ml of the conidial suspension of *Colletotrichum* sp. (10^6 conidia/ml), maintained under agitation (27 rpm) and incubated at 27ºC for 120 h. The mycelium were harvested by filtration, washed with sterile-distilled water and stored at -20ºC until use. Total genomic DNA was extracted as described by Kuramae and Izioka (1997). The mycelium was ground into the fine powder under liquid nitrogen and suspended in 700 µL extraction buffer (1M Tris-HCl pH 8.0; 5M NaCl; 0,5mM EDTA pH 8.0; 10% SDS). Upon homogenization, the tubes were incubated for 30 minutes at 65ºC.

DNA samples were purified with equal volumes of chloroform: isoamyl alcohol (24:1) mixture (1X), and precipitaded with isopropanol. The tubes were centrifuged at 15400g (Eppendorf® centrifuge) for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried, suspended in TE buffer (pH 8.0) and stored at 4°C until use.

DNA amplification with *primers* CgInt/ITS4

For specific confirmation of the *C. gloeosporioides* isolates, species-specific *primers* CgInt-ITS4 was used. Amplification reactions were prepared to final volume of 25 µL containing 1x *Taq* buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 25 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and CgInt (5'-GGCCTCCCGCCTCCGGGCGG-3') and 0.04U *Taq* DNA polymerase (Invitrogen), as described by Freeman et al (2000). Thermal cycling consisted of initial denaturation of 5 minutes at 95ºC, followed by 40 cycles of 30 seconds at 95ºC, 30 seconds at 45ºC and 1 minute and 30 seconds at 72ºC. The amplicons were visualized in 1.0 % (w/v) agarose gel at 3 V/cm in TBE buffer (pH 8.0) after ethidium bromide staining using 100-pb ladder marker (Invitrogen).

RFLP-ITS

Amplification reactions were prepared to final volume of 25 µL containing 1x *Taq* buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 25 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAA-3') and 0.04U *Taq* DNA polymerase (Invitrogen), as described by White et al (1990). Thermal cycling consisted of initial denaturation of 5 minutes at 95ºC, followed by 40 cycles of 30 seconds at 95ºC, 30 seconds at 50ºC and 1 minute and 30 seconds at 72ºC. Amplification products were visualized in 1.4 % (w/v) agarose gel at 3 V/cm in Tris-Borato-EDTA (TBE) buffer (pH 8.0) after ethidium bromide staining. Aliquots of 4 µL of the amplicons were subjected to enzymatic digestion with *Dra*I, *Msp*I or *Hae*III, according to manufacturer instructions. Fragments were separated in 1.5% (w/v) agarose gel and their molecular weights were determined using to 100-pb ladder marker (Invitrogen).

ISSP

Fingerprinting analysis were performed with EI1 type I Intron Splice Site Primer (5'-CTGGCTTGGTGTATGT-3') as described by De Barros Lopes et al (1996). The amplification reactions contained 1x *Taq* buffer (20 mM Tris-

HCl pH 8.4, 50mM KCl), 25 ng template DNA, 1.5 mM $MgCl_2$, 0.25mM dNTP, 0.5 µM of EI1 (5'-CTGGCTTGGTGTATGT-3') primer and 0.04U *Taq* DNA polymerase (Invitrogen) into final volume of 25 µL. Thermal cycling consisted of initial denaturation of 5 minutes at 95ºC, followed by 40 cycles of 30 seconds at 95ºC, 30 seconds at 45ºC, 1 minute and 30 seconds at 72ºC, with final extension of 5 minutes at 72ºC. The amplicons were visualized in 1.0 % (w/v) agarose gel at 3 V/cm in TBE buffer (pH 8.0) after ethidium bromide staining.

Genetic analysis

The variable binary similarity matrix was prepared using Jaccard coefficient by the NTSYS program (Numerical Taxonomy System of Multivariate program) version PC 2.1 (Rohlf, 1988). Dendrogram were prepared by UPGMA (Underweight Pair Group Method with Arithmetical Average) analysis.

Amplification of the *cap*20 gene by PCR

Primers for the PCR reactions were constructed from the sequences of *C. gloeosporioides* selected from GenBank (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov). The amplification reactions were prepared to final volume of 25 µL with following conditions: 1x *Taq* buffer (Tris-HCl 20mM pH 8.4; 50mM KCl), 1.5 mM $MgCl_2$, 0.3 mM dNTP, 0.2 µM of each primer P1 (5'-GCAACATCTCGTCCGCTCT-3') and P2 (5'-TGAAGTGGGGAGAAGGGAA-3'), 0.04U *Taq* DNA polimerase (Invitrogen Life Tecnologies) and 25 ng of DNA. The PCR reaction involved an initial step of denaturation of 5 minutes at 95°C, followed by 10 cycles involving denaturation of 30 seconds at 95 °C; anelament of 30 seconds at 50°C and extension of 30 seconds at 72°C, and a second step with 30 cycles involving initial denaturation of 30 seconds at 95°C, anelament of 30 seconds at 47°C and extension of 30 seconds at 72°C. These reactions were repeated four times (4x) to get the generated confirmation results. The products amplified were visualized in 1.0 % (w/v) agarose gel at 3 V/cm in TBE buffer (pH 8.0) after ethidium bromide staining and their molecular weights were determined using 100-pb ladder marker (Invitrogen).

Enzimatic digestion of the amplified *cap*20 gene products

Aliquots of 6 µl of the amplicons were subjected to enzymatic digestion with *Msp*I (Invitrogen), according to manufacturer instructions. The restriction fragments were separated on 1.5% (w/v) agarose gel, with 0.5x TBE buffer. The gel was stained with ethidium bromide (0.5mg/mL), and the DNA fragments visualized under UV light, using marker of molecular weight 1-kb plus (Invitrogen).

RESULTS AND DISCUSSION

Specific characterization with *primers* CgInt-ITS4

The amplification using the *primers* CgInt-ITS4 (Fig. 1) was positive for all of the *C. gloeosporioides* isolates, generating fragments of approximately 450-bp and negative for of the *C. acutatum* and *C. sublineolum* isolates.The use of these primers confirmed the identity of the isolates of the *C. gloeosporioides* identified by traditional methods. Mills et al. (1992) and Freeman et al. (2000) used these markers for the identification of the *C. gloeosporioides* isolates, which showed fragments of 450-bp. The results were similar to those found here.

Polymorphism of fragments digestion of the ITS rDNA regions of *C. gloeosporioides*

The amplification of the products ITS rDNA regions using *primers* ITS4 and ITS5 revealed fragments around the 600-pb for *Colletotrichum* sp. isolates. The ITS rDNA region was not informative to differentiate the *Colletotrichum* species (Fig. 2A).

Several authors working with primers ITS1 and ITS2 observed that the majority of isolates identified as *C. gloeosporioides*, showed a single fragment of approximately 590-600 bp (Abang, 2002; Martínez-Culebraz et al., 2000; Saha, 2002). The digestion of the fragments of the ITS with *Dra*I produced a monomorphic fragment of approximately 400-bp, for all *Colletotrichum* sp. isolates that made impossible to differentiate the species (Fig. 2B). *Msp*I generated three distinct fragments of 300, 150 and 100-bp for all of the *C. gloeosporioides* isolates, except for the

URM4900 isolate which presented fragments of 350, 150 and 100-bp; fragments of 300, 170 and 100-bp for URMPI15 and URMAcu of *C. acutatum* isolates and fragments of 300, 130 and 100-bp for URMSub of the *C. sublineolum* isolate (Fig. 2C). The restriction of the products amplified with the *Hae*III enzyme generated three fragments distinct in sizes of 280, 180 and 150-bp for all the *C. gloeosporioides* and *C. acutatum* isolates, and fragments of 300, 180 and 170-bp for URMSub of the *C. sublineolum* isolate, distinguishing only this last specie (Fig. 2D) (Table 3).

In this study, the *Msp*I and *Hae*III enzymes showed polymorphism in the number and length of the resulting fragments, supporting the idea of the existence of intraspecific genetic diversity among different *C. gloeosporioides* isolates.

The *Msp*I restriction enzyme better separated the three of the *Colletotrichum* species, in comparison with the other enzymes tested; however was not efficient in separating the *C. gloeosporioides* isolates from the host and geographic region, except for URM4900 isolate. The URM4900 isolate from cashew leaf of the *C. gloeosporioides* showed different amplification profiles of the other isolates of the same host.

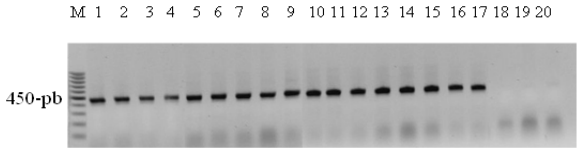

Figure 1 - *Colletotrichum gloeosporioides.* Isolates profiles obtained with *primers* Cgint-ITS4. M – Molecular weight size markers 100-bp. Lanes 1-20 corresponding to strains URM4626, URM4627, URM4628, URM4629, URM4894, URM4896, URM4900, URM4905, URM4908, URM4852, URM4854, URM4856, URM4857, URM4858, URM4859, URMCi, URMPI13, URMPI15, URMAcu and URMSub, respectively**.**

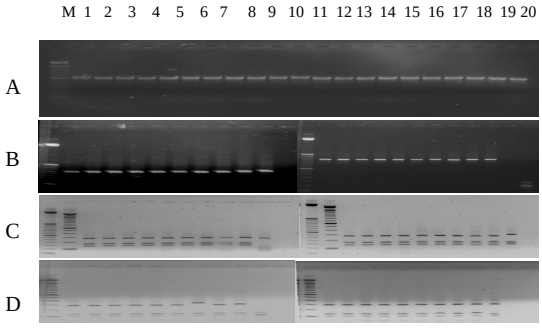

Figure 2 - *Colletotrichum gloeosporioides* ITS-RFLP profiles of the isolates, obtained with *primers* ITS4 and ITS5 (A), restriction enzymes (B) *Dra*I, (C) *Msp*I and (D) *Hae*III. M – Molecular weight size markers 100-bp. Lanes 1-20 corresponding to strains URM4626, URM4627, URM4628, URM4629, URM4894, URM4896, URM4900, URM4905, URM4908, URM4852, URM4854, URM4856, URM4857, URM4858, URM4859, URMCi, URMPI13, URMPI15, URMAcu and URMSub, respectively**.**

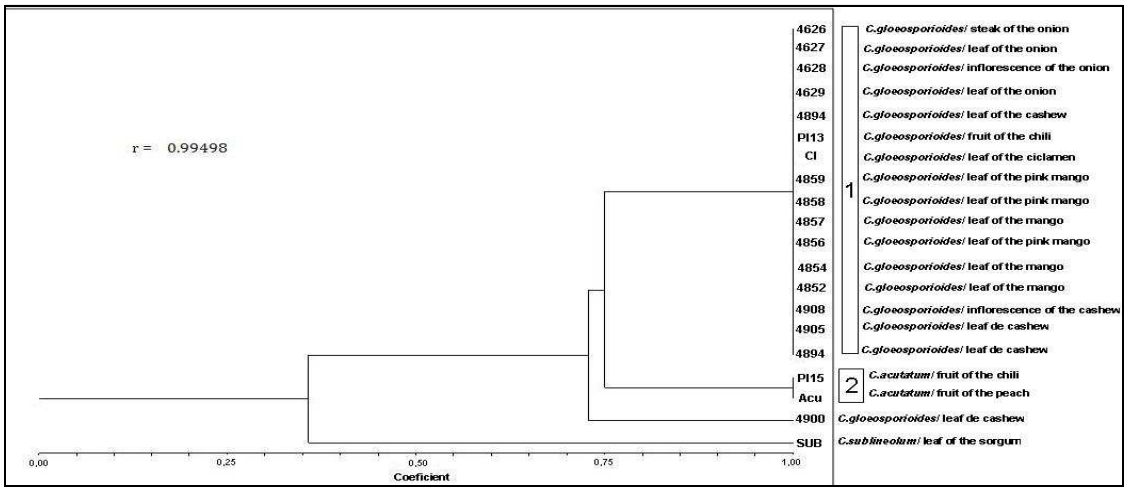

Figure 3 - Dendrogram of relationships between isolates of the *C. gloeosporioides* basead on UPGMA clustering of the matrix obtained by Jaccard coefficient (J).

Table 3 - ITS length and restriction fragments from ITS products generated.

Isolates	**ITS (pb)**	***Dra*I**	***Msp*I**	***Hae*III**
4626	600	400	300, 150 e 100	280, 180 e 150
4627	600	400	300, 150 e 100	280, 180 e 150
4628	600	400	300, 150 e 100	280, 180 e 150
4629	600	400	300, 150 e 100	280, 180 e 150
4894	600	400	300, 150 e 100	280, 180 e 150
4896	600	400	300, 150 e 100	280, 180 e 150
4900	600	400	300, 150 e 100	280, 180 e 150
4905	600	400	300, 150 e 100	280, 180 e 150
4908	600	400	300, 150 e 100	280, 180 e 150
4852	600	400	300, 150 e 100	280, 180 e 150
4854	600	400	300, 150 e 100	280, 180 e 150
4856	600	400	300, 150 e 100	280, 180 e 150
4857	600	400	300, 150 e 100	280, 180 e 150
4858	600	400	300, 150 e 100	280, 180 e 150
4859	600	400	300, 150 e 100	280, 180 e 150
Ci	600	400	300, 150 e 100	280, 180 e 150
PI13	600	400	300, 150 e 100	280, 180 e 150
PI15	600	400	300, 170 e 100	280, 180 e 150
Acu	600	400	300, 170 e 100	280, 180 e 150
Sub	600	400	300, 130 e 100	300, 180 e 170

This could be due to the great morphological plasticity, adaptability and specificity that existed among the isolates of the *C. gloeosporioides* specie or by pathogenic specialization caused by the mutations, requiring further studies. Results obtained with the *Hae*III restriction enzyme suggested that *C. gloeosporioides* and *C. acutatum* species presenting the same profile of digestion, could be related and that these species probably differed recently.

The analysis of the grouping profiles generated from three enzymes allowed the construction of the dendrogram where two distinct groups could be observed in the level of similarity of size of fragments of 85%. The first group contained all of the *C. gloeosporioides* isolates with 100% of similarity between themselves (except the URM4900 isolate), and the second group with the two isolates of *C. acutatum* also with 100% of similarity between themselves. The URM4900

isolate showed similarity of size of fragments with the others two groups in amount around 73%. The URM4900 of the *C. gloeosporioides* isolate and URMSub of *C. sublineolum* isolate were different (Fig. 3).

Martínez-Culebras et al (2000), studying the genetic variability of *Colletotrichum* sp. isolates from the strawberry separated the isolates of *C. gloeosporioides* from the other species examined for the restriction profiles of ITS rDNA region with nine restriction enzymes whose specific profiles of *C. gloeosporioides* was observed with the MvnI enzyme. Martínez-Culebras (2003) differentiated 80 species of the *Colletotrichum* genus by ITS1 and ITS2 of the region 5.8S rDNA, which showed that the *C. acutatum* isolates of the same geographical region belonged to the same group. Vinnere (2002), using profiles of restriction of the fragments of ITS region distinguished the *C. acutatum*, *C. gloeosporioides* and *C. dematium* species between the isolates which were identified as a classical form of *C. gloeosporioides*. The ITS-RFLP technique was useful for the identification of the *Colletotrichum* species, whose specific profile was observed with the *Msp*I enzyme and efficient for the analysis of intraspecific genetic diversity between *C. gloeosporioides* isolates using the *Msp*I and *Dra*I enzymes.

Analysis of the intron site of splicing

The amplification profiles of the region of intron using the *primer* EI1 in *Colletotrichum* sp. isolates and dendrogram generated are illustrated in the Figs. 4 and 5, respectively. The dendrogram generated from the amplification profiles revealed the formation of three distinct groups with 85% similarity in the size of fragments, indicating high intraspecific genetic diversity among the *C. gloeosporioides* isolates. The first group was represented by four isolates of *C. gloeosporioides* from the onion (URM4626, URM4627, URM4628 and URM4629); the second group was formed by two isolates of *C. gloeosporioides* from the mango (URM4852 and URM4858), and the third group was represented by three isolates from the mango (URM4854, URM4857 and URM4859) of *C. gloeosporioides*. The groups formed presented 100% similarity in the size of fragments for the isolates between themselves.

In this study, all the *C. gloeosporioides* isolates from the onion showed the same profile of bands; however, the cashew isolates of the species (URM4894, URM4896, URM4900, URM4905, URM4908) were more heterogeneous in the number, and the length of the bands generated showed high genetic diversity. It was observed that the URM4856 of the *C. gloeosporioides* isolate from the pink mango did not form group with other isolates of the same host, and the URMCi and URMPI13 isolates were different.

The ISSP marker showed high intraspecific genetic diversity among the *C. gloeosporioides* isolates, presenting certain degree of grouping for the host, but the grouping for the species was not evidenced (Fig. 5). This is the first report on the use of primer EI1 for genetic analysis of the *Colletotrichum* species.

Brasileiro et al (2004) had used marker EI1 to analyze the intraspecific polymorphism in *Fusarium solani* isolates. They reported that some *F. solani* isolates understood the I-EI1 group that represented a clonal strain. Moreover, other isolates of the same species showed high genetic divergence to the primer EI1, detected by the analysis of ITS region.

Medeiros (2008), studying the genetic characterization by ISSR molecular markers of 20 isolates of *Colletotrichum* sp. from the various hosts, found that this technique was able to group the isolates of different species according to the origin host. Similar, in this study the homology observed between the isolates from the onion and the grouping between some isolates of *C. gloeosporioides* from the mango and cashew.

In this work, the ITS-RFLP and ISSP techniques were effective to demonstrate the intraspecific genetic characterization between the *C. gloeosporioides* isolates; however, the ISSP molecular marker was more informative to separate the intraspecific isolates, generating a higher polymorphism and consequently, greater genetic diversity in relation to ITS-RFLP technique, which was a very important method to differentiate the species of fungi. Moreover, these two techniques are appropriate tools to discriminate all the species at the interspecific level.

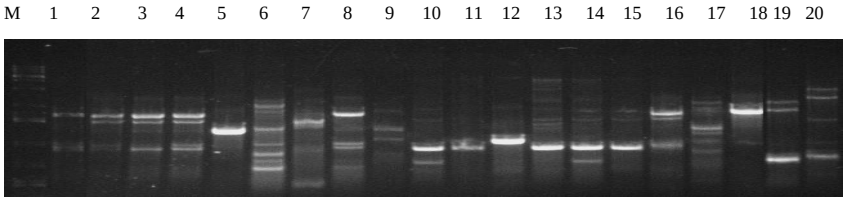

Figure 4 - Profiles of intron of the *Colletotrichum gloeosporioides* isolates with the *primer* EI1. M – Molecular weight size markers 100-bp. Lanes 1-20 corresponding to strains URM4626, URM4627, URM4628, URM4629, URM4894, URM4896, URM4900, URM4905, URM4908, URM4852, URM4854, URM4856, URM4857, URM4858, URM4859, URMCi, URMPI13, URMPI15, URMAcu and URMSub, respectively.

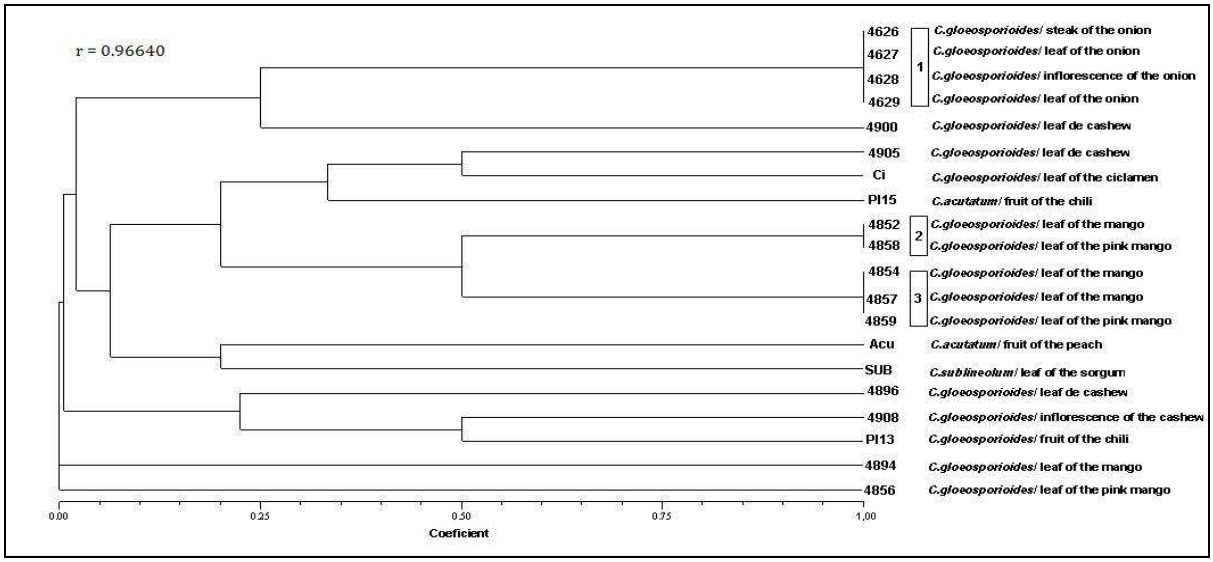

Figure 5 - Dendrogram of relationships between 20 isolates of *C. gloeosporioides* based on UPGMA clustering of the matrix obtained by Jaccard coefficient (J).

Amplification of the *cap*20 gene by PCR

The amplification of *cap*20 gene fragments, using the specific *primers* P1 and P2 for the *Colletotrichum* sp., is illustrated in Fig. 6. Between the *C. gloeosporioides* isolates, the tests with *primers* constructed indicated a variation in the structure of *cap*20 gene, therefore 12 of the *C. gloeosporioides* isolates amplified one fragment of 950-bp, and of the URM4905 isolate proceeding from the cashew presented consistently two bands in all the repetitions of the reaction, one common to the isolates of the *C. gloeosporioides* (950-bp) and other of size lesser (700-bp) similar to the band generated in *C. boninense*, *C. capsici* and *C. dematium*. Moreover, all mango isolates (except URM4856) and *C. gloeosporioides* URM1633 isolate from the chilli, did not present amplification. Also there was variation in the structure of the gene between the species, therefore, *C. boninense*, *C. capsici* and *C. dematium* presented the bands of different sizes of the *C. gloeosporioides* from majority of the isolates. Hwang et al (1995) found that the *cap*20 gene in *C. gloeosporioides* was a apressorium functional gene whose expression was clearly induced by the wax present in the fruits of avocado and tomato, and its delection caused drastic reduction in the pathogenicity to these fruits. However this gene presented only one copy in the genome.

This work showed the amplification of the *cap*20 gene in *C. gloeosporioides* isolates from the onion,

cashew, mango, chilli and ciclamen related to the host of origin and in *C. boninense*, *C. capsici*, *C. dematium* and *C. gossypii* var. *cephalosporioides* species and absence in *C. sublineolum*, *C. graminicola*, *C. acutatum* and *C. coccodes*. The majority of *C. gloeosporioides* isolates derived from the mango did not present amplification with *primers* tested. This suggested that *primers* constructed had a structural difference in the isolates of this host or that *primers* had not been adjusted for the amplification of all the isolates.

This is the first report on the presence of *cap*20 gene in *C. gloeosporioides* isolates of other hosts, except the avocado, and other species of *Colletotrichum*.

Analysis of the digestion fragment of amplification *cap*20 gene with the *Msp*I restriction enzyme

The amplification products of *cap*20 gene of the *C. gloeosporioides* isolates amplified with *primers* P1 and P2 were submitted to the digestion with the *Msp*I enzyme. The digestion generated two fragments of approximately 500-bp and 250-bp for all the *C. gloeosporioides* isolates (Fig. 7), not showed differences in the number and length of this gene among the isolates tested.

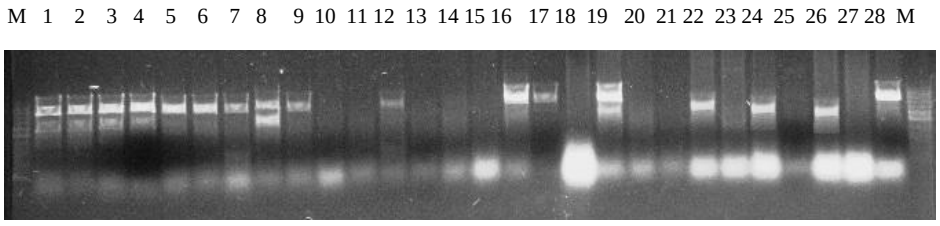

Figure 6 - Amplification of the pathogenicity *cap*20 gene in 28 *Colletotrichum* isolates with the *primers* P1 and P2. M – Molecular weight size markers of the 1Kb; Lanes 1-19 corresponding to *C. gloeosporioides* isolates (URM4626, URM4627, URM4628, URM4629, URM4894, URM4896, URM4900, URM4905, URM4908, URM4852, URM4854, URM4856, URM4857, URM4858, URM4859, URMCi, URMPI13, URM1633 and *C. gloeosporioides* "esalq"); lanes 20-21 corresponding to *C. acutatum* isolates (URMPI15 and URMAcu); lanes 22-28 corresponding to *C. boninense* (esalq), *C. coccodes* (esalq), *C. capsici* (URM1245), *C. sublineolum* (URMSub), *C. dematium* (URM1023), *C. graminicola* (URM1041) and *C. gossypii* var. c*ephalosporioides* (URM1147) isolates, respectively.

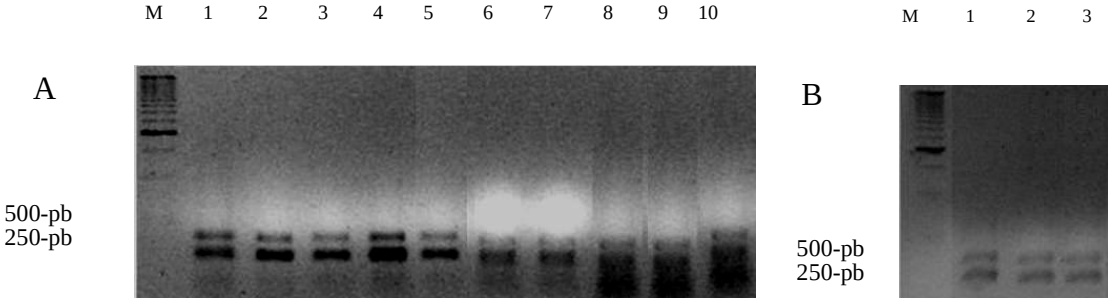

Figure 7 - Restriction profiles of the products of the amplification of the gene *cap*20 with restricition enzyme *Msp*I (A e B). M – Molecular weight size markers of the 1Kb; The samples from to 1-10 corresponding to *C. gloeosporioides* isolates 1 to 10 (A) they are DNAs from *C. gloeosporioides* (URM4626, URM4627, URM4628, URM4629, URM4894, URM4896, URM4900, URM4905, URM4908 e URM4856, respectively) isolates and the samples from 1 to 3 (B) they are DNAs *C. gloeosporioides* (URMCi, URMPI13 e *C. gloeosporioides* "Esalq", respectivamente) isolates.

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RESUMO

Estudos foram realizados para analisar a caracterização genética usando marcadores de RFLP-ITS e ISSP e a amplicação do gene de patogenicidade cap20 por PCR em isolados de Colletotrichum gloeosporioides de diferentes hospedeiros. Primers para o gene cap20 foram construídos a partir de seqüências selecionadas do GenBank (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov) com o programa Primer 3. A análise dos dendrogramas revelou que o marcador RFLP-ITS foi mais informativo em separar as espécies de Colletotrichum, e que o primer EI1 evidenciou maior diversidade genética. A amplificação do DNA dos isolados de Colletotrichum para o gene cap20 com os primers P1 e P2 indicou que este gene pode apresentar variações dentro de C. gloeosporioides relacionada ao hospedeiro, e que também está presente em outras espécies de Colletotrichum.

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