Mol Biotechnol (2008) 40:293–298
DOI 10.1007/s12033-008-9093-0

RESEARCH

# PCR-Based Detection and Characterization of the Fungal Pathogens *Colletotrichum gloeosporioides* and *Colletotrichum capsici* Causing Anthracnose in Papaya (*Carica papaya* L.) in the Yucatan Peninsula

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Published online: 1 August 2008


**Abstract** *Colletotrichum gloeosporioides* is the common causal agent of anthracnose in papaya (*Carica papaya* L.) fruits, and infection by this fungal pathogen results in severe post-harvest losses. In the Yucatán peninsula (Mexico) a different *Colletotrichum* species was isolated from papaya fruits with atypical anthracnose lesions. The DNAs from a variety of *Colletotrichum* isolates producing typical and atypical lesions, respectively, were amplified by PCR with *C.gloeosporioides*-specific primers. All isolates from typical anthracnose lesions yielded a 450 bp PCR product, but DNAs from isolates with atypical lesions failed to produce an amplification product. For further characterization, the rDNA 5.8S-ITS region was amplified by PCR and processed for sequencing and RFLP analysis, respectively, to verify the identity of the papaya anthracnose pathogens. The results revealed unequivocally the existence of two *Colletotrichum* species causing anthracnose lesions on papaya fruits: *C. gloeosporioides* and *C. capsici*. PCR-RFLP using the restriction endonuclease *Msp*I reliably reproduced restriction patterns specific for *C. capsici* or *C. gloeosporioides*. The generation of RFLP patterns by *Msp*I (or *Alu*I or *Rsa*I) is a rapid, accurate, and unequivocal method for the detection and differentiation of these two *Colletotrichum* species.

**Keywords** *Colletotrichum capsici* · *Carica papaya* · *Colletotrichum gloeosporioides* · PCR-RFLP · Restriction patterns · Sequencing of rDNA ITS region · Anthracnose

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## Introduction

*Colletotrichum* is the most important and most common fungal genus causing anthracnose in temperate, subtropical, and tropical areas, with an extremely wide host range including vegetable, forage crops, fruit trees, and ornamentals [1]. Numerous cases have been reported in which several *Colletotrichum* species or biotypes are associated with a single host [2–5], making their identification by morphological and physiological methods more difficult. The use of molecular marker techniques has improved the accuracy and speed of identification and classification of phytopathogenic fungi [6, 7]. Among these molecular techniques, DNA fragment analysis [e.g. randomly amplified polymorphic DNA (RAPD), and arbitrarily primed (AP)-PCR] has been extensively used to investigate relationships among isolates of many fungal genera including *Colletotrichum* spp [8, 9]. Similarly, nucleotide sequence information for the 5.8S gene and the internal transcribed spacer [ITS] region of ribosomal DNA (rDNA) has been used to design *Colletotrichum* species-specific primers for diagnostic purposes and for phylogenetic analysis [5, 10].

Anthracnose causes significant economic losses in papaya (*Carica papaya* L.) fruits, and *Colletotrichum gloeosporioides* is the primary causal agent of this disease [11, 12] which is characterized by the typical lesions shown

in Fig. 1b. In addition, however, in papaya orchards in the Yucatan peninsula of Mexico, atypical anthracnose lesions were observed. Therefore, the objective of this study was to use PCR-based molecular tools for the rapid and reliable identification and characterization of the pathogen(s) involved in atypical lesion development.

## Materials and Methods

### Fungal Isolates

The *Colletotrichum* isolates were taken at different locations in the state of Yucatan and obtained from naturally infected papaya fruits showing atypical or typical anthracnose lesions (Table 1). The fungi were isolated and cultured on potato dextrose agar (PDA) at 28°C. For DNA extraction, 150 ml of nutrient broth was inoculated with two plugs of 5-mm diameter for each isolate, and cultures were incubated in an orbital shaker at 100 rpm at 28°C. The mycelial mass of each isolate was harvested with a glass hook and deposited on conical corning sterile tubes.

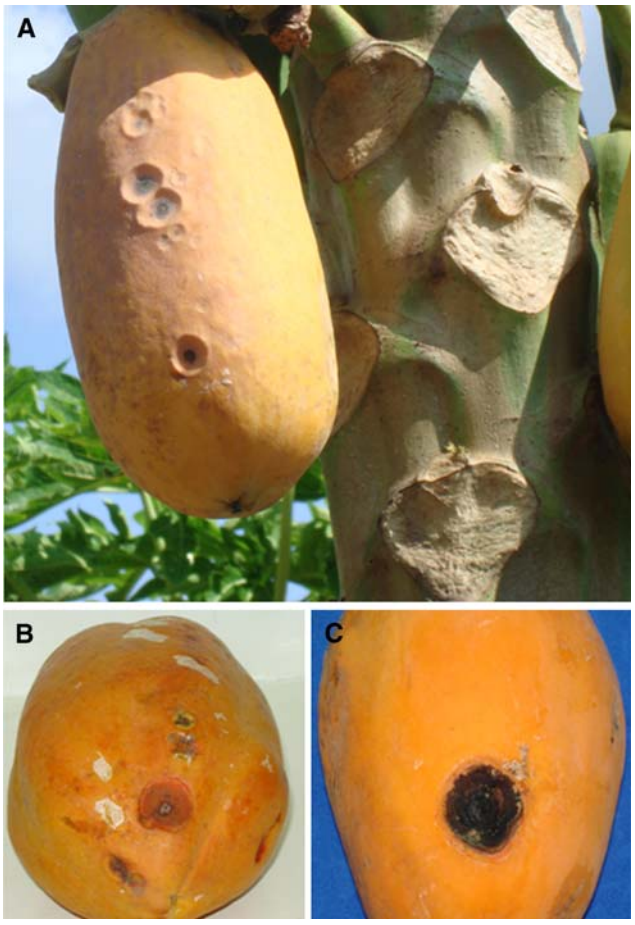


**Fig. 1** (**a**) Papaya fruit with atypical anthracnose lesions caused by *Colletotrichum* species on the field, (**b**) typical, and (**c**) atypical anthracnose lesions

### Isolation of DNA

Total genomic DNA was extracted according to a method developed in the GeMBio laboratory and this was used in all tests performed [13].

**Table 1** *Colletotrichum* spp isolates from papaya orchards in Yucatan Peninsula

| Isolate code | Location | Lesion type |
|---|---|---|
| gb1 | Colonia Yucatan | Atypical |
| gb2[a] | Colonia Yucatan | Atypical |
| gb3 | Colonia Yucatan | Atypical |
| gb4 | Colonia Yucatan | Typical |
| gb5 | Colonia Yucatan | Atypical |
| gb6 | Colonia Yucatan | Atypical |
| gb7 | Colonia Yucatan | Atypical |
| gb8 | Colonia Yucatan | Atypical |
| gb9 | Colonia Yucatan | Atypical |
| gb10 | Colonia Yucatan | Atypical |
| gb11 | Dzidzantun | Atypical |
| gb12[b] | Dzidzantun | Atypical |
| gb13 | Dzidzantun | Atypical |
| gb14 | Dzidzantun | Typical |
| gb15 | Dzidzantun | Atypical |
| gb16 | Dzidzantun | Atypical |
| gb17 | Nohuayun | Atypical |
| gb18 | Nohuayun | Atypical |
| gb19 | Nohuayun | Typical |
| gb20 | Nohuayun | Typical |
| gb21 | Tekax | Atypical |
| gb22 | Tekax | Typical |
| gb23 | Tekax | Atypical |
| gb24 | Tekax | Atypical |
| gb25 | Tekax | Typical |
| gb26 | Tizimin | Atypical |
| gb27 | Tizimin | Atypical |
| gb28 | Tizimin | Typical |
| gb29 | Tizimin | Atypical |
| gb30 | Tizimin | Atypical |
| gb31 | Tizimin | Atypical |
| gb32 | Tizimin | Typical |
| gb33 | Tizimin | Atypical |
| gb34 | Tizimin | Atypical |

[a] NCBI accession no. EU056739

[b] NCBI accession no. EU056740

### Diagnostic PCR with Species-Specific Primers

The primer *CgInt* (5′-GGCCTCCCGCCTCCGGGCGG-3′) specific for *C. gloeosporioides* [10, 14] was used in conjunction with the conserved primer ITS4 [10, 15] for rDNA amplification. Amplification reactions without DNA template were used as negative controls as well as the strain Cgb1 (NCBI accession no. EU056738) of *Colletotrichum capsici*. The PCR reaction (25 µl final volume) contained 25 ng of DNA, 1× PCR buffer (10×: 200 mM Tris-HCl, 500 mM KCl, pH 8.4; Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM primers and 1U *Taq* polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and consisted of an initial denaturing step at 95°C for 5 min, followed by 25 cycles of 30 s at 94°C, 2 min at 62°C, and 2 min at 72°C, and a final extension step of 5 min at 72°C [10]. PCR products were visualized by electrophoresis in 1.5% (wt/vol) agarose gels run 1× Tris-Borate-EDTA [TBE] buffer and stained with ethidium bromide.

### PCR Amplification and Sequencing of 5.8S-ITS of rDNA

The *Colletotrichum* isolates were further characterized by nucleotide sequence analysis. For this, the 5.8S-ITS regions were amplified with the universal primers ITS1 and ITS4 [15], and the strain Cgb1 of *C. capsici* was used as positive control. PCR reactions were performed in reaction volumes of 50 µl containing 25 ng of genomic DNA, 1× PCR buffer (Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM primers, and 1U *Taq* polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and the program consisted of an initial denaturing step at 94°C for 1 min, followed by 30 cycles of 60 s at 94°C, 2 min at 58°C, and 60 s at 72°C; and a final extension step of 5 min at 72°C [16]. PCR products (10 µl aliquots) were separated by electrophoresis in 1.5% (wt/vol) agarose gels and visualized by ethidium bromide staining.

DNA sequencing was performed with two primers (ITS1 and ITS4) in both directions to ensure that there was no misreading. PCR products were purified and sequenced by Macrogen Inc, Korea. Alignment and edition were carried out with the BioEdit Program v 7.0.5 [17] and visually corrected. Sequences were then compared against those available in the GenBank database.

### PCR-RFLP Analysis of rDNA

The 5.8S-ITS amplification products (10 µl aliquots) of 12 selected *Colletotrichum* strains (including the Cbg1 control strain) were digested with the restriction enzymes *Alu*I, *Hae*III, *Hind* III, *Mse*I, *Msp*I, *Rsa*I, and *Taq*I, respectively, in a volume of 20 µl. Digestion products were separated on a 2% agarose gel (NuSieves 3:1) using 0.5× TBE buffer, and stained with ethidium bromide. Digestion patterns were visualized under a UV transilluminator and images were taken with a UVP BioImaging Systems.

## Results

Thirty-four *Colletotrichum* isolates were obtained from papaya fruits with atypical and typical anthracnose lesions, respectively (Fig. 1). Typical lesions produced by *C. gloeosporioides* are round, water soaked, and sunken spots with pinkish-orange areas are formed by the conidial masses, that cover the lesion center and sometimes produce a concentric rings pattern (Fig. 1b). In the case of atypical lesions, the main difference with typical ones is that the lesion areas formed are totally covered by brownish-black conidial masses (Fig. 1c).

### PCR Amplification and Sequencing of 5.8S-ITS of rDNA

In first PCR tests with the *C. gloeosporioides*-specific primer *CgInt* used in conjunction with the conserved primer ITS4, eight isolates (all from typical anthracnose lesions) yielded the expected 450-bp product, while PCR products were not obtained for the other isolates from atypical lesions (Fig. 2). The DNAs of fungal isolates with typical and atypical anthracnose lesions were then used in PCR with the general primers ITS1 and ITS4 for the amplification of the rDNA region comprising the two non-coding internal transcribed spacers ITS1 and ITS2 and the 5.8S rRNA gene. All isolates amplified a PCR product of approximately 600 bp. Sequences were determined of each isolate, and blast analyses were carried out for eight isolates, previously identified with species-specific primers as *C. gloeosporioides*. The results showed a 100% homology with DNA sequences from other *C. gloeosporioides* strains deposited in the GenBank, while the blast analysis of the remaining isolates (atypical symptoms) produced a 99% homology with DNA sequences from *C. capsici* strains registered in the same database (data not shown). Some of these DNA sequences were registered in NCBI GenBank database.

### PCR-RFLP Analysis of rDNA

The PCR amplification products were then processed for RFLP analysis and digested with seven different endonucleases each (Table 2). With the exception of *Hind*III and

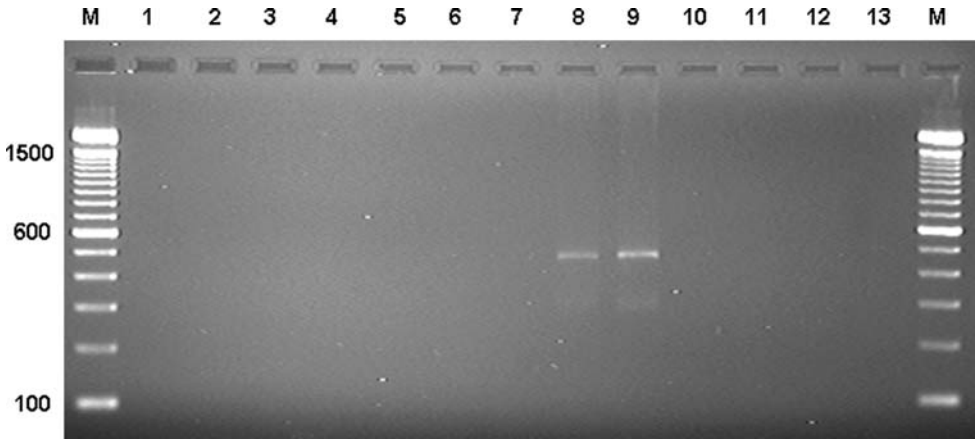


**Fig. 2** Amplification products obtained in the species-specific PCR test (a selection of isolates is shown). All test isolates were analyzed using primer specific for *C. gloeosporioides*. M: molecular marker (100 bp DNA ladder), lane 1: Cgb1 (accession no. EU056738), lane 2: gb2, lane 3: gb9, lane 4: gb12, lane 5: gb15, lane 6:gb16, lane 7: gb21, lane 8: gb22, lane 9: gb32, lane10: gb23, lane 11: gb30, lane12: gb34, lane 13: negative control

**Table 2** PCR-RFLP of the ITS1-5.8S-ITS2 region of *Colletotrichum spp*. Fragment lengths (bp) obtained from endonucleases digestion with *Alu*I, *Hae*III, *Hind*III, *Mse*I, *Msp*I, *Rsa*I and *Taq*I

| Species | Fragment size (bp) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Alu*I | *Hae*III | *Hind*III | *Mse*I | *Msp*I | *Rsa*I | *Taq*I |
| *C. capsici* | 380 | 300 | Nd | Nd | 450 | Nd | 250 |
| | 200 | 170 | | | 135 | | 230 |
| | | 145 | | | | | 80 |
| *C. gloeosporioides* | 380 | 280 | Nd | Nd | 300 | 380 | 240 |
| | 190 | 170 | | | 130 | 180 | 230 |
| | | 140 | | | | | 80 |

Nd: not digested

*Mse*I that did not cut the DNA, the digestions resulted in clear and reproducible restriction patterns as depicted in Fig. 3. The enzyme *Alu*I (Fig. 3a) produced fragments around 190 and 380 bp specific for all *C. gloeosporioides* isolates, and fragments around 200 and 380 bp specific for all *C. capsici*. While *Hae*III digestion generated specific bands around 140 and 280 bp for *C. gloeosporioides* and 145 and 300 bp for *C. capsici* (Fig. 3b). In the case of *Msp*I specific bands at 130 and 300 bp for *C. gloeosporioides* and 135 and 450 bp for *C. capsici* were visualized (Fig. 3c). *Rsa*I has no recognition site for this region of the *C. capsici* rDNA, while specific bands around 180 and 380 bp were obtained for *C. gloeosporioides* (Fig. 3d). In the cases of *Taq*I and *Hae*III only slight differences among the banding patterns for *C. capsici* and *C. gloeosporioides* were discernible.

## Discussion

The main aim of this research was to use PCR-based methods to identify rapidly and accurately the species of *Colletotrichum* responsible for typical and atypical anthracnose lesions on fruits in papaya fields in Yucatán. This article presents the first report of molecular detection of *C. capsici* affecting *C. papaya* fruits in Yucatan, Mexico.

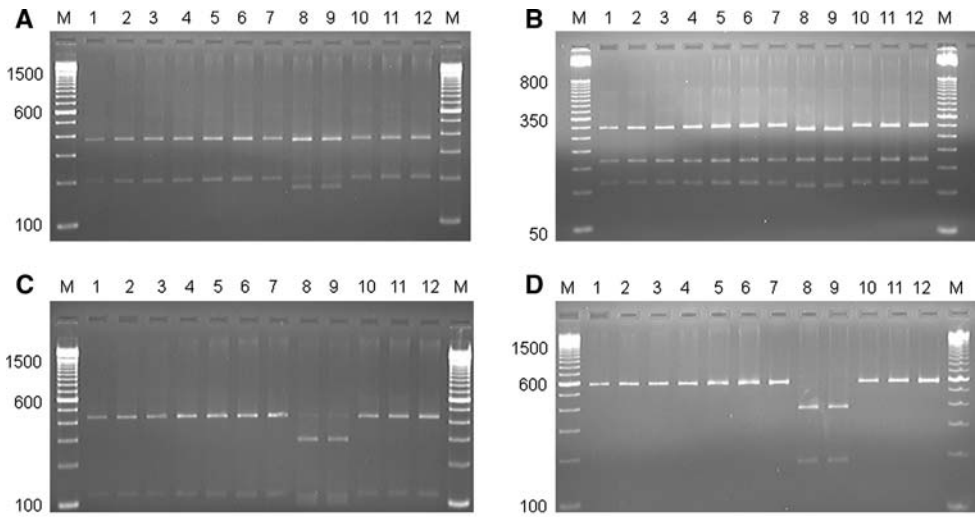


**Fig. 3** PCR-RFLP profiles of rDNA obtained with restriction enzymes: *AluI* (**a**), *Hae*III (**b**), *Msp*I (**c**), and *Rsa*I (**d**). M: molecular marker (100 bp DNA ladder for **a**, **c**, and **d**; 50 bp DNA ladder for **b**), lane 1: Cgb1 (accession no. EU056738), lane 2: gb2, lane 3: gb6, lane 4: gb9, lane 5: gb12, lane 6: gb15, lane 7: gb16, lane 8: gb22, lane 9: gb32, lane 10: gb21, lane11: gb23, lane 12: gb30

For several years molecular techniques have been widely used to differentiate the *Colletotrichum* genus at species or race level [5, 14, 18, 19]. In this study and based on PCR amplification with species-specific primers, all isolates from typical anthracnose lesions were identified as *C. gloeosporioides.* The size of the amplification product (450 bp) confirmed other reports for the same species [10], and subsequent rDNA sequence analysis corroborated these results.

PCR with species-specific primers as well as PCR amplification of the 5.8S-ITS region of DNA, subsequent sequence analysis and PCR-RFLP analysis of the rDNA product revealed unequivocally the existence of two species causing anthracnose lesions on papaya fruits: *C. gloeosporioides* and *C. capsici*. The species *C. capsici* is found throughout the world causing anthracnose in Habanero pepper (*Capsicum chinense* Jacquin) and pepper (*Capsicum annuum* L.) [20–22]. *C. capsici* has also been reported as a pathogen for several other hosts such as cowpea (*Vigna unguiculata*), bean (*Phaseolus vulgaris*), and betle vine (*Piper betle*) [23]. In the Yucatan peninsula it is common to find papaya orchards side by side to Habanero pepper fields. This together with the broad range of host plants that are infected by *Colletotrichum* species [1] could account for the infection of papaya by *C. capsici*. RFLP patterns generated from the rDNA region spanning the ITS and the 5.8S, made it possible to differentiate between *C. gloeosporioides* and *C. capsici*, since each one has a characteristic banding pattern. The ITS region has already shown before to be useful for *Colletotrichum* species identification [5, 18, 24]. The *C. gloeosporioides* restriction patterns obtained with *Alu*I, *Hae*III, *Msp*I, and *Rsa*I were similar to those reported previously for the same species [25].

It is interesting to note that, with the use of one endonuclease *Msp*I, a reliable and reproducible distinction between *C. capsici* and *C. gloeosporioides* was obtained. Sequence data undoubtedly offer more precise information than the restriction analysis, but the procedure is time-consuming and cost-intensive. PCR-RFLP in contrast with any of the three enzymes *Alu*I, *Msp*I, or *Rsa*I achieves a rapid and unequivocal identification of *Colletotrichum* isolates derived from papaya fruits and their assignation to *C. capsici* and *C. gloeosporioides* species. This technique will thus facilitate routine work in phytopathogen diagnostics. In any laboratory providing the services of phytopathogen detection, it is essential to have the support of these kind of techniques which will facilitate the correct and rapid identification of the agents causing the diseases, and to be able to provide the crop producers with this response so that they can take the necessary steps towards the control of these diseases and reduce crop yield losses.

**Acknowledgements** The authors are grateful to Dr. Jairo Alejo for providing some of the samples of papaya fruits with anthracnose used in this study; and especially to Prof. Dr. Wolfgang Rohde, from Max Planck Institute for Plant Breeding Research, for his complete review of this manuscript and his time and effort in providing comments.

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