PCR-RFLP BASED GENETIC DIVERSITY OF COLLETOTRICHUM MUSAE ISOLATES OF BANANA IN JAFFNA DISTRICT

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ABSTRACT

Anthracnose disease is a major post-harvest disease of banana in Sri Lanka. The present study was conducted to determine the genomic variations among Colletotrichum musae isolates, causing anthracnose disease of banana, by PCR-RFLP analysis. Morphologically-different six colletotrichum musae isolates were isolated from 3 different banana local cultivars ('Kathali', 'Kappal', 'Etharai'). Genomic DNA was extracted from each purified isolate using a modified CTAB method. PCR amplification was done with ITS1 and ITS 4 primers to amplify the 5.8S-ITS subunit. For fungi, the internal transcribed spacer (ITS) region in the ribosomal RNA (rRNA) operon has been accepted as the formal fungal barcode. A 590 bp PCR product were resulted by two Colletotrichum musae isolates (Cm2 and Cm5). Colletotrichum musae isolates (Cm1, Cm2, Cm3, Cm4, Cm5 and Cm6) showed two polymorphic groups based on PCR-RFLP by HaeIII. The same isolates showed no polymorphism based on PCR-RFLP by RsaI and MspI. Amplification of 5.8S-ITS region and subsequent PCR-RFLP by HaeIII is an effective and reliable method to determine genomic variation among Colletotrichum musae isolates causing anthracnose disease in banana fruits.

Keywords: PCR-RFLP, Genomic polymorphism, ITS region, Colletotrichum musae

1. INTRODUCTION

Anthracnose is the most important post-harvest disease of Banana in Sri Lanka. Most of the commercial banana varieties grown in Sri Lanka are susceptible to anthracnose causing post-harvest losses (Adikaram, 1986-87; Perera and Karunaratne, 1995). Anthracnose becomes severe when the banana fruits are wounded by scratches during handling and transportation and causing the fruit unmarketable. Symptoms of anthracnose include black and sunken lesions with spore masses or acervuli in the lesion (Latiffah *et al*., 2009). Infection on banana usually starts during the development of fruit but remains quiescent until the fruit ripens; symptoms often manifest during storage and marketing (Prusky and Plumbley, 1992). Although *C. musae* is the most common species associated with anthracnose of banana, *C. gloeosporioides* has also been reported to be associated with banana anthracnose (Wijesundera, 1994; Duduk *et al*., 2009). Most studies related to banana anthracnose have used morphological characteristics to identify *Colletotrichum* spp., which may not be sufficient to confidently use in species delimitation, especially to differentiate closely related species. Moreover, *C. musae* is a member of the *C. gloeosporioides* species complex, which indicates that *C. musae* and *C. gloeosporiodes* are closely related. Internal transcribed spacer (ITS) regions are useful to define the relationships of *Colletotrichum* spp. within a species complex in phylogenetic analysis (Phoulivong *et al*., 2010). The objective of this study was to determine

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the pcr-rflp based genetic diversity of *colletotrichum musae* isolates of banana fruits in jaffna district

2. METHODOLOGY

2.1 Isolation of fungi

Three banana cultivars (local names in Jaffna district: 'Katali', 'ethereal' and 'kappal') with symptom of anthracnose were collected from Thirunelvely vegetable market in Jaffna district of Sri Lanka. Fungi were isolated from anthracnose lesion on three varieties of bananas sold in the market. The infected fruits which showed typical symptoms were surface sterilized in 1 % Clorox solution for three minute and washed repeatedly thrice in sterile distilled water then air-dried on a clean bench. Then surface sterilized peel of fruits were cut into small bits measuring about 1 cm and transferred to sterile petri plates containing PDA medium with streptomycin 100 µg/mL under aseptic condition then incubated at room temperature. The fungal colonies that appeared on the banana peel bits after incubation period were isolated in fresh sterile PDA medium with streptomycin 100 µg/mL to prepare pure culture and identified.

2.2 Extraction of DNA

The genomic DNA were extracted from 6 days old fungal cultures and purified by the DNA extraction protocol described by Mc Gravey and Kaper (1991). The concentration of DNA were assessed by Quantus Fluorometer and the quality of the DNA were assessed by gel electrophoresis. The samples were diluted to a final concentration of below 500ng/µl.

2.3 PCR amplification and PCR-RFLP

The 5.8S-ITS region was amplified by using universal primers ITS1 (GCCGTAGGTGAACCTGCGG) and ITS4 (GCCTCCGCTTATTGATATGC) for *Colletotrichum* spp. (Pedro *et al*., 2000). PCR reactions were performed in a total volume of 50µl containing 2µl of template DNA, 25 µl Marster mix (Promega, USA), 5µl of forward primer, 5µl of reverse primer and 13µl of nuclease free water. The reaction mixtures were incubated in a thermocyclear during 40 cycles, each consisting 1 min at 95ºC, 1 min at 52ºC and 1 min at 72ºC. PCR products were digested with the restriction enzymes *Rsa* I, *Hae* III and *Msp* I. PCR products and their restriction fragments were separated on 1 and 2 % agarose gels with a 100 bp DNA size marker, respectively. After electrophoresis, gels were stained with ethidium bromide and the DNA bands were visualized by gel documentation system.

3. Results and discussion

3.1 Variation of colony morphology

Six *Colletotrichum* isolates were isolated from anthracnose infected banana fruits (2 isolates per each variety). Isolates cm1 & cm2, cm3 & cm4 and cm5 &

cm6 were isolated from banana variety 'Kathali', 'Kappal' and 'Etharai' respectively. Identification of Colletotrichum isolates was based on morphological characters such as colony characters and size and shape of conidia (Rasangi and Vengadaramana, 2015). Growth rates showed a significant difference among six *Colletotrichum musae* isolates in PDA media at 6 days after incubation at room temperature. Characteristic symptoms of anthracnose were developed by all the isolates of *Colletotrichum musae* on banana fruits after wound inoculation of the pathogen and the diameter of the anthracnose lesions varied among the six *Colletotrichum musae* isolates significantly (Rasangi and Vengadaramana, 2015).

3.2 Genomic variation of the fungal isolates

When the 6 *Colletotrichum musae* isolates were amplified by ITS1/ITS4 primer pair *C. musae* isolates cm3 and cm5 resulted PCR products of 590 bp (Figure 1) and cm1, cm2, cm4 and cm6 resulted the size of PCR products more than 590 bp (Figure 1)

The PCR-RFLP profiles of all *Colletotrichum musae* isolates given by the three restriction endonucleases (i.e. *Rsa*I *Hae*III and *Msp*I) are shown respectively in Figures 2, 3 and 4. Based on PCR-RFLP analysis, 2 polymorphic groups were observed among the isolates of *Colletotrichum musae*. When PCR products were digested with *Hae*III two polymorphic groups were given (Figure 3). However, no polymorphism was observed among the isolates when digested with *Rsa*I and *Msp*I (Figures. 2 and 4).

Table 4.1. Quantity of fungal DNA isolates

Isolates	Quantity
cm1	61ng/µL
cm2	76 ng/µL
cm3	89 ng/µL
cm4	67 ng/µL
cm5	39 ng/µL
cm6	91 ng/µL

General fungal primers, ITS1 and ITS4 (White *et al*., 1990), were used to selectively amply the fungal ITS region of the rRNA operon, including the ITS1 region, 5.8S rRNA gene, and ITS2 region. Restriction endonuclease digest patterns of PCR amplified ITS region using *Hae*III, *Msp*I and *Rsa*I were able to give further clarity on the existence of genetic variability of isolates based on the ITS region and gave more detailed information within the morphogroups. In this study, 2 *Colletotrichum musae* isolates resulted in the expected sized PCR products of 590 other 4 isolates resulted more than 600 bp when amplified by ITS1 & ITS4 primers.

Figueiredo *et al*., (2012) reported that the amplification of the ITS1-5.8S-ITS2 of the rDNA region using the universal primers ITS1 and ITS4 produced a fragment of approximately 590 bp for *C. gloeosporioides* isolates and his report also confirming previously reported results (Abang *et al*., 2002). Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species. Amplification of target DNA through PCR with taxon-specific primers is a potentially more sensitive and accurate approach than conventional microscopic techniques (Kamel *et al*., 2003).

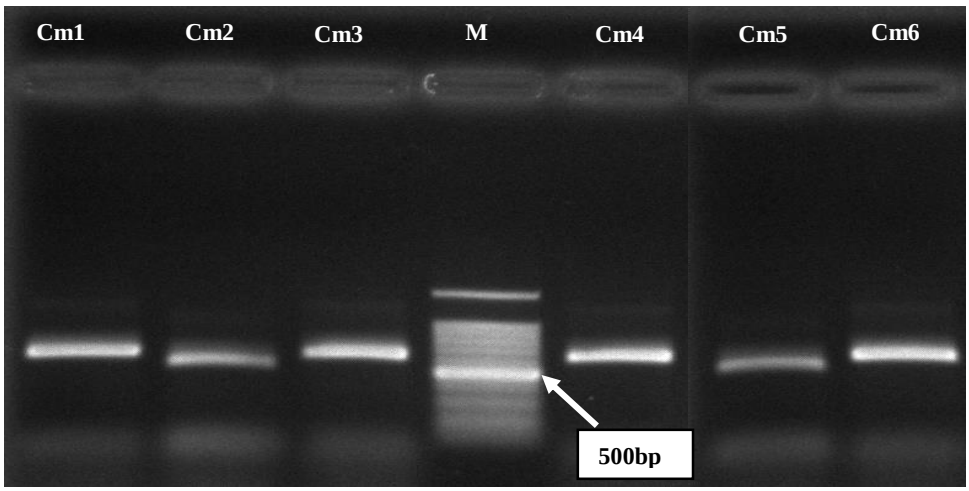

Figure 1. PCR products of six Colletotrichum musae isolates amplified by ITS1/ITS4 primers. M, molecular-weight markers (100bp ladder, Promega, USA)

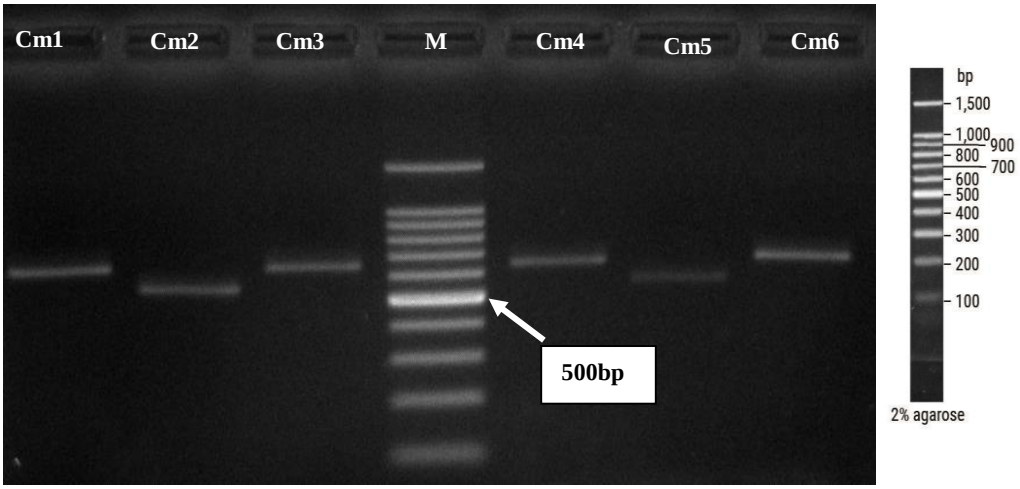

Figure 2. PCR-RFLP pattern exhibited by 6 Colletotrichum musae isolates after digestion with restriction endonuclease MspI and M- Molecular weight markers (100bp ladder, Promega, USA)

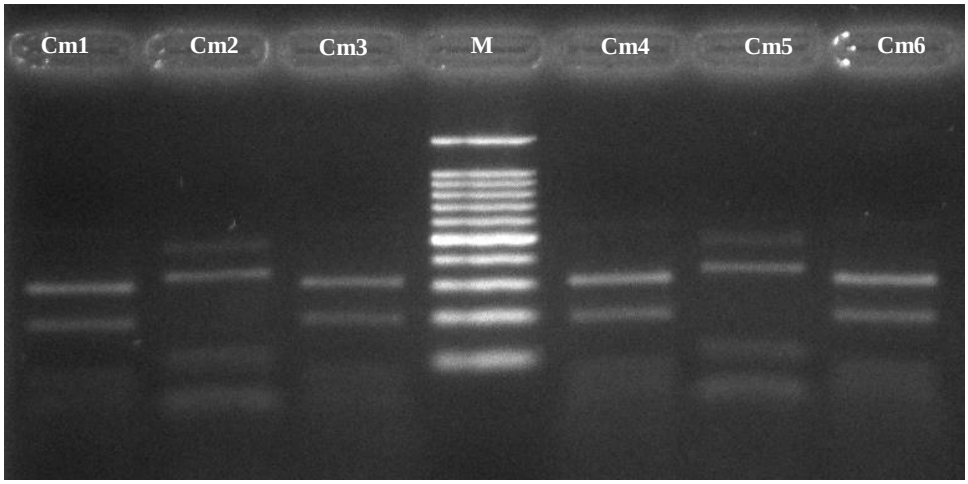

Figure 3. PCR-RFLP pattern exhibited by 6 Colletotrichum musae isolates after digestion with restriction endonuclease HaeIII and M- Molecular weight markers (100bp ladder, Promega, USA)

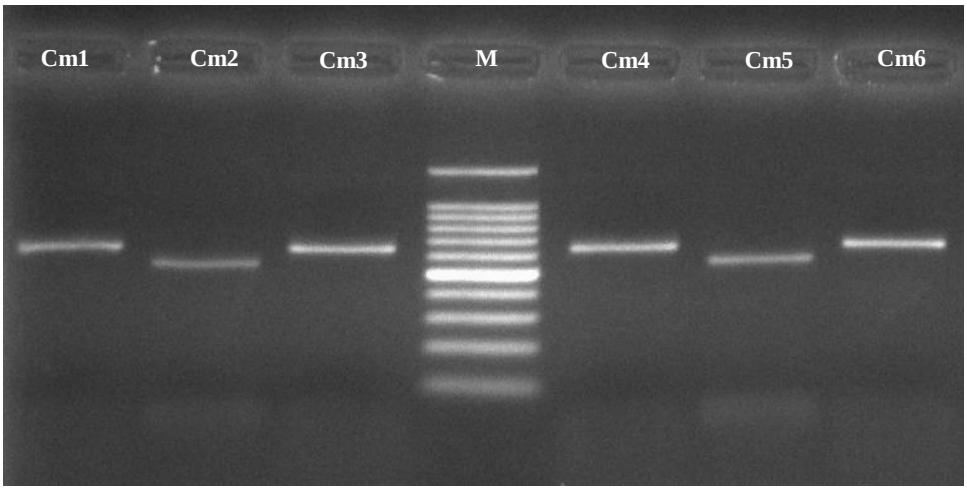

Figure 4. PCR-RFLP pattern exhibited by 6 Colletotrichum musae isolates after digestion with restriction endonuclease RsaI and M- Molecular weight markers (100bp ladder, Promega, USA)

Molecular identification by ribosomal DNA sequencing is based on the analysis of gene sequences typically stored in 16S and 5.8S ribosomal RNA by amplification of the Internal Transcribed Spacer (ITS). This one is used for the genomic variation of *Colletotrichum musae* isolates isolated from anthracnose disease of banana fruit in Jaffna district. The six morphologically and

physiologically different *Colletotrichum musae* isolates showed speci¢c pattern with the endonuclease *Hae*III. The fragments resulting from the digestion with *Hae*III could be used as a method to identify the species. The digestion with this enzyme can also be used for differentiating the species.

4. CONCLUSION

The used primer sets are successful in amplifying the target region. a reasonable level of genomic variation could be identified when the PCR-RFLP was done with *Rsa*I rather than using *Pvu*II. Hence, further analyses on PCR-RFLP have to be done with several other restriction endonucleases. Findings of PCR-RFLP of the present study generated sufficiently detailed restriction profiles for detection, preliminary differentiation and typing of most common *Colletotrichum musae*' The PCR products given by ITS1 / ITS4 primers were digested with *Hae*III.

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