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Differentiating Races of Corynespora cassiicola *Using RAPD and Internal Transcribed Spacer Markers*

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Nine isolates of Corynespora cassiicola *from* Hevea brasiliensis *were analysed using two molecular marker techniques: random amplified polymorphic DNA (RAPD); and the amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA. RAPD distinguished two groups of isolates* i.e. *isolates infectious to RRIM 2020 clones, and isolates that infected RRIM 600 and several other clones of* H. brasiliensis. *The ITS region of eight out of nine isolates were found to be monomorphic.*

Key words: *Corynespora cassiicola*; *Hevea brasiliensis*; molecular marker; internal transcribed spacer; RAPD; RRIM 2020; RRIM 600

Corynespora leaf fall disease caused by *Corynespora cassiicola* [(Berk. & Curt) Wei] was first detected in Malaysia in 1960. It was thought at the time to be a minor leaf disease as it only attacked certain clones in budwood nurseries[1], but there has been increasing incidences of the disease in Malaysia. RRIM 600 and GT 1 were the clones most commonly affected by the disease[2] and these clones were among the most widely planted clones throughout Malaysia in the 1960s. Previous approaches of controlling the disease were to avoid planting susceptible clones and by chemical control[3]. Combating the disease calls for the identification of the susceptible clones and can be done with the use of molecular markers. In a preliminary study, random amplified polymorphic DNA (RAPD) technique was used to differentiate between 15 *C. cassiicola* fungal isolates[4].

Two races of *C. cassiicola* from soyabean were identified[5]. It was suspected that physiological strains of *C. cassiicola* also exist based on different disease reactions observed on different *Hevea* clones[6,7]. The existence of physiological races was later confirmed by Ismail and Jeyanayagi[8] using a set of differential clones.

This paper reports a further study on the use of molecular markers to differentiate races of *C. cassiicola* that infect *Hevea*

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clones RRIM 600 and the RRIM 2000 series of clones and to determine whether susceptibility of *Hevea* clones is specific to the race of the micro-organism.

MATERIALS AND METHODS

Fungal Isolates and Culture Conditions

The *C. cassiicola* were isolated from infected rubber leaves and twigs showing typical symptoms. Nine isolates isolated from clones RRIM 600, RRIM 2020, RRIM 2009 and a clone not identified from various locations were used (*Table 1*).

Infected leaves with general 'fish bone' symptoms were used for fungal isolation. Square pieces of leaf (2 mm × 2 mm) cut from the edge of a lesion, were surface sterilised with 1% mercuric chloride solution for 1 min and rinsed five times in sterile distilled water. The pieces were then placed aseptically in petri plates containing 15 mL of Potato Dexrose Agar (PDA)[9] and incubated at room temperature (28°C ± 2°C) in darkness for three days. Emerging colonies were re-isolated on fresh PDA plates.

Pure culture of the pathogen was subcultured by placing a segment of agar medium into Erlmeyer flasks containing 200 mL potato dextrose broth. The flasks were shaken at 250 r.p.m. using an orbital shaker at room temperature. After 7–10 days mycelia were harvested by vacuum filtration using a Buchner funnel and Whatman paper No.1, washed with sterile water, lyophilised and stored in a freezer at –20°C.

Isolation of Fungal DNA

The method used for extracting *C. cassiicola* fungal DNA was adopted from Raeder and Broda[10]. The final concentration of total DNA was determined by running 5 µL of fungal DNA along side with lambda DNA at concentrations of 10 ng/ µL, 20 ng/ µL, 50 ng/ µL and 100 ng/ µL. Crude fungal DNA (5 µL) was digested with *Eco*R1 at 37°C overnight and electrophoresed on 0.8% agarose gel in 1 × TBE to verify the extent of DNA digestion.

TABLE 1. LIST OF *CORYNESPORA CASSIICOLA* ISOLATES USED IN THE STUDY AND THEIR ORIGIN

Isolate	Host	Location
CSB 1	RRIM 600	Sg. Buloh, Selangor
CL 1	RRIM 600	Mentakab, Pahang
CUN 1	RRIM 600	Segamat, Johor
CSS 1	RRIM 600	Segamat, Johor
CSB 6	RRIM 2009	Sg. Buloh, Selangor
CPEN 1	RRIM 2020	Pendang, Kedah
CLN 11	RRIM 2020	Lanchang, Pahang
CSD 1	Not identified	Sg. Durian, Perak
CSB 4	Not identified	Sg. Buloh, Selangor

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Random Amplified Polymorphic DNA (RAPD)

The protocol used was adopted from Williams and co-researchers[11]. Short oligonucleotide primers (10-mer) obtained from Operon Technologies, (USA) were used. The amplified products were then electrophoresed through a 1.5% agarose gel and visualised under UV light.

ITS Region of rDNA

Two primers (ITS1: 5'-GGGAATTCTCCGTAGGTGAACCTGCGG and ITS2: 5'-GCAAGCTTTCCTCCGCTTATTGATATGC) that amplify the ITS region of the rDNA were used[12] and the cycling conditions were adopted from Silva and co-researchers[13]. Amplification products were then restricted with *Msp*I, *Hae*III, *Hinf*I and *Eco*RI and electrophoresed through a 1.5% agarose gel.

RESULTS AND DISCUSSION

Isolation of Fungal DNA

The quality of fungal DNA extracted was good and the isolation procedure was satisfactory. All isolates of fungal DNA could be digested with *Eco*RI (*Figure 1*).

Molecular Identification of *C. cassiicola* Using RAPD

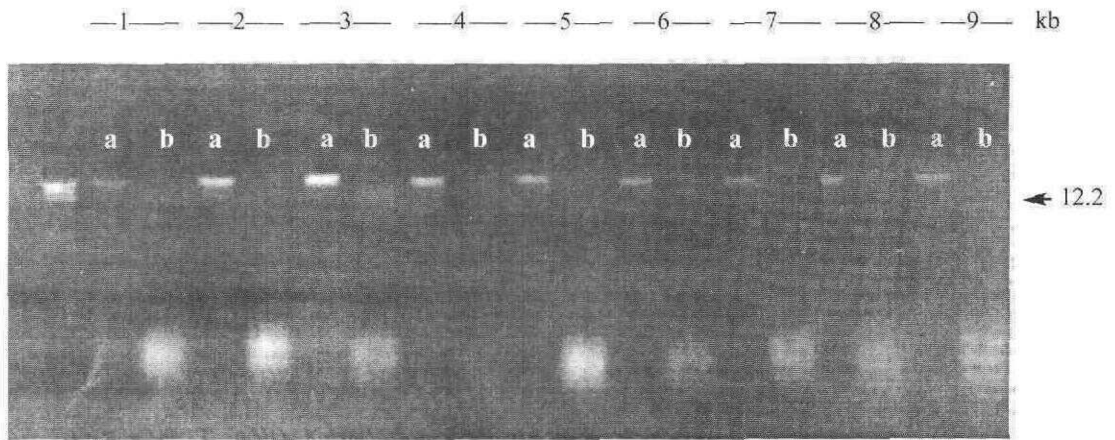
Twelve 10-mer primers were used in the initial screening of the fungal DNA and four were selected for detailed analysis (*Table 2*). These four primers amplified reproducible banding patterns in four separate experiments. Isolates CPEN 1 and CLN 11, which infected *Hevea* clone RRIM 2020, consistently showed different fingerprinting patterns as compared to the rest of the isolates. The remaining six isolates, which infected *Hevea* clones RRIM 600 (isolates CSB 1,CL 1. CUN 1, CSS 1) and clones that have not been identified (CSD1 and CSB 4), displayed similar fingerprinting patterns. It was also observed that the banding patterns of isolates CPEN 1 and CLN 11 were different though both isolates specifically infected *Hevea* clone RRIM 2020 (*Figure 2*).

Molecular Identification of *C. cassiicola* Isolates by Amplification of the ITS Region of rDNA

When the ITS region of rDNA was amplified, a single DNA fragment of 500 bp was obtained in all isolates except for isolate CSB 6 (lane 5) which had no amplified product (*Figure 3*). The amplified fragment was subsequently digested with four restriction enzymes to reveal potential fingerprints. Isolate CPEN 1 (lane 6) produced a fragment at 340 bp after digestion with *Hae*III in contrast with the other eight isolates which yielded 390 bp fragment each. On the other hand, digestion with the other three restriction enzymes, yielded monomorphic patterns with all nine isolates (*Figure 4*).

The advent of molecular marker techniques provides another sensitive tool for understanding genetic diversity and epidemiology of fungal pathogens[14]. Two such molecular marker techniques *i.e.* RAPD and amplification of the ITS region of rDNA were used as rapid methods for differentiation of *C. cassiicola* of *Hevea*.

Genetic analysis with RAPD indicated the presence of the two groups of *C. cassiicola* (CPEN 1 and CLN 11). One group specifically infected clone RRIM 2020 and that could be



Fungal isolates: Lane 1= CSB 1; Lane 2= CL 1; Lane 3= CUN 1; Lane 4= CSS 1; Lane 5= CSB 6; Lane 6= CPEN 1; Lane 7= CLN 11; Lane 8= CSD 1; Lane 9= CSB 4.

Figure 1. Restriction enzyme digestion of C. cassiicola *gDNA. Isolated fungal gDNA before (a) and after digestion with EcoRI (b).*

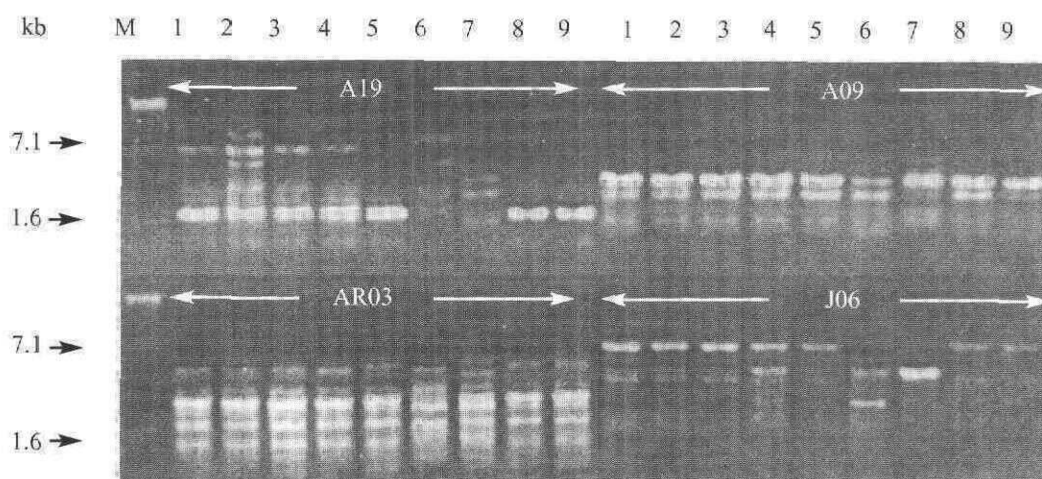
TABLE 2. SEQUENCE OF OLIGONUCLEOTIDE PRIMERS USED TO ELICIT REPRODUCIBLE FINGERPRINTING PATTERNS WHICH COULD DIFFERENTIATE TWO *C. CASSIICOLA* ISOLATES FROM NINE EXAMINED

Primer	Sequence (5' – 3')
A-19	CAAACGTCGG
A-09	GGGTAACGCC
AR-03	GTGAGGCGCA
J-06	TCGTTCCGCA

one race, and another group which did not infect RRIM 2020 but infected RRIM 600 and the other clones.

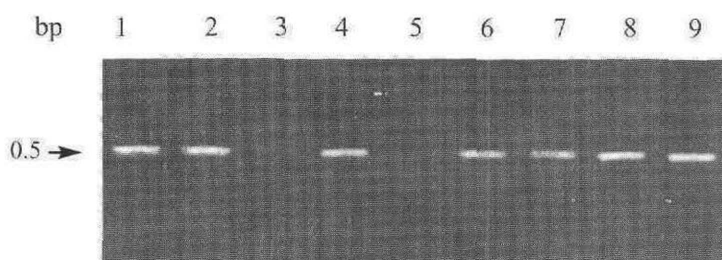
Earlier study[8] showed the existence of two races of *C. cassiicola* using the conventional method *i.e.* using a set of differential clones. The current study using the same isolates used by them, confirmed the existence of the races of *C. cassiicola*.

ITS region of the nuclear ribosomal unit has been used in the study of evolution and population genetics of many species and is



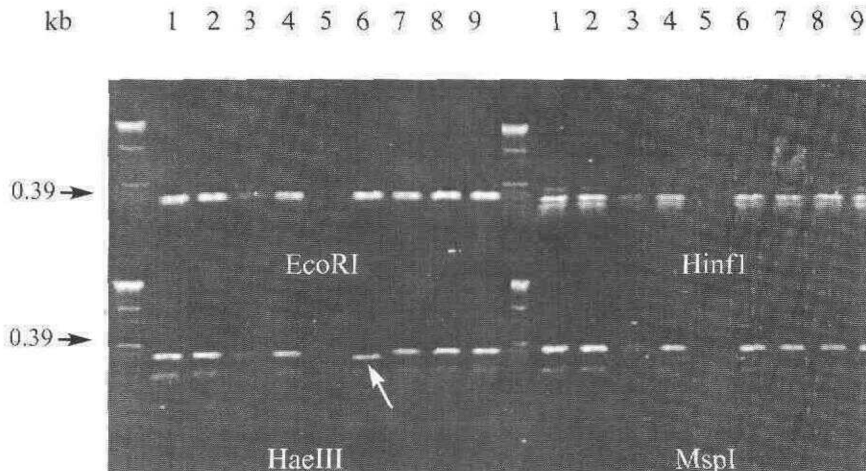
Fungal isolates: Lane 1= CSB 1; Lane 2= CL 1, Lane 3= CUN 1; Lane 4= CSS 1, Lane 5= CSB 6; Lane 6= CPEN 1, Lane 7= CLN 11; Lane 8= CSD 1; Lane 9= CSB 4.

Figure 2. Polymorphic banding patterns are observed between isolates in lane 6 and 7 when fungal DNAs were amplified with primers A19, A09, AR03 and J06.



Fungal isolates: Lanes 1= CSB 1; Lane 2= CL 1; Lane 3= CUN 1; Lane 4= CSS 1; Lane 5= CSB 6; Lane 6= CPEN 1; Lane 7= CLN 11; Lane 8= CSD ; Lane 9= CSB 4.

Figure 3. Amplified rDNA ITS fragments from nine C. cassiicola *isolates.*



Fungal isolates: Lane 1= CSB 1; Lane 2= CL 1; Lane 3= CUN 1; Lane 4= CSS 1; Lane 5= CSB 6; Lane 6= CPEN 1; Lane 7= CLN 11; Lane 8= CSD 1; Lane 9= CSB 4.

Figure 4. Amplified rDNA ITS fragments were digested with EcoR*I*, Hinf*I*, Hae*III and* Msp*I*. *Isolate CPEN 1 (lane 6) showed a lower molecular weight when restricted with* Hae*III*.

found to be species specific. Analysis of the ITS region in this study revealed that there were differences in this region for isolates CPEN 1 and CLN 11, both of which infected clone RRIM 2020 and not the other clones. Cloning and sequencing of PCR fragments from both isolates have to be done before further conclusions could be made.

CONCLUSION

Using molecular techniques, the existence of two races of *C. cassiicola* was confirmed. This study identified *Race 1* as isolates that infected RRIM 600, RRIM 2009 and unidentified *Hevea* clones; and *Race 2* as isolates that infected *Hevea* clone RRIM 2020.

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