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Identification of *Colletotrichum acutatum* from rubber using random amplified polymorphic DNAs and ribosomal DNA polymorphisms

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The fungal pathogen responsible for Colletotrichum leaf disease of *Hevea*, develops three different disease symptoms: raised spots, anthracnose and papery lesions. These diseases have been attributed to *Colletotrichum gloeosporioides* (teleomorph *Glomerella cingulata*) and the fungi involved are morphologically indistinguishable. Twenty-five *Colletotrichum* isolates, which originated from three different disease symptoms, were characterized initially using RAPD markers. Two major RAPD profiles were detected which were related to the type of disease symptom developed. *Colletotrichum* isolates causing raised spot symptom were easily distinguished from isolates originating from either anthracnose or papery lesions. Restriction analysis of the PCR amplified 5.8S ribosomal DNA (rDNA), including both the flanking internal transcribed spacers (ITS) of representative isolates from the three different disease symptoms, also revealed two distinct RFLP patterns reflecting the same groupings as detected through RAPDs. Both molecular approaches suggested that there were two species of *Colletotrichum* associated with *Hevea* inciting the development of three different symptoms: *Colletotrichum acutatum* causing raised spot symptom, and *C. gloeosporioides* causing both anthracnose and papery lesions. This is the first record of *Colletotrichum acutatum* on *Hevea* in India.

INTRODUCTION

Colletotrichum leaf disease in rubber (*Hevea brasiliensis*) has been recorded from most rubber growing countries. The fungus causing the disease was originally identified as *Glomerella cingulata* the teleomorph of '*Gloeosporium*' and *Colletotrichum* (Carpenter & Stevenson 1954). These anamorphic genera have been merged into one as differences between the two are not consistent or significant. The only difference between *Colletotrichum* and '*Gloeosporium*' strains infecting rubber is the development of setae in the acervuli of the former (Ramakrishnan & Radhakrishna Pillay 1961). This character is unreliable as it seems to be determined, at least partially, by the environment (Alexopoulos, Mims & Blackwell 1996). Several species of *Colletotrichum* and '*Gloeosporium*' have been recorded from *Hevea* (Petch 1906, Altson 1950a, b). Carpenter & Stevenson (1954) considered that all reported species infecting *Hevea* are closely related to *C. gloeosporioides* and could not be maintained as distinct from it. All these represent anamorphs of *Glomerella cingulata*. However, in rubber, the symptoms of infection by '*Gloeosporium alborubrum*' and *C. gloeosporioides* differ, though both species are considered to be synonymous. Symptoms caused by '*G. alborubrum*' are characterized by the appearance of numerous minute circular brown lesions on the leaflets. Later, they develop a thick brown margin and are raised above the surface as conical projections or raised spots (Fig. 1). The pathogen mainly infects tender and immature leaves. *C. gloeosporioides* was reported to cause anthracnose disease of rubber. The anthracnose spots appear as concentric rings that occur generally along the margins and occasionally in the middle of leaflets (Fig. 2). The lesions are large, 1–5 cm diam, and may coalesce to form larger spots. The central portion of the spot is light brown, papery and necrotic. Another leaf spot symptom of almost circular papery lesions with a dark brown centre surrounded by a yellow halo (Fig. 3) was reported in rubber and the pathogen identified as *C. gloeosporioides* (Rajalakshmy & Joseph 1988).

Given the occurrence of different types of disease symptoms, such as raised spots, anthracnose and papery

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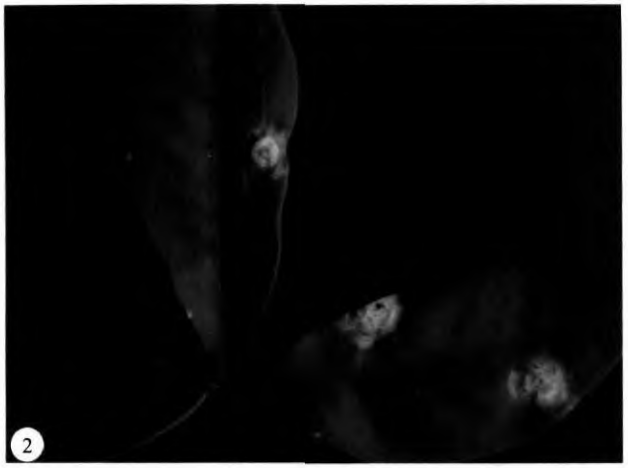


Figs 1–3. Three different disease symptoms caused by *Colletotrichum* spp. on *Hevea* leaf: raised spots (Fig. 1), anthracnose (Fig. 2), and papery lesions (Fig. 3).

lesions on *Hevea*, the present work aimed to characterize the pathogen from all three symptoms using molecular techniques. Sutton (1992) suggested that relationships within the genus *Colletotrichum* were unlikely to be resolved using morphology alone. Morphological plasticity and overlapping phenotypes make traditional taxonomic criteria unreliable for the accurate delineation of *Colletotrichum* species. On the other hand, genetic markers generated by random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990) have been proved to be useful in determining the genetic structure and evaluating relationships amongst *Colletotrichum* populations (Freeman, Pham & Rodriguez 1993, Sreenivasaprasad, Mills & Brown 1993), including species identification (Freeman & Katan 1997). Use of ribosomal DNA (rDNA) restriction digest analysis and sequence data as molecular methods for species delineation in *Colletotrichum* have been reported (Sreenivasaprasad *et al.* 1993, 1994, Sherriff *et al.* 1994, 1995, Freeman, Katan & Shabi 1996, Buddie *et al.* 1999) and these methods are now well established. The internal transcribed spacers ITS1 and ITS2 in the ribosomal RNA gene block, displaying high rates of genetic drift, may also be useful for understanding phylogenetic relationships at a sub-generic level (Bruns, White & Taylor 1991, Gardes *et al.* 1991, Lee & Taylor 1992, Sherriff *et al.* 1994, Sreenivasaprasad, Brown & Mills 1992, Sreenivasaprasad *et al.* 1996).

In the present study, RAPD markers and restriction fragment length polymorphisms of internal transcribed spacers (ITS) of ribosomal DNA were used to investigate molecular variation among *Colletotrichum* isolates associated with *Hevea*. The objectives of this investigation were to verify pathogen identity based on disease symptoms, investigating relationships between them, and to determine the presence of any subpopulation of the pathogens.

MATERIALS AND METHODS

The pathogen

The isolates from three typical symptoms, raised spots, anthracnose, and papery lesions on leaflets of different clones of *Hevea brasiliensis*, were collected from rubber plantations located at the Rubber Research Institute of India (RRII), Kottayam and other locations in Kerala state (Table 1). Twenty-five isolates (14 from raised spots, six from anthracnose, and five from papery lesions) were used in the present study. Isolations were made by plating surface-sterilized pieces of diseased tissue on oat meal agar (OMA). All isolates were purified by single-spore culture, maintained on OMA and stored at 5 °C in darkness. Fungal isolates were identified based on cultural characteristics and microscopic morphology, and representative cultures preserved in the collections of the Plant Pathology Division of the Rubber Research Institute of India (RRII).

Genomic DNA extraction

For extraction of genomic DNA, three mycelial plugs (5 mm diam) were removed from the advancing margins of 5 d old cultures of each isolate, transferred to oat meal broth and incubated at 25 ° on an orbital shaker (100 rpm) for 4 d. Mycelium (*ca* 300–500 mg) was harvested by filtering through Whatman No. 1 filter paper under vacuum, washed three times with sterile distilled water, immediately frozen in liquid nitrogen

Table 1. Source of *Colletotrichum* isolates from *Hevea* clones.

Code no.	Host genotype	Location	Symptom
CG_1	BD 10	RRII Farm, Kottayam	Raised spots
CG_2	BD 10	RRII Farm, Kottayam	Raised spots
CG_3	GT 1	RRII Farm, Kottayam	Raised spots
CG_4	GT 1	RRII Farm, Kottayam	Raised spots
CG_5	RRII 208	RRII Farm, Kottayam	Raised spots
CG_6[1]	RRII 105	RRII Farm, Kottayam	Raised spots
CG_7	PB 280	RRII Farm, Kottayam	Raised spots
CG_8	PB 310	RRII Farm, Kottayam	Raised spots
CG_9	PB 217	RRII Farm, Kottayam	Raised spots
CG_{10}	PB 217	RRII Farm, Kottayam	Raised spots
CG_{11}	RRII 105	RRII Farm, Kottayam	Raised spots
CG_{12}	RRII 105	Cheruvally Estate	Raised spots
CG_{13}	RRII 105	Cheruvally Estate	Raised spots
CG_{14}	RRII 105	Manickal Estate	Raised spots
CG_{15}	Seedling	RRII Farm, Kottayam	Anthracnose
CG_{16}	Seedling	RRII Farm, Kottayam	Anthracnose
CG_{17}	Seedling	RRII Farm, Kottayam	Anthracnose
CG_{18}[2]	Seedling	RRII Farm, Kottayam	Anthracnose
CG_{19}	PR 255	RRII Farm, Kottayam	Papery lesions
CG_{20}[3]	RRII 300	RRII Farm, Kottayam	Papery lesions
CG_{21}	RRII 300	RRII Farm, Kottayam	Papery lesions
CG_{22}	PB 235	RRII Farm, Kottayam	Papery lesions
CG_{23}	Seedling	Kaliyar Estate	Anthracnose
CG_{24}	PB 260	Manickal Estate	Anthracnose
CG_{25}	RRII105	Manickal Estate	Papery lesions

[1] Culture preserved as IMI 383015.
[2] Culture preserved as IMI 383016.
[3] Culture preserved as IMI 383017.

Table 2. Details of random decamer oligonucleotide primers used in this study.

Primer code	Nucleotide sequence (5′ to 3′)	Number of amplified loci
OPA-18	AGGTGACCGT	25
OPB-10	CTGCTGGGAC	21
OPB-12	CCTTGACGCA	9
OPB-17	AGGGAACGAG	20
OPB-18	CCACAGCAGT	19
OPD-11	AGCGCCATTG	16
OPE-18	GGACTGCAGA	20
OPI-06	AAGGCGGCAG	28
OPJ-20	AAGCGGCCTC	26

and powdered Extraction and purification of total genomic DNA were carried out following a modified CTAB (Hexadecyl trimethyl ammonium bromide, Sigma, St Louis) method optimized for *Corynespora cassiicola* (Saha *et al.* 2000).

RAPD analysis

Nine decamer primers (Table 2) selected at random from Operon primer kits (Operon Technology, USA) were used for PCR amplifications. Amplifications were performed in a total volume of 25 µl by mixing 50 ng of template DNA with 10 picomoles of single primer, 0.2 mM of each dNTP, 0.7 unit of Taq DNA polymerase (Promega, USA), 2.5 µl 10 × DNA polymerase buffer (100 mM Tris–HCl (pH 9.0), 500 mM KCl, 20 mM $MgCl_2$). Amplifications were performed in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, USA) with an initial denaturation at 94 ° for 3 min, followed by 40 cycles of 30 s at 94 °, 1 min at 37 °, and 2 min at 72 ° with a final extension at 72 ° for 7 min. Amplified products were analysed along with a DNA size marker, by electrophoresis on a 1.4 % agarose gel in 1 × TAE buffer. The gels were stained with ethidium bromide and viewed on a UV transilluminator. To check the reliability of the method, the amplification reactions were performed twice. Amplification products were scored on the basis of their presence or absence and variations in the intensity of the same band across the isolates were not considered in the analysis. Pairwise comparisons of the RAPD profiles of the isolates, based on both unique and shared amplification products, were employed to calculate genetic distance (GD) (Link *et al.* 1995):

$$GD_{xy} = (N_x + N_y)/(N_x + N_y + N_{xy}), \quad (1)$$

where N_x is the number of bands in genotype x and not in genotype y, N_y is the number of bands in genotype y and not in genotype x and N_{xy} is the number of bands in genotypes x and y. The data were subsequently used for cluster analysis to construct a dendrogram. All calculations were made using the TREECON programme (van de Peer & de Wachter 1994).

Ribosomal DNA RFLP analysis

Amplification of rDNA

Amplification of the internal transcribed spacer regions between the small (18S) and large (28S) nuclear rDNA was achieved using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990) supplied by Sigma Genosys, USA. The amplified fragment included the 5.8S rDNA gene and the internal transcribed spacers (ITS1 and ITS2). PCR amplification was performed in a total volume of 50 µl containing 100 ng of template DNA with 0.5 µM of each primer, 0.2 mM of each dNTP, 2 units of Taq DNA polymerase (Promega, USA), and 5 µl of 10 × DNA polymerase buffer (100 mM Tris–HCl (pH 9.0), 500 mM KCl, 20 mM $MgCl_2$). Amplifications were performed in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, USA) with an initial denaturation at 94 ° for 3 min, followed by 45 cycles of 30 s at 94 °, 1 min at 55 ° and 2 min at 72 ° with a final extension at 72 ° for 7 min. Amplified products were separated on 1 % agarose gel in 1 × TAE buffer. The gels were stained with ethidium bromide and viewed on a UV transilluminator.

Restriction enzyme digestion of amplified rDNA

PCR amplified rDNA products were purified and digested with the restriction enzymes *Acc*I, *Alu*I,

*Sau*3AI, *Eco*RI (Boehringer Mannheim), *Taq*I and *Xho*I (Promega) using the buffers and conditions recommended by the suppliers. The digested fragments were separated on a 4% agarose gel, stained and visualized as described above.

RESULTS AND DISCUSSION

Comparison of Colletotrichum *isolates by RAPDs*

Two major types of RAPD profiles were obtained among the three groups of isolates, raised spots (R), anthracnose (A), and papery lesions (L) (Figs 4–5) that clearly indicated the fungal isolates causing raised spot symptom in *Hevea* reacted differentially from the other two symptoms. With all primers tested, 14 isolates from raised spot symptom were easily distinguished from 11 isolates originating from anthracnose and papery lesion type symptoms and these formed two genetically distinct groups. All isolates, irrespective of their symptoms, were variable in their morphological features. There were, however, significant differences in the growth patterns and conidial characteristics between these two groups. Anthracnose-causing fungal isolates showed rapid radial growth in contrast to isolates originating from raised spots (A. Kumar, unpubl.). RAPD profiles also revealed genetic diversity within the sub-groups (Figs 4–5). A dendrogram illustrating genetic relationships among the fungal isolates (Fig. 6) was constructed using the RAPD data, comprising 184 amplified loci (Table 2). Although the isolates clustered into two major groups, all were found to be genetically different. Wide genetic variability with distance coefficients of 0.41 to 0.78 was detected among isolates from anthracnose and papery lesions, whereas isolates originating from raised spots showed comparatively less variation with distance coefficients of 0.04 to 0.57 (Table 3). More than 90% genetic dissimilarity was recorded between these two groups. Although current fungal taxonomy suggests that the genera '*Gloeosporium*' and *Colletotrichum* are synonymous, the molecular characterization of fungal isolates through RAPD markers in the present study supports the existence of two distinct groups at the species level. Grouping of *Colletotrichum* isolates causing anthracnose and papery lesions together on the basis of RAPD profiles, suggested that in both cases symptoms developed due to infection caused by *Colletotrichum gloeosporioides*, previously identified from papery lesions in *Hevea* (Rajalakshmy & Joseph 1988).

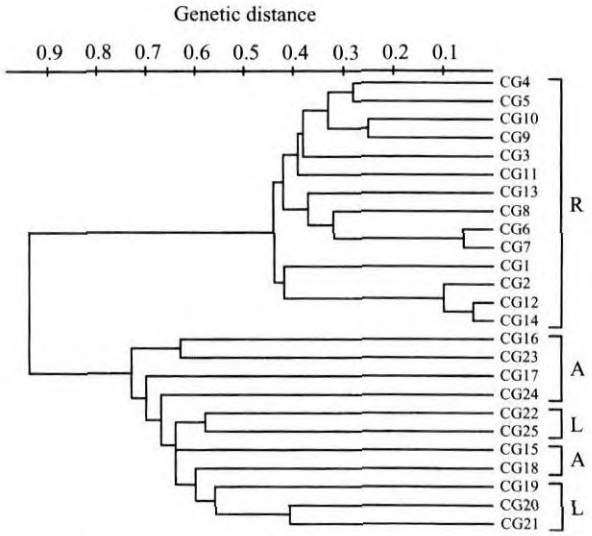
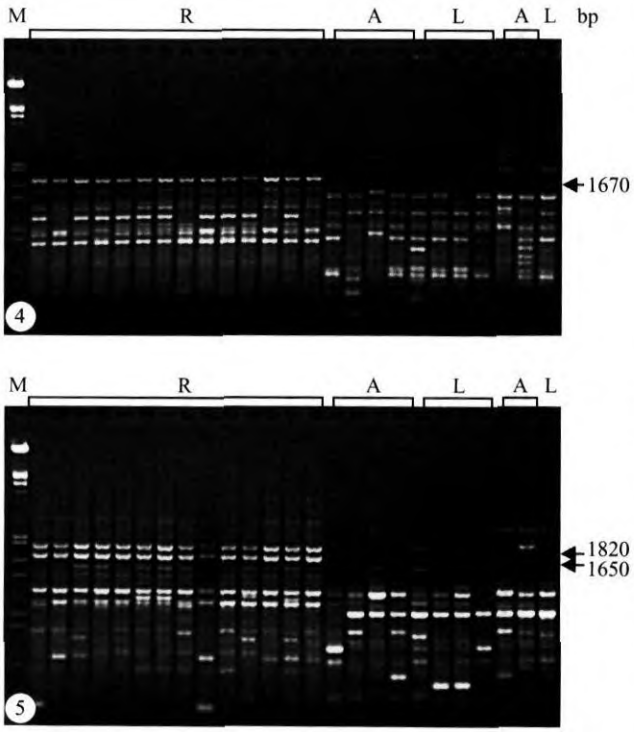


Fig. 6. Dendrogram resulting from a cluster analysis of genetic distance data based on RAPD profiles, generated with nine primers, demonstrating genetic relationships among 25 *Colletotrichum* isolates from three different disease symptoms, raised spot symptom (R), anthracnose (A) and papery lesions (L).



Figs 4–5. RAPD fingerprints of 25 *Colletotrichum* isolates generated with primers OPI-06 (**Fig. 4**) and OPJ-20 (**Fig. 5**). Lanes are according to the serial order of isolates given in Table 1. *Colletotrichum* isolates causing raised spot symptom (R), anthracnose (A) and papery lesions (L) are marked. Lane M: molecular weight marker (λ DNA/*Eco*RI + *Hin*dIII).

rDNA RFLPs

The suggested involvement of two distinct groups of pathogens based on RAPD profiling was further tested by ribosomal DNA analysis of all isolates of *Colletotrichum*. The length of the ITS4/5 amplified product (internal transcribed spacers including 5.8S rDNA) was ~ 0.6 kb and length polymorphism was not detected among the isolates, as also reported in *Colletotrichum* isolates from strawberry (Buddie *et al.* 1999). Amplified rDNA products were subjected to digestion with six

Table 3. Genetic distance matrix of *Colletotrichum* isolates based on RAPD data.

	CG1	CG2	CG3	CG4	CG5	CG6	CG7	CG8	CG9	CG10	CG11	CG12	CG13	CG14	CG15	CG16	CG17	CG18	CG19	CG20	CG21	CG22	CG23	CG24	CG25
CG1	0.000																								
CG2	0.393	0.000																							
CG3	0.436	0.500	0.000																						
CG4	0.474	0.483	0.389	0.000																					
CG5	0.296	0.345	0.386	0.278	0.000																				
CG6	0.397	0.460	0.424	0.407	0.322	0.000																			
CG7	0.379	0.444	0.433	0.417	0.305	0.058	0.000																		
CG8	0.431	0.417	0.458	0.467	0.356	0.310	0.322	0.000																	
CG9	0.418	0.404	0.389	0.370	0.309	0.484	0.516	0.467	0.000																
CG10	0.392	0.459	0.364	0.315	0.316	0.356	0.393	0.390	0.250	0.000															
CG11	0.517	0.450	0.411	0.448	0.362	0.400	0.436	0.379	0.393	0.339	0.000														
CG12	0.439	0.102	0.492	0.475	0.362	0.452	0.436	0.433	0.421	0.475	0.441	0.000													
CG13	0.491	0.550	0.434	0.526	0.492	0.302	0.346	0.456	0.473	0.446	0.407	0.567	0.000												
CG14	0.431	0.100	0.483	0.492	0.356	0.444	0.429	0.426	0.414	0.468	0.433	0.042	0.557	0.000											
CG15	0.942	0.960	0.927	0.912	0.932	0.947	0.962	0.946	0.943	0.945	0.914	0.944	0.939	0.946	0.000										
CG16	0.964	0.966	0.951	0.977	0.966	0.978	0.978	0.978	0.952	0.954	0.928	0.953	0.963	0.954	0.763	0.000									
CG17	0.913	0.929	0.885	0.914	0.919	0.907	0.921	0.918	0.914	0.904	0.861	0.916	0.880	0.918	0.724	0.775	0.000								
CG18	0.938	0.941	0.938	0.951	0.942	0.943	0.943	0.954	0.925	0.928	0.914	0.927	0.949	0.929	0.667	0.651	0.697	0.000							
CG19	0.914	0.943	0.900	0.901	0.920	0.921	0.933	0.893	0.928	0.918	0.890	0.929	0.910	0.931	0.661	0.706	0.672	0.662	0.000						
CG20	0.926	0.929	0.885	0.927	0.906	0.907	0.921	0.892	0.927	0.929	0.846	0.916	0.880	0.918	0.604	0.721	0.687	0.590	0.541	0.000					
CG21	0.951	0.941	0.910	0.951	0.954	0.955	0.967	0.941	0.938	0.941	0.886	0.927	0.935	0.929	0.615	0.750	0.635	0.552	0.574	0.407	0.000				
CG22	0.958	0.960	0.958	0.958	0.947	0.948	0.949	0.947	0.958	0.960	0.931	0.945	0.940	0.947	0.581	0.767	0.771	0.648	0.667	0.558	0.596	0.000			
CG23	0.925	0.916	0.951	0.952	0.930	0.943	0.956	0.942	0.939	0.941	0.915	0.915	0.949	0.917	0.783	0.635	0.739	0.692	0.706	0.757	0.750	0.724	0.000		
CG24	0.924	0.941	0.924	0.951	0.929	0.930	0.943	0.941	0.925	0.928	0.900	0.940	0.907	0.941	0.714	0.750	0.697	0.688	0.682	0.656	0.667	0.673	0.672	0.000	
CG25	0.973	0.974	0.973	0.973	0.975	0.975	0.976	0.988	0.973	0.974	0.961	0.974	0.972	0.975	0.714	0.774	0.717	0.684	0.678	0.672	0.611	0.578	0.667	0.636	0.000

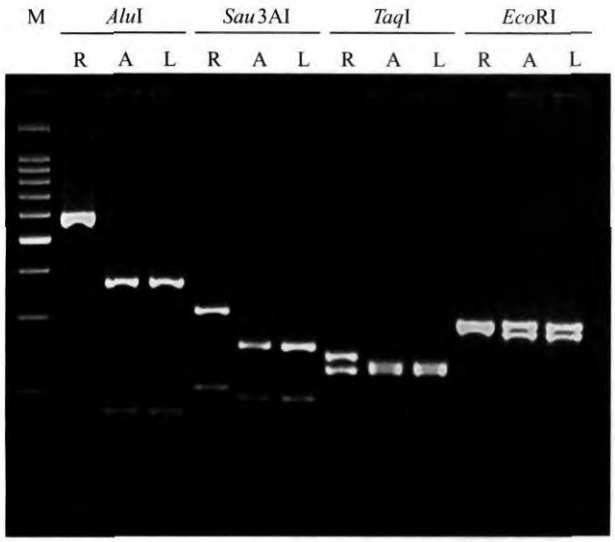


Fig. 7. Restriction fragment length polymorphisms of PCR amplified rDNA from three representative isolates of *Colletotrichum* causing three different disease symptoms: raised spots (R), anthracnose (A) and papery lesions (L) on *Hevea*. Restriction enzymes are indicated on the gel photograph. Lane M: 100 bp ladder molecular weight marker.

different restriction endonucleases (*Acc*I, *Alu*I, *Sau*3AI, *Eco*RI, *Taq*I and *Xho*I) to identify fragment length polymorphisms. Restriction digestion was obtained with only four enzymes, *Alu*I, *Sau*3AI, *Eco*RI, and *Taq*I. Fig. 7 shows the RFLP profiles of three representative isolates with each of the four restriction enzymes used. Restriction patterns of the isolates causing raised spots were clearly differentiated from other *Colletotrichum* isolates originating from anthracnose and papery lesions. *Colletotrichum* rDNA RFLPs reflected the same grouping as revealed from RAPD studies. The rDNA restriction length polymorphisms detected among the fungal isolates were due to sequence variations in ITS regions, which are rapidly evolving regions of ribosomal DNA/rRNA gene sequences and are often studied for comparing species and closely related genera. Thus, molecular evidence suggested that the fungus causing raised spot type of symptom in rubber is distinct from that causing anthracnose and papery lesions, the later two being similar.

RAPDs appeared to be very useful in distinguishing fungal pathogens in the present investigation, although this method is generally considered to be discriminatory at the population level rather than the species level (Kohn 1992). Some of the RAPD fragments (OPI-06_{1670} (Fig. 4), OPJ-20_{1820} and OPJ-20_{1650}, Fig. 5) identified among the fungal isolates, could be used as markers for the identification of fungal pathogen causing raised spots symptom on rubber. The International Mycological Institute, UK, confirmed the identification of the isolate causing raised spots (IMI 383015) as *Colletotrichum acutatum* (P. F. Cannon, pers. comm.), which is the first report of the species as a pathogen of rubber in India. Two isolates causing anthracnose (IMI 383016) and papery lesions (IMI 383017) were identified as *Colletotrichum gloeosporioides*, as expected. Distinguishing these two polymorphic taxa by morphological studies only (especially of conidium shape and size) is not easy as many *Colletotrichum* isolates produce secondary conidia directly from germinating primary spores. These secondary spores are generally smaller and more variable in shape and cannot be distinguished reliably (Buddie *et al.* 1999). On the other hand, *C. acutatum* and *C. gloeosporioides* revealed considerable levels of variation in rDNA RFLPs and random amplified polymorphic DNA that could successfully be used in identifying *Colletotrichum* isolates from rubber, at the species level.

The importance of *C. acutatum* as a pathogen was first recognized in strawberry anthracnose (Simmonds 1965, 1968), and it was subsequently identified from a wide range of crop plants (Buddie *et al.* 1999). However, *C. acutatum* was unknown in rubber until it was reported from Sumatra and Sri Lanka (Brown & Soepena 1994, Jayasinghe, Fernando & Priyanka 1997). Although *C. acutatum* was reported only recently from rubber, the common occurrence of raised spots in Indian rubber plantations predated the identification of the pathogen on strawberry plants, but it was described as '*Gloeosporium alborubrum*' (Ramakrishnan & Radhakrishna Pillay 1961). '*G. alborubrum*', identified as the causal organism of raised spots, was suggested to be synonymous with *C. gloeosporioides* (Carpenter & Stevenson 1954) and it is now understood that *C. acutatum* is the major cause of Colletotrichum leaf disease, until now known as Gloeosporium leaf disease (Edathil, Jacob & Joseph 2000). RAPD and rDNA analysis could thus help in the successful identification of *Colletotrichum acutatum* from rubber for the first time in India. As different species of *Colletotrichum* are reported to show differential response to fungicides (Bernstein *et al.* 1995, Brown, Sreenivasaprasad & Timmer 1996, Liyanage, McMillan & Kistler 1992), proper identification of the fungal pathogen associated with raised spots in *Hevea* could be of great help in developing meaningful disease management strategies for the pathogen.

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