Indian Journal of Natural Rubber Research, 13(1&2) : 1-10, 2000

GENETIC VARIABILITY OF *CORYNESPORA CASSIICOLA* INFECTING *HEVEA BRASILIENSIS* ISOLATED FROM THE TRADITIONAL RUBBER GROWING AREAS IN INDIA

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Saha, T., Kumar, A., Sreena, A.S., Joseph, A., Jacob, C.K., Kothandaraman, R. and Nazeer, M.A. (2000). Genetic variability of *Corynespora cassiicola* infecting *Hevea brasiliensis* isolated from the traditional rubber growing areas in India. *Indian Journal of Natural Rubber Research*, 13(1&2) : 1-10

Molecular characteristics of 20 isolates of *Corynespora cassiicola* from 16 different locations of two rubber growing states of southern India *viz.*, Kerala and Karnataka, were investigated using random amplified polymorphic DNA (RAPD) markers. RAPD analysis clearly indicated the existence of at least seven different genotypes of *C. cassiicola*. Considerable genetic variations were detected among the Kerala isolates and three of them, KL01/97, KL04/98 and KL06/97 showed completely different RAPD profiles from others with each of the primers tested. Putative virulence specific RAPD profile of *Corynespora* was identified among the isolates where the disease became epidemic in Karnataka. Genetic relationships were established among the *Corynespora* isolates.

Key words : *Corynespora cassiicola*, Genetic diversity, *Hevea brasiliensis*, Leaf disease, RAPD.

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INTRODUCTION

Corynespora cassiicola (Berk. & Curt.) Wei. causing *Corynespora* leaf disease was recognized earlier as a weak pathogen on *Hevea brasiliensis* (Ramakrishnan and Pillai, 1961; Ellis and Holliday, 1971). It became a major pathogen in South and South-East Asian rubber growing countries since 1980 and the disease attained an epidemic scale affecting vast areas of rubber plantations in Indonesia, Sri Lanka, Malaysia and Thailand (Jacob, 1997). In Sri Lanka, the disease developed into devastating proportions affecting about 4600 ha of rubber plantations (Liyanage *et al.*, 1989; Jayasinghe and Silva, 1996). *C. cassiicola* was reported for the first time in India in 1958 as a pathogen on *Hevea* (Ramakrishnan and Pillai, 1961). Since then the disease has been observed in various parts of Kerala and Karnataka states. Generally, *Corynespora* infection appears on a mild scale, confined to the nursery seedlings and budwood plants. Incidence of *Corynespora* infection on mature trees, causing severe damage, has been reported recently from Karnataka (Rajalakshmi and Kothandaraman, 1996). This disease is of serious concern as high

yielding clones like RRII 105 have become susceptible to *Corynespora* infection.

Severity of the disease in different rubber growing regions is highly variable, which may be attributed to several factors like climate, existence of virulent pathotypes and susceptibility of the host genotype under cultivation. Development of new races of the pathogen could be suspected where a mild disease turns out to be an epidemic within a short time. Morphologically, the races/pathotypes of the *C. cassiicola* are not distinguishable. Moreover, identification of races/pathotypes by cultural characteristics may not be accurate as it is based on morphological criteria that are often dependent on cultural conditions, leading to misidentification (Faris-Mokaiesh *et al.*, 1996). Development of molecular techniques, such as PCR-based methods, has provided powerful tools for fungal diagnosis and taxonomy (McDonald, 1997; Bridge and Arora, 1998). Random amplified polymorphic DNA (RAPD) assay is being extensively used in fungal studies to distinguish within and between species (Goodwin and Annis, 1991; Guthrie *et al.*, 1992; Raina *et al.*, 1997). It is considered to be an efficient technique as it could detect more genetic variability and distinguish more unique DNA profiles than the restriction fragment length polymorphism (RFLP) analysis of internal transcribed spacer (ITS) or inter-genic spacer (IGS) of rDNA (Silva *et al.*, 1998; Hsiang and Mahuku, 1999). However, there are only few published reports on molecular characterisation of *C. cassiicola* isolates on rubber (Darmono *et al.*, 1996; Silva *et al.*, 1995; Silva *et al.*, 1998) as the pathogen is a relatively less known fungus. Studies on genetic variability of the pathogen from the rubber growing regions of India have not been attempted so far. Such studies are essential for understanding the genetic structure of the populations, genetic control for pathogenesis and identification of molecular marker for virulence which are very important for effective disease management. Hence, the present study was undertaken as a preliminary investigation for the assessment of molecular variability among the isolates of *C. cassiicola* from the rubber plantations of major rubber growing areas in South India in relation to disease incidence in rubber.

MATERIALS AND METHODS

Corynespora isolates

Leaves of *H. brasiliensis* showing typical symptoms of *Corynespora* infection were collected from various rubber plantations and nurseries located at 16 different places in Kerala and Karnataka states of India (Fig. 1; Table 1). Isolations were made by plating surface-sterilized pieces of dis-

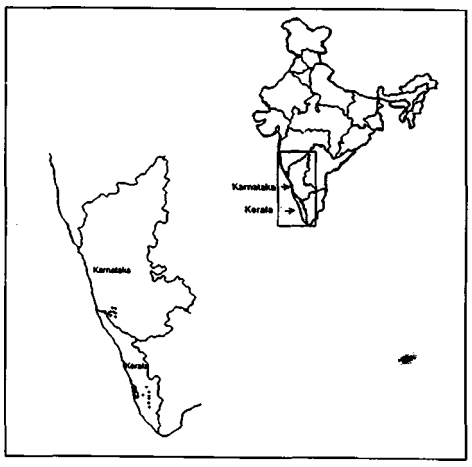

Fig. 1. Map of the two rubber growing states of southern India showing the locations (*) from where populations of *C. cassiicola* were sampled

Table 1. **Source of *Corynespora cassiicola* isolates**

Code[a]	Location	Year	Host genotype	% infection
Kerala				
KL01/97	Mundakayam	1997	RRII 105[b]	ns[c]
KL02/98	Mallappally	1998	RRII 105	05
KL03/98	Rajagiri	1998	RRII 105	30
KL04/98	Teekoy	1998	RRII 105	40
KL05/97	Vakayar	1997	RRII 105[b]	ns
KL06/97	Karikattoor	1997	Nursery	ns
KL07/99	Karikattoor	1999	Nursery	ns
KL08/99	Chethackal	1999	Germplasm	ns
Karnataka				
KR01/98	Kadaba	1998	RRII 105	50
KR02/98	Eswaramangala	1998	RRII 105	70
KR03/98	Sullia	1998	RRII 105	50
KR04/96	Nettana	1996	RRII 105	10
KR05/98	Jalsur	1998	RRII 105	30
KR06/98	Sampaje	1998	RRII 105	50
KR07/98	Nettana	1998	RRII 105	10
KR08/98	Devachalla	1998	RRII 105	70
KR09/98	Guthigar	1998	RRIM 600	10
KR10/98	Ivernadu	1998	RRII 105	15
KR11/99	Nettana	1999	RRII 105	07
KR12/99	Sampaje	1999	RRII 105	25

[a] First two letters of the code designate the state, next digits are the isolate number and the last two digits indicate the year of collection

[b] Isolates from polybag plants [c] ns, not scored

eased tissue on potato dextrose agar (PDA). All isolates were purified by single-spore culture, maintained on PDA and stored at 5°C in the dark. Twenty isolates of *Corynespora* were used in the present study.

Genomic DNA extraction

Three mycelial plugs (5 mm diameter) were removed from the advancing margins of 5 day old culture of each isolate transferred to modified Czapek's broth and incubated at 25°C on an orbital shaker (100 rpm) for 4 days for the extraction of genomic DNA. About 300-500 mg of mycelium was harvested by filtering through Whatman No.1 filter paper under vacuum, washed three times with sterile distilled water, immediately dried in liquid nitrogen and powdered. Extraction and purification of the total genomic DNA of 20 *Corynespora* isolates were carried out following a modified CTAB (Hexadecyl trimethyl ammonium bromide, Sigma Co., St. Louis, USA) method (Doyle and Doyle, 1990) optimized for *Hevea* leaf tissue. However, two additional precipitation steps with 2M NaCl were carried out to remove polysaccharides from the DNA (Fang *et al.*, 1992). Cell lysis was achieved by the

addition of extraction buffer pre-heated to 60°C containing 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0) and 0.3% β-mercaptoethanol (added just before use). After incubation for 45 min at 60°C with occasional shaking, equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed gently by inversion for 10 min. The mixture was then centrifuged at room temperature for 10 min at 6000 x g. The aqueous phase was then transferred to a new tube and the chloroform-isoamyl alcohol extraction was repeated. Genomic DNA was precipitated by the addition of 0.6 volume of ice-cold isopropanol and centrifuged at 4°C for 10 min at 6000 x g. The pellet was washed in pre-chilled 75% ethanol, centrifuged as above, air dried and dissolved in 1 ml TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. RNase (10 mg/ml) was added to a final concentration of 150 µg/ml and incubated at 37°C for 1 h. DNA was isolated by chloroform-isoamyl alcohol extraction, as described above and precipitated by the addition of NaCl to a final concentration of 2M followed by 2.5 volume of ice-cold dehydrated ethanol. This precipitation step with NaCl was repeated once. The DNA pellet was washed twice in pre-chilled 75% ethanol, air dried and finally dissolved in TE buffer. The DNA concentration and the purity were determined spectrophotometrically (Beckman DU 640B). DNA stock (0.5 mg/ml) and working solutions (10 ng/µl) were adjusted for assays and stored at 4°C.

RAPD analysis

Twenty arbitrary decamer primers (OPA01–OPA20; Operon technology Inc. USA) were screened for PCR amplification and seven were used for the assays. Amplifications were performed in a total volume of 25 µl by mixing 50 ng of template DNA with 10 pmole of single primer, 0.2 mM of each dNTP, 0.7 unit of Taq DNA polymerase (Promega, USA), 2.5 µl of DNA polymerase buffer 10X (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 step at 94°C for 3 min, followed by 40 cycles of 30 sec at 94°C, 1 min at 37°C and 2 min at 72°C with a final extension at 72°C for 7 min. Amplified products were analysed along with a DNA marker, as molecular size reference, by electrophoresis on a 1.4% agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide and viewed on an 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. Pair-wise comparisons of the RAPD profiles of the isolates, based on both unique and shared amplification products, were employed to calculate genetic distance (GD). GD was calculated according to the following equation (Link *et al.*, 1995) :

$$GDxy = (Nx + Ny) / Nx + Ny + Nxy)$$

when Nx is the number of bands in line x and not in line y, Ny is the number of bands in line Y and not in line x and Nxy is the number of bands in lines x and y. The data was subsequently used for cluster analysis to construct a dendrogram. All calculations were made using the TREECON programme (Van de Peer and De Wachter, 1994).

RESULTS AND DISCUSSION

The modified CTAB procedure for DNA isolation from *Hevea* leaf tissue, had been the method of choice as it was very rapid and yielded sufficiently pure DNA from fungal mycelia, which gave reproducible PCR amplification. The NaCl precipitation steps could remove the polysaccharides from the DNA sample and appeared very useful in isolating DNA from other fungi also (unpublished). RAPD analysis provided a rapid method to distinguish *Corynespora* isolates and to assess the variability existing among them. Of the 20 primers screened, amplifications of the fungal DNA were obtained with only 12 primers. Out of the 12 primers, seven primers which gave reproducible RAPD fragments (Table 2) were selected for the assay of all the 20 *Corynespora* isolates. Amplification of a total of 66 loci/band positions having 0.36 to 2.74 kb size were recorded with the above seven primers. Banding patterns generated were assessed through 'Gene Profiler' software (Scanalytics, USA). Polymorphisms were detected among the isolates collected from Kerala for each primer (Figs. 2A, 2B, 3A, 4A and 4B). However, the isolates from Karnataka showed uniform amplification profiles except with the primer OPA18 (Fig. 3B), which could detect polymorphisms among the Karnataka isolates as the presence of a 1.06 kb amplicon in eight isolates;

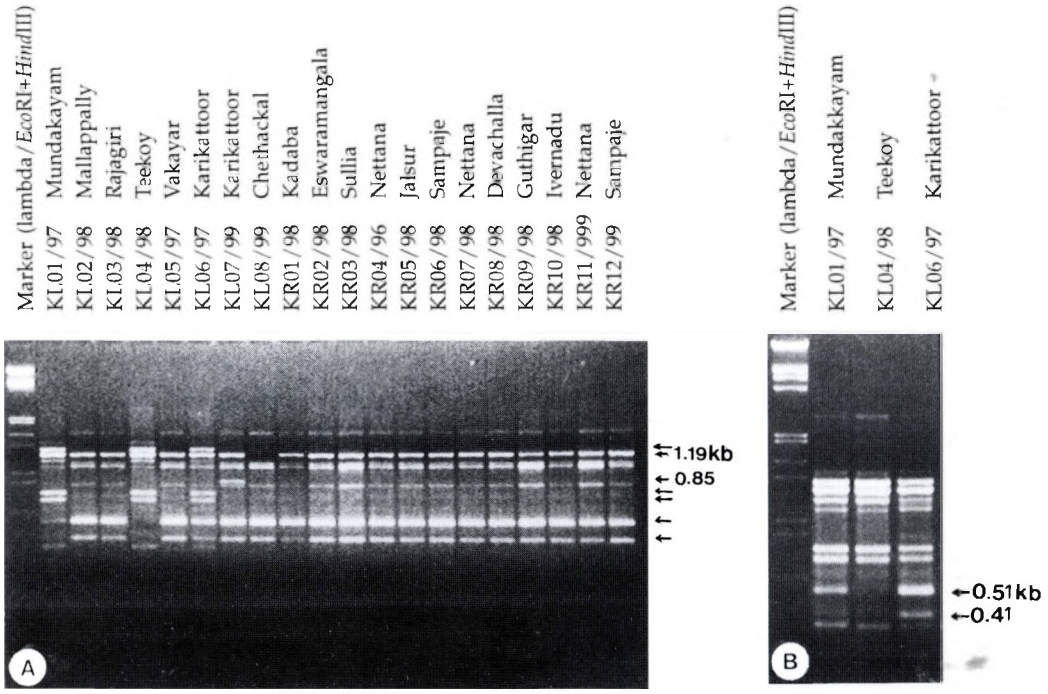

Fig. 2. RAPD profiles of *Corynespora* isolates generated by the primer OPA04; (A) : Six types of DNA fingerprints detected among the isolates. Five Kerala isolates, KL01/97, KL04/98, KL06/97, KL07/99, KL08/99, showed distinct patterns and could be identified individually; (B) : Reproducible amplification profile of the three Kerala isolates, KL01/97, KL04/98 and KL06/97, with OPA04 primer showing distinct isolate specific patterns. Discriminating polymorphic markers are indicated.

Table 2. **Details of decamer oligonucleotides used as primers in the RAPD assay**

Primer code	Nucleotide sequence (5′ to 3′)	G+C content (%)	No. of amplified fragments
OPA01	CAGGCCCTTC	70	8
OPA02	TGCCGAGCTG	70	9
OPA03	AGTCAGCCAC	60	13
OPA04	AATCGGGCTG	60	13
OPA10	GTGATCGCAG	60	5
OPA18	AGGTGACCGT	60	10
OPA20	GTTGCGATCC	60	8

KR02/98, KR04/96, KR05/98, KR07/98, KR08/98, KR10/98, KR11/99 and KR12/99; and absence of the same in the remaining four isolates; KR01/98, KR03/98, KR06/98 and KR09/98. Based on the amplification profile developed by the primer OPA04, Kerala isolates could be distinguished into six categories (Figs. 2A and 2B). No polymorphism could be detected among the Karnataka isolates with the same primer and the amplification profiles were very similar to that of three isolates from Kerala, KL02/98, KL03/98 and KL05/97. Three of the Kerala isolates, KL01/97, KL04/98 and

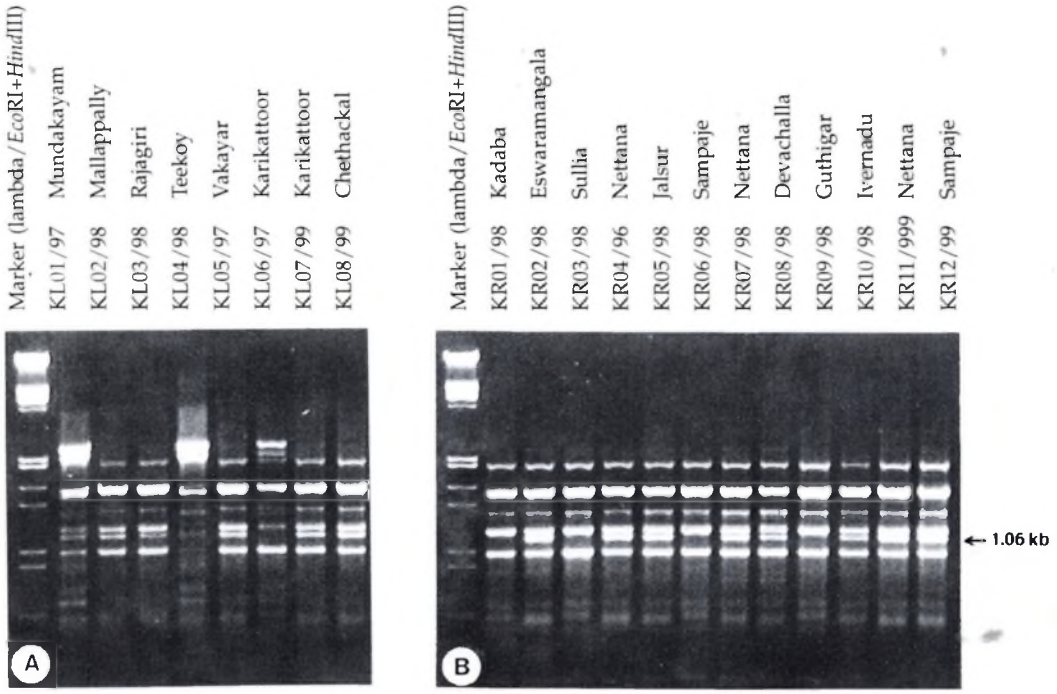

Fig. 3. RAPD profiles of *Corynespora* isolates generated by the primer OPA18; (A) : Extensive polymorphisms noticed in three Kerala isolates : KL01/97, KL04/98 and KL06/97; (B) : Amplification profile of 12 Karnataka isolates with the same primer (OPA18). The isolates are differentiated into two groups. Differentiating RAPD marker of 1.06 kb size is indicated.

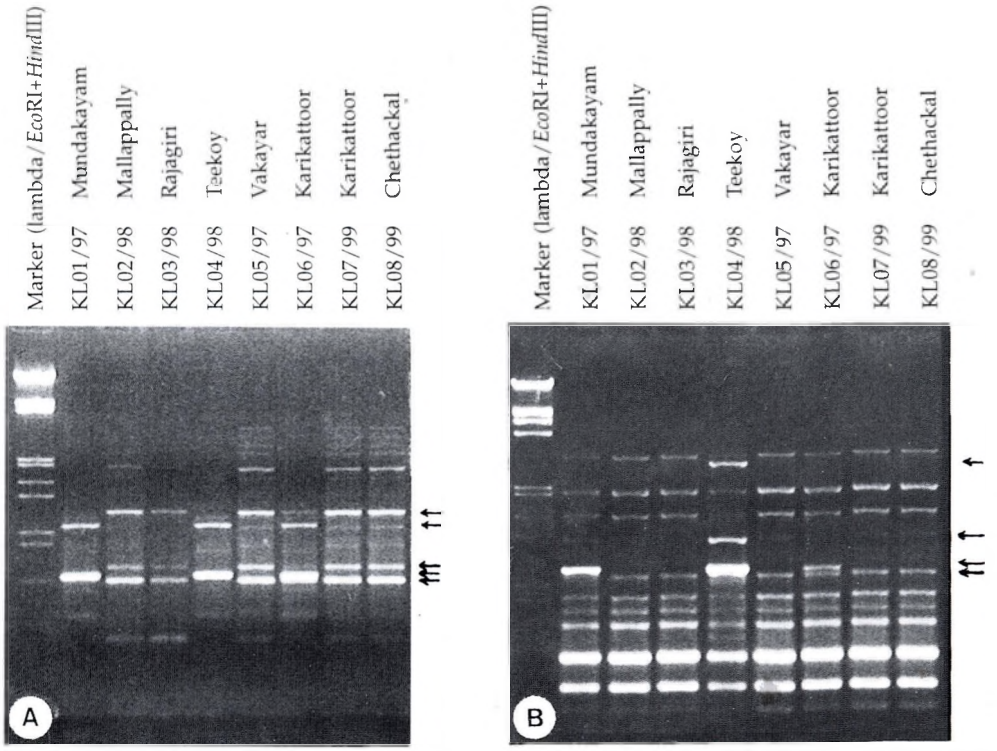

Fig. 4 : RAPD profiles of eight Kerala isolates generated by the primers OPA02 (A) and OPA03 (B). The three Kerala isolates are clearly discriminated. The isolates can be identified individually with OPA03 primer. Polymorphic bands are indicated.

KL06/97, always showed banding pattern distinct from the others (Figs. 2A, 3A, 3B, 4A and 4B) with all the primers tested and could be identified individually with the primers OPA03 (Fig. 4B), OPA04 (Fig. 2B) and OPA18 (Fig. 3A). Two Kerala isolates, KL07/99 and KL08/99, collected very recently from a seedling nursery at Karikattoor and wild *Hevea* germplasm at Chethackal respectively, could also be distinguished from all other isolates based on the amplification profile generated by OPA04 (Fig. 2A). Presence of an additional band of 0.85 kb size could easily identify KL07/99 from all other isolates, whereas the absence of a common band of 1.19 kb size had been marked as the characteristic feature of the isolate KL08/99. Ultimately, seven genotypes could be identified among the 20 isolates through RAPD profiling.

Genetic relationships among the isolates were analysed using genetic distance data based on pair-wise comparison of the RAPD profiles. A dendrogram (Fig. 5), predicting the genetic relationships among the 20 isolates analysed, was constructed by the UPGMA clustering method. Four distinct clusters were observed with a distance coefficient of 0.3. Cluster 1 included 17 isolates under 4 sub-clusters from Kerala and Karnataka, while the clusters 2, 3 and 4 contained single isolates from Kerala only. The dendrogram clearly illustrates the wide divergence among the Kerala isolates, specially with KL01/97, KL04/98 and KL06/97. Cluster 4 with a single isolate,

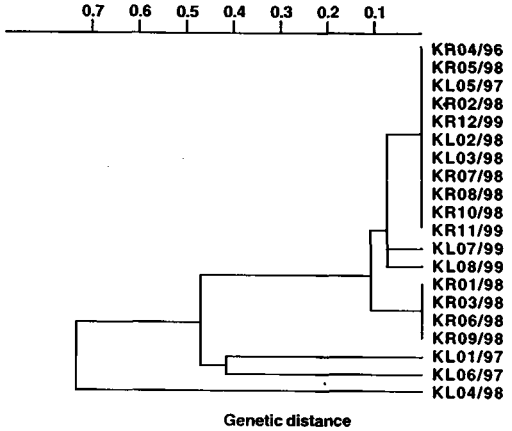

Fig. 5. Dendrogram showing genetic relationships among the *Corynespora* isolates collected from several locations of Kerala and Karnataka states.

KL04/98, showed maximum distance coefficient of 0.75 with the other three clusters.

Existence of different races of the pathogen could be evidenced by the genetic variability detected among the isolates from both the states. However, more number of races have been identified in Kerala compared to Karnataka. The reason may be attributed to the long history of rubber cultivation in Kerala compared to that in Karnataka, and genetic modification of the pathogen could have taken place over the years in old rubber plantations as the different host populations act as a powerful selection force on pathogen population and vice-versa (Burden, 1987). Existence of several pathotypes of *C. cassiicola* on rubber was reported from Sri Lanka (Silva *et al.*, 1998) and Indonesia (Darmono *et al.*, 1996), where the disease became epidemic. RAPD profiles proved to be useful in detecting polymorphisms among the *Corynespora* isolates.

Selection intensities on virulent and avirulent genotypes on susceptible hosts are likely to vary spatially and temporarily within and between years because plant pathogen populations are very sensitive to host genotypes and environmental variations. Variability among the two isolates, KL06/97 and KL07/99, collected in two different years, from the same location, Rubber Board Central Nursery, Karikattoor, Kerala state, could be attributed to the highly heterozygous nature of the seedling populations raised each year in the nursery. The greater uniformity observed among the three isolates, KR04/96, KR07/98 and KR11/99 collected in three consecutive years from an isolated region at Nettana of Karnataka, might be due to the same host genotype (Table 1). Three isolates from Kerala *viz.* KL01/97, KL04/98 and KL06/97 having very similar morphological features with other isolates, revealed high degree of genetic variability in the present RAPD studies. This proves the notion that taxonomic consideration, based solely on phenotype, may be subjected to ambiguities as it is induced by the environmental conditions. One of the significant observations is the detection of unique RAPD profile among the three isolates, KR01/98, KR03/98 and KR06/98 (Fig. 3B) from Kadaba, Sullia and Sampaje in Karnataka respectively, where the disease incidence was very high during 1998, based on a visual grading (Table 1). Similar RAPD profile with low disease incidence was observed for the isolate KR09/98 from the clone RRIM 600, a relatively less susceptible host compared to RRII 105. These observations could lead to a marker assisted screening of virulent races of *Corynespora* on rubber. However, there might be limitations to this method as isolates KR02/98 and KR08/98 collected from severely infected areas could not be differentiated with

the primers used. Since effective disease management requires information about the variations in genetic constitution and virulence of the pathogen *vis-a-vis* host genotype, further studies are required on pathogenic diversity.

In conclusion, the present study revealed for the first time, the existence of seven different genotypes of C. *cassiicola* associated with rubber, in India. Specific RAPD profile/fingerprints generated for each individual genotype could effectively be used in rapid screening of the pathogen. Putative virulence specific RAPD profile in *Corynespora* was identified which needs further confirmation through pathogenesis study of the isolates.

ACKNOWLEDGEMENTS

The authors wish to thank the World Bank for financial assistance, Dr. M.R. Sethuraj, former Director, RRII, for guidance to set up the Genome Analysis Laboratory and Dr. N.M. Mathew, Director, RRII for constant encouragement. The authors are also thankful to Mr. M.J. Manju and Mr. Sabu P. Idicula, Scientists, Plant Pathology Division, RRII, for collection of samples and Dr. Y.A. Varghese, Dy. Director (Germplasm) for her constructive suggestions.

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