27 Phylogenetic Relationships of *Colletotrichum* Species Infecting Rubber (*Hevea brasiliensis*) based on Nuclear Ribosomal DNA Spacer Sequences

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ABSTRACT

Colletotrichum leaf fall disease in rubber caused by the *Colletotrichum spp.* produces three different disease symptoms, namely, raised spots, anthracnose and circular papery lesions. Involvement of two different species of the pathogen namely *C. gloeosporioides* and *C. acutatum* could be established through rDNA-ITS-RFLP analysis of the fungal isolates from rubber. Ribosomal DNA spacer sequences from both the *Colletotrichum* species infecting rubber were compared to get an idea about the nucleotide divergence existing among them in the spacer regions including 5.8 S gene. Aligned sequence data of the spacer regions revealed the existence of more nucleotide divergence including base substitutions and indels in the ITS 1 compared to the ITS 2 region. Phylogeny of the rubber pathogen with the closely related fungal isolates from different hosts, based on the rDNA spacer sequences, clearly revealed their uniqueness. The bootstrapped consensus tree derived through neighbour-joining method comprised of two major branches having the fungal isolates belonging to two different species indicating clear species delineation of *Colletotrichum*. The fungal isolates belonging to *C. gloeosporioides* and *C. acutatum* infecting *Hevea* appeared to be closely related to the pathogens infecting fragaria and cyclamen respectively.

KEYWORDS : *Hevea brasiliensis*, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, ITS-RFLP, rDNA spacers, phylogenetic relationship.

The fungal genus *Colletotrichum* Corda includes several important plant pathogens that infect a wide range of both tropical and temperate crop plants (Jeffries *et al.*, 1990). These fungi cause diseases commonly known as anthracnose of grasses, legumes, vegetables, small fruits and perennial tree crops (Waller, 1992). Colletotrichum leaf disease is one of the major causes of declining yields of para rubber (*Hevea brasiliensis*) in the South East Asia. The Colletotrichum leaf disease in rubber was

reported first in the year 1906 from Ceylon and *Colletotrichum heveae* (Synonym: *Colletotrichum gloeosporioides*) was identified as the causative agent of this disease (Petch, 1906). Later the disease was reported from many parts of the world. Two different species of *Colletotrichum* have been reported on rubber causing three different disease symptoms: *C. gloeosporioides* (teleomorph: *Glomerella cingulata*) for anthracnose and papery lesions on mature rubber leaves, and *C. acutatum* for raised spot symptoms (Saha *et al.*, 2002). From India as well as Sri Lanka it has been reported that the main pathogen of Colletotrichum leaf disease of rubber is *C. acutatum* (Jayasinghe *et al.*, 1997; Kumar *et al.*, 2002; Saha *et al.*, 2002).

In several *Colletotrichum* species, conventional taxonomic characters such as conidial shape, size, appressorium morphology as well as pathogenicity vary widely (Sreenivasaprasad *et al.*, 1996). Morphologically indistinguishable *Colletotrichum* isolates have been assigned different species names based on their host origin. The existence of more than one species on a single host, like rubber, causing disease complexes, further complicates the situation. Sutton (1992) suggested that relationships within the genus *Colletotrichum* were unlikely to be resolved using morphology alone as morphological plasticity and overlapping phenotypes make traditional taxonomic criteria unreliable for accurate delineation of *Colletotrichum* species. Molecular techniques exploiting variations in the ribosomal DNA (rDNA) are being used extensively for systematic and phylogenetic studies in fungal pathogens (Sreenivasaprasad *et al.*, 1994; Johnston and Jones, 1997; Martin and Garcia-Figueres, 1999; Green *et al.*, 2004). Different regions of the rDNA diverged at different rates allowing the regions to be exploited at different taxonomic levels (Bruns *et al.*, 1991). The non-coding rDNA regions, especially the internal transcribed spacer regions (ITS 1 and ITS 2), are generally more variable than the rRNA genes and also be useful for understanding phylogenetic relationships at a sub-generic level. Use of rDNA restriction digest analysis and sequence data as molecular methods for species delineation in *Colletotrichum* have been reported (Sreenivasaprasad *et al.*, 1994; Sreenivasaprasad *et al.*, 1996; Saha *et al.*, 2002). The ITS sequence data offers potentially enough characters for phylogenetic reconstruction and one of the advantages of ITS is that it is flanked by regions that are highly conserved among genera and species making PCR analysis and sequencing straight forward.

In rubber, two species of *Colletotrichum* were identified through molecular analysis including RAPD markers and restriction fragment length polymorphism (RFLP) of ribosomal DNA spacers (Saha *et al.*, 2002). In the present study, we have revalidated the use of rDNA-ITS RFLPs for species delineation of *Colletotrichum* pathogens infecting *Hevea* and also determined the nucleotide sequences of ribosomal ITS regions including the 5.8 S ribosomal gene from both the species, *C. gloeosporioides* and *C. acutatum*, to evaluate divergence of the nucleotide sequences in this region between these two species. The sequence data were also used to infer the phylogeny of the *Colletotrichum* pathogens from rubber with the same species from other crops.

Materials and Methods

Colletotrichum Isolates and Genomic DNA Extraction

The isolates from three typical symptoms, raised spots, anthracnose and papery lesions on leaflets of different clones of *H. brasiliensis* were collected from rubber plantation of the Rubber Research Institute of India (RRII), Kottayam and other locations in Kerala (Table 1). Twenty-five isolates (14 from raised spots, 6 from anthracnose and 5 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 the dark. Fungal isolates were identified based on culture characteristics and microscopic morphology. Three mycelial plugs (5 mm diameter) were removed from the advancing margins of 5-day-old culture of each isolate, transferred to oat meal 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 vacuum filtering through Whatman No.1 filter paper, washed three times with sterile distilled water and were ground to powder in liquid nitrogen. Extraction and purification of the total genomic DNA were carried out following a modified CTAB (hexadecyl trimethyl ammonium bromide, Sigma Co., St. Louis, USA) method optimised for *Corynespora cassiicola* (Saha *et al.*, 2000).

PCR Amplification of rDNA-ITS Regions

Amplification of the internal transcribed spacer regions between the small (18S) and large (28S) nuclear rDNA was carried out using primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) synthesised at Sigma Genosys, UK. The amplified fragment includes the 5.8S rDNA gene and the internal transcribed spacers (ITS1 and ITS2). PCR amplification was performed in a total volume of 50 ml containing 100 ng of template DNA with 0.5 mM of each primer, 0.2 mM of each dNTP, 2 units of Taq DNA polymerase (Promega, USA), 5 ml of 10X 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 step at 94°C for 3 min, followed by 45 cycles of 30 sec at 94°C, 1 min at 55°C and 2 min at 72°C with a final extension at 72°C for 7 min. Amplified products were separated on a 1% agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide and viewed on a UV transilluminator.

Restriction Analysis of the Amplified rDNA-ITS Regions

PCR amplified rDNA products were purified and digested with the restriction enzymes *Acc* I, *Alu* I, *Sau* 3A I, *Eco*R I (Roche), *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.

Table 1 Source of *Colletotrichum* isolates

Isolate's code	Host genotype	Symptom	Location
CG1	BD 10	Raised spots	RRII
CG2	BD 10	Raised spots	RRII
CG3	GT 1	Raised spots	RRII
CG4	GT 1	Raised spots	RRII
CG5	RRII 208	Raised spots	RRII
CG6[1]	RRII 105	Raised spots	RRII
CG7	PB 280	Raised spots	RRII
CG8	PB 310	Raised spots	RRII
CG9	PB 217	Raised spots	RRII
CG10	PB 217	Raised spots	RRII
CG11	RRII 105	Raised spots	RRII
CG12	RRII 105	Raised spots	Cheruvally Estate
CG13	RRII 105	Raised spots	Cheruvally Estate
CG14	RRII 105	Raised spots	Manickal Estate
CG15	Seedling	Anthracnose	RRII
CG16	Seedling	Anthracnose	RRII
CG17	Seedling	Anthracnose	RRII
CG18[2]	Seedling	Anthracnose	RRII
CG19	PR 255	Papery lesions	RRII
CG20[3]	RRII 300	Papery lesions	RRII
CG21	RRII 300	Papery lesions	RRII
CG22	PB 235	Papery lesions	RRII
CG23	Seedling	Anthracnose	Kaliyar Estate
CG24	PB 260	Anthracnose	Manickal Estate
CG25	RRII 105	Papery lesions	Manickal Estate

[1] Culture preserved as IMI 383015

[2] Culture preserved as IMI 383016

[3] Culture preserved as IMI 383017

Cloning and sequencing of rDNA-ITS regions

The amplified rDNA from both the species of *Colletotrichum*, based on their restriction profiles, were purified using GFX column and ligated into pGEM-T vector. The ligated products were subsequently used for transformation of *E. coli* cells (JM 109). Nucleotide sequences of the cloned PCR products from each of the species were determined by sequencing reaction using a BigDye Terminator Cycle Sequencing kit and the products were run with an Applied Biosystems ABI 3700 Sequencer at the Microsynth GmbH, Switzerland. Sequencing reactions were primed on both strands using either the T7 or SP6 promoter sequences of the pGEM-T vector.

Sequence Analysis

Nucleotide sequences obtained in this study were compared with the rDNA-ITS sequences of several *Colletotrichum* isolates belonging to two different species *C. gloeosporioides* and *C. acutatum* infecting different host plants retrieved from the NCBI GenBank Nucleotide Sequence Database (Table 2) (http://www.ncbi.nlm.nih.gov). Initially, the sequences from the rubber pathogens were subjected to homology search with the nucleotide sequences in the GenBank through BLASTN programme available at the web server http://www.ncbi.nlm.nih.gov. The sequences belonging to *C. gloeosporioides* and *C. acutatum* from other crops that made significant alignments with the rubber pathogens were selected and retrieved for further analysis. For each *Colletotrichum* species, sequences were selected from ten different hosts ignoring the sequences from other *Colletotrichum* species although they showed significant homology with the rubber pathogen. For sequence comparison, *Colletotrichum* ITS sequences including 5.8 S rDNA regions were analysed after trimming the original sequences for only ITS and 5.8 S rDNA regions. Sequence alignment was done using the CLUSTALW (Thompson *et. al.*, 1994) alignment programme available on the web-server http://www.ebi.ac.uk/clustalw. Clustal alignments were further used for inferring phylogenetic relationships among the *Colletotrichum* isolates. The neighbour-joining analyses were performed using Treecon 1.15 (Van de Peer and De Wachter, 1994). Distances were calculated under the Kimura two-parameter with insertions separately accounted for. All bootstrap analyses were performed with 1000 replications. The bootstrap replicate trees were re-rooted using the sequences from *C. gloeosporioides* infecting lentil (AF451905) as the outgroup before the bootstrap consensus was computed.

Table 2 Ribosomal DNA spacer sequences (ITS 1-5.8S-ITS2) of the *Colletotrichum* spp. retrieved from the database for the study.

Colletotrichum gloeosporioides		*Colletotrichum acutatum*	
GenBank accession number	Host plant	GenBank accession number	Host plant
AF 488777*	Rubber	AF 488778*	Rubber
AF 090855	Olive	AF 207794	Almond
AF 207792	Almond	AF 411765	Rhododendron
AF 411769	Rhododendron	AJ 301911	Vaccinium
AF451905	Lentil	AJ 301915	Primula
AJ 301907	Hypericum	AJ 301921	Capsicum
AJ 301909	Mango	AJ 301922	Anemone
AJ 301919	Fragaria	AJ 301924	Coffee
AJ 301977	Palm	AJ 301950	Fragaria
AJ 301988	Citrus	AJ 301964	Lupinus
AJ 311884	Yam	AJ 301982	Cyclamen

* Sequence data generated in this study and registered with the GenBank.

Results and Discussion

Ribosomal DNA-ITS RFLPs

The entire ITS region including the 5.8 S ribosomal RNA gene (Fig. 1a), were amplified using the universal primers ITS 1 and ITS 4 (White *et. al.*, 1990). The amplified product was approximately 0.6 kb (Fig. 1b) and there was no detectable length polymorphism among the *Colletotrichum* isolates from rubber on agarose gel as described earlier (Saha *et. al.*, 2002). Amplified rDNA-ITS fragments were subjected to digestion with four different restriction endonucleases (*Alu* I, *Sau* 3A I, *EcoR* I and *Taq* I) to identify rDNA-ITS groups through fragment length polymorphisms, which yielded two specific restriction profiles (Fig. 2a-c). Restriction patterns of the isolates causing raised spots were different from the other two populations originating from anthracnose and papery lesions indicating the involvement of two different species as reported earlier (Saha *et. al.*, 2002).

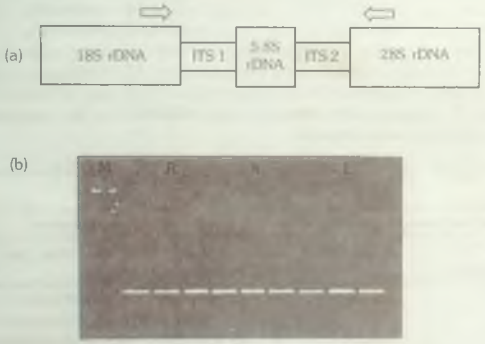

Figure 1 (a) Schematic diagram of repeat unit of nuclear ribosomal DNA showing the internal transcribed spacer regions ITS 1 and ITS 2. Arrowhead indicates the primers (ITS 1/ ITS 4) used for PCR amplification of the spacer regions along with 5.8 S rDNA.

(b) Representative gel photograph showing the amplification of ITS region including 5.8 S rDNA (~0.6 kb) of *Colletotrichum* isolates causing raised spots (R), anthracnose (A) and papery lesion (L) symptoms on *Hevea*. Ribosomal DNA-ITS length polymorphisms could not be detected among the isolates. Lane M: molecular weight marker (I-DNA marker/*Eco* RI + *Hin*d III).

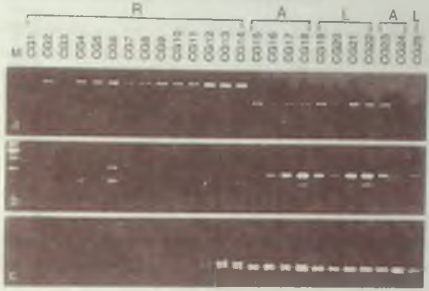

Figure 2 Representative gel photograph of the amplified rDNA-ITS fragments subjected to digestion with three different restriction endonucleases: *Alu* I (a), *Sau* 3A I (b) and *Taq* I (c) to identify rDNA-ITS groups through fragment length polymorphisms. Restriction patterns clearly showed that the isolates causing raised spots (R) were different from the other isolates causing anthracnose (A) and papery lesions (L). The isolates (CG1 to CG25) are indicated in the corresponding lanes. Lane M: molecular weight marker: 100 bp ladder (Promega).

Inter-specific variability in rDNA-ITS sequences of *Colletotrichum* species from rubber

Two rDNA spacer sequences derived from *C. gloeosporioides* and *C. acutatum* infecting *Hevea* were aligned to identify the degree of nucleotide variations in the ITS regions including 5.8 S rDNA (Fig. 3). These two species showed substantial nucleotide divergence in both the spacer regions with a lesser degree of variation in the 5.8 S rDNA as expected. The length of the spacer regions (ITS 1 and ITS 2) including 5.8 S was found to be 481 and 490 bp in *C. gloeosporioides* and *C. acutatum* respectively. ITS 1 of *C. acutatum* contained 185 bp whereas *C. gloeosporioides* had 171 bp. The number of base pairs in ITS 2 (152bp) and 5.8 S (158 bp) regions were found to be the same in both the species. However, sequence alignment of rDNA spacers from both the species resulted in 494 bases/characters as several gaps were introduced indicating the possible insertions/deletions (indels) (Fig. 3). A total 47 base substitutions (transitions and transversions) along with 7 indels ranging from 1 to 5 bases were noticed in the ITS regions including 5.8 S. Out of 47 base substitutions, 29 and 15 base substitutions were noticed in ITS 1 and ITS 2 regions respectively, whereas only 3 base substitutions were detected in the 5.8 S gene, which is highly conserved than the spacer regions. One of the general features noticed in the aligned sequences was the presence of more number of C↔T transitions compared to any other base substitution. All the seven indels were noticed in the spacer regions of which, 5 were concentrated in the ITS 1 region with a wide range of variation in the size of the indels.

Figure 3 Alignment of the rDNA-ITS sequences of *Colletotrichum gloeosporioides* and *C. acutatum* causing Colletotrichum leaf disease in rubber. Sequences were aligned by the CLUSTALW program. Shaded region of the sequence indicates 5.8 S gene. Asterix symbol (*) indicates the complete match whereas, dash (-) for the insertions/deletions.

Intra-specific variability based on rDNA-ITS sequences

Entire ribosomal spacer sequences of *C. acutatum* and *C. gloeosporioides* infecting different crop species were retrieved from the GenBank database and aligned with the respective *Colletotrichum* isolate from rubber to get an insight of the nucleotide variations existing within the species.

C. gloeosporioides

Spacer regions including 5.8 S rDNA of *C. gloeosporioides* infecting rhododendron, fragaria, almond, citrus, yam, mango, hypericum, lentil, olive and palm, retrieved from the database were aligned with the sequence belonging to *C. gloeosporioides* infecting rubber (Fig. 4). The multiple alignments of the sequences showed the nucleotide variability existing among the isolates from different hosts. Base substitutions along with small indels (1-2 bases) were identified in these sequences. Eight nucleotide substitutions and two indels were noticed in the ITS 1 region. Of the eight substitutions, the most prevalent one was the C↔T transition. Two indels noticed in this region was due to the additional bases present in the isolate infecting yam and lentil. In 5.8 S rDNA sequences, a single base polymorphism was noticed in the form of an indel among the isolates, which was due to an additional base present in the isolates infecting olive and lentil. Compared to the ITS 1 region, less number of polymorphic bases was noticed in the ITS 2 region, which was characterised by the presence of only four substitutions and one indel.

C. acutatum

The ten rDNA spacer sequences that produced significant alignment with the rubber isolates belonging to *C. acutatum* were from lupinus, almond, anemone, fragaria, capsicum, rhododendron, cyclamen, primula, vaccinium and coffee. In the aligned sequences of all isolates of the pathogen from different hosts, only two substitutions and one indel were found to exist in the ITS 1 region, whereas, ITS 2 appeared to be relatively more variable than the ITS 1 containing 7 nucleotide substitutions (Fig. 5) in their spacer regions. Only a single base substitution was noticed in the 5.8 S rDNA region and that was due to C↔T transition present in rubber pathogen, otherwise this region appeared to be highly conserved while considering the same gene sequences from the other ten *C. acutatum* isolates. Another unique substitution was noticed in the ITS 2 region of the rubber pathogen only. From the alignment result, it is evident that the isolates belonging to *C. acutatum* infecting different hosts are closely related compared to the *C. gloeosporioides* isolates.

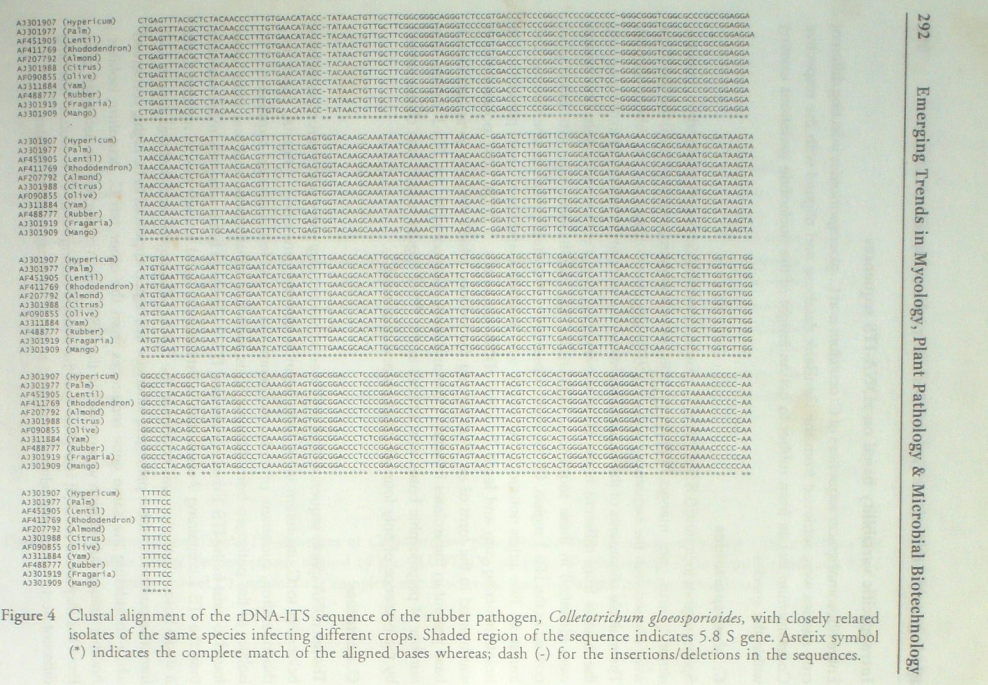

Figure 4 Clustal alignment of the rDNA-ITS sequence of the rubber pathogen, *Colletotrichum gloeosporioides*, with closely related isolates of the same species infecting different crops. Shaded region of the sequence indicates 5.8 S gene. Asterix symbol (*) indicates the complete match of the aligned bases whereas; dash (-) for the insertions/deletions in the sequences.

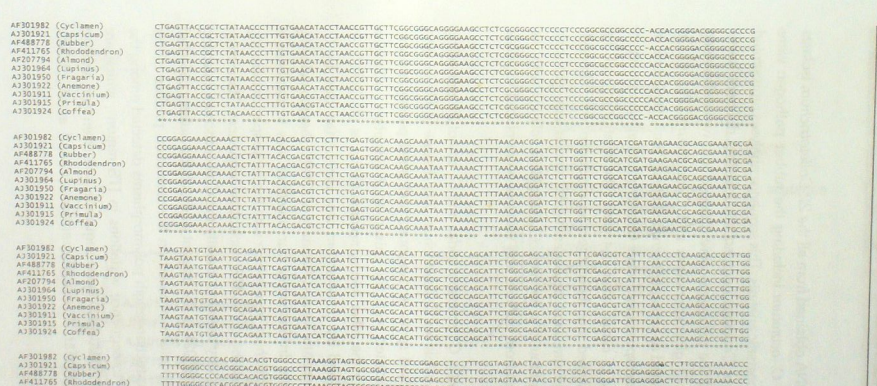

Figure 5 Clustal alignment of the rDNA-ITS sequences of the fungal isolates belonging to *Colletotrichum acutatum* infecting different crops including rubber. Shaded region of the sequence indicates 5.8 S gene. Asterix symbol (*) indicates the complete match of the aligned bases whereas; dash (-) for the insertions/deletions in the sequences.

Phylogenetic Analysis

ITS sequences along with the 5.8 S rDNA obtained from all the 22 *Colletotrichum* isolates belonging to *C. gloeosporioides* and *C. acutatum* including the rubber pathogen were aligned and the data was used for phylogenetic analysis. This sequence analyses represented a reliable insight into the phylogeny of *Colletotrichum* isolates infecting different hosts. The phylogram clearly showed that all the pathogens were grouped into two delineating the two *Colletotrichum* species viz. *C. gloeosporioides* and *C. acutatum* (Fig. 6). In *C. gloeosporioides*, each isolate appeared as separate branch showing the uniqueness of the pathogen. Phylogenetically, *C. gloeosporioides* isolate infecting rubber was closer to the isolate infecting fragaria than that of citrus, olive and yam. The relationship of the isolates belonging to *C. acutatum* revealed that those infecting rhododendron, almond, lupinus, anemone, fragaria and capsicum were phylogenetically similar and grouped together. The isolates from rubber were found to be distinct having close relation with the isolate infecting cyclamen. The isolates from primula and vaccinia were placed together depicting the closeness of these two.

Two species of *Colletotrichum* namely *C. gloeosporioides* and *C. acutatum* prevailing in Indian rubber plantations are responsible for Colletotrichum leaf disease in *Hevea* and they produce different symptoms: raised spots, anthracnose and small papery lesions. *C. acutatum* was found to be associated with the raised spot symptom, whereas *C. gloeosporioides* was responsible for the other two symptoms (Saha *et al.*, 2002). In rubber plantations, the damage caused by *C. acutatum* was more than that by *C. gloeosporioides* as the former infect immature leaves (Kumar *et al.*, 2002) thereby delaying the apical growth of the young plants. In this study, our objective was to establish a phylogeny of the two species of *Colletotrichum* from rubber with the same species infecting other crops based on the homology of the rDNA-ITS nucleotide sequences. Restriction analysis of the rDNA was found to be very useful to identify the *Colletotrichum* infecting rubber at the species level (Saha *et al.*, 2002). In the present study, all the isolates collected from rubber plants could be grouped into two based on the restriction profiles of the amplified rDNA spacers. The rDNA restriction length polymorphisms detected among the fungal isolates, were generated due to sequence variations in ITS regions, which are rapidly evolving regions of ribosomal DNA/rRNA gene sequence and often best studied for comparing species and closely related taxa (Sreenivasaprasad *et al.*, 1996; Freeman *et al.*, 2000).

Figure 6 Neighbour-joining tree of the fungal isolates belonging to the species *C. gloeosporioides* and *C. acutatum* from different hosts including rubber reveals the relationships of the *Colletotrichum* isolates under study. These isolates form two major groups based on their ribosomal spacer sequences (ITS 1 - 5.8 S - ITS 2) representing two *Colletotrichum* species. The uniqueness of the rubber pathogens is also evident from the tree. Bootstrap values above 50% are indicated for the corresponding branches.

Nucleotide sequence analysis of the ITS 1 region showed a greater degree of inter-specific divergence compared to ITS 2 in the form of base substitutions as well as insertion/deletion. Among the base substitutions, transitions were found to occur at higher frequencies than transversions as noticed in any other genome which is considered to be a general property of DNA sequence evolution (Wakeley, 1996; Yang and Yoder, 1999). At the intra-species level, considerably more nucleotide variations were detected in ITS 1 than in ITS 2 regions of *C. gloeosporioides*. Therefore in *C. gloeosporioides*, ITS 1 sequence was proved to be more useful for understanding the phylogeny as reported earlier (Sreenivasaprasad *et al.*, 1996; Freeman *et al.*, 2000 Abang *et al.*, 2002). In the case of *C. acutatum*, ITS 2 was found to be more variable containing more number of nucleotide substitutions compared to ITS 1. Sequence alignment of the entire ITS region including the 5.8 S gene sequences from both the species infecting several crops was used to infer phylogenetic relationship among them. The *Colletotrichum* isolates infecting different hosts including rubber could clearly be separated into two groups belonging to two different species in the phylogram, which is supported by a bootstrap value of 100% indicating a high reliability. The *Colletotrichum* isolates infecting rubber appeared to be different from that of the other hosts based on the ITS sequence divergence.

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