

Genetic diversity study of *Cercospora canescens* (Ellis & Martin) isolates, the pathogen of *Cercospora* leaf spot in legumes

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Genetic diversity was studied in eleven different isolates of *Cercospora canescens* (Ellis & Martin), the causative agent of *Cercospora* leaf spot in legumes. The isolates, which were obtained from different geographical locations, had different morphological and pigment production characteristics. The polymorphism at the molecular level was studied by random amplified polymorphic DNA (RAPD) marker technique and variation in the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). RAPD profiling clustered all the isolates into three clusters. Considerable genetic diversity was observed in the isolates from the same geographical location. rDNA analysis showed length variation in ITS of two isolates from mungbean, with one 600 bp band common to both. Restriction analysis could differentiate between the common 600 bp bands of the two isolates. The present study indicates that compared to restriction analysis of the ITS region, the RAPD technique is better suited for determining the genetic diversity and differentiation of *C. canescens* isolates.

Keywords: *Cercospora*, rDNA, ITS, legumes, RAPD.

CERCOSPORA canescens (Ellis & Martin) is the principal pathogen causing leaf spotting and defoliation in several legumes including mungbean (*Vigna radiata*) and black gram (*Vigna mungo*)¹, especially in humid tropical areas of southeast Asia². Losses caused by the disease are reported to be around 40% (ref. 3). The fungus belongs to the group of imperfect fungi or deuteromycetes, in the order Moniliales⁴. Most of the 3000 named species in the genus *Cercospora* have no known sexual stage. But a few species of *Cercospora* have been identified for which a sexually reproducing stage (telomorph) called *Mycosphaerella* has been identified⁵.

So far, the identification of *C. canescens* is based on fungal morphology. The pathological characteristics, important from a disease point of view, are not given due attention in the identification of the pathogen. The pathogen is reported to infect a large number of legume species. However, there are reports indicating the pathological speciali-

zation in *C. canescens* population^{6,7}. These findings could be validated by extending the studies to molecular characterization of pathogen population of different species of the *Vigna*. Molecular analysis of pathogen would ultimately help in developing new resistant plant type by making the interspecific crosses carrying resistant genes.

RAPD markers have been widely used for assessing genetic diversity, genome mapping and molecular diagnostics of many fungal species. The technique is simple, does not require any prior knowledge of DNA sequences and often yields a large number of discriminating markers⁸. Regions of ribosomal DNA (rDNA) also have been used in phylogenetic studies of fungal genomes^{9,5,10}. These regions are highly conserved and can easily be investigated using PCR amplification. Out of the various regions of rDNA, the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the nuclear rDNA repeat units have been reported to evolve fast and may vary among species within a genus or among populations¹¹ and hence can be used for phylogenetic studies at these taxonomic levels.

The present study was aimed to assess the genetic diversity of different isolates of *C. canescens* using RAPD and rDNA region variations. The details of morphological and cultural characteristics, geographical location and their host plant are given in Table 1. All the isolates included in this study were previously tested for their pathogenicity on their respective host¹². The pure cultures were maintained on potato dextrose agar (PDA) (HiMedia, laboratories Ltd. Bombay, India) at 21–23°C.

Fungal mycelia was cut from the PDA plate with a sterile knife, and used for inoculating 50 ml of Richard's broth (1% KNO₃, 0.5% KH₂PO₄, 0.25% MgSO₄, 0.002% FeCl₂, & 0.5% sucrose) in 250 ml conical flasks and were incubated with shaking (120 rpm) at 21–23°C for 7 days. Mycelia from 50 ml broth were harvested by filtration through 4 layers of sterile muslin cloth, blotted dry and immediately used for DNA extraction.

The mycelia were ground using a pre-chilled mortar and pestle, to a fine powder in liquid nitrogen, and DNA was isolated using a previously described method¹³. The DNA was quantified using spectrophotometric analysis, diluted to a final concentration of 25 ng/μl and used in polymerase chain reactions (PCR).

RAPD amplification was done using decamer primers obtained from Operon Technologies, Inc. Alameda, CA, USA. Amplification was performed in a 25 μl reaction volume containing, *Taq* polymerase assay buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl₂ and 0.01% gelatin), 0.2 mM of each dNTP, 0.5 units of *Taq* polymerase (Bangalore Genei Pvt Ltd, Bangalore, India), 0.2 μM of random primer and 50 ng of DNA. Amplification was performed using Eppendorf Master Cycler gradient (Eppendorf Netheler-Hinz GMBH, Hamburg, Germany), programmed for initial denaturation at 94°C for 2 min and 45 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. The amplification was completed with a 5 min final

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Table 1. Isolates of *Cercospora canescens* used in the present study

Isolates	Host (ICMP No.)	Place of collection	Characteristics
CC(NU)*	<i>Vigna radiata</i>	Nagpur	Non pigmented, greenish-black mycelia
B4-96	<i>Vigna radiata</i>	Varanasi	Non pigmented, white mycelia
B6-20	<i>Vigna radiata</i>	Varanasi	Red pigmented, greenish brown mycelia
B2-95	<i>Vigna radiata</i>	Varanasi	Non pigmented, white mycelia
B5-97	<i>Vigna radiata</i> (ICMP 13648)	Gujarat	Red pigmented, reddish mycelia
B3-96	<i>Vigna radiata</i>	Varanasi	Red pigmented, white mycelia
B8-97	<i>Vigna sylvestris</i> (ICMP 13854)	Varanasi	Red pigmented, white mycelia
B10-97	<i>Vigna mungo</i> (ICMP 13656)	Varanasi	Black pigmented, white mycelia
B9-96	<i>Vigna unguiculata</i>	Varanasi	Red pigmented, white mycelia
B7-97	<i>Lablab niger</i> (ICMP 13855)	Varanasi	Black pigmented, white mycelia
B12-98	F ₁ (Mung × black gram)	Varanasi	Red pigmented, white mycelia

*Isolate designation given by authors. Other isolate numbers are as maintained at the Department of Mycology and Plant Pathology, BHU, Varanasi, India.

ICMP: International culture collection centers of plant microbes, Auckland, New Zealand.

extension at 72°C. Amplified products were resolved in 1.5% agarose gel electrophoretically at 75 V, using 1X TBE buffer. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light¹⁴. Lambda DNA/*Hind*III digest served as the standard molecular weight marker (Bangalore Genei Pvt Ltd, Bangalore, India).

Internal transcribed spacer (ITS) region was amplified using the universal primers previously described¹¹. The primers were synthesized by Board of Radiation and Isotope Technology, Mumbai, India. The forward and reverse primers, viz. ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTTATTGATATG 3') were based on conserved 18s and 28s coding regions of the nuclear rDNA¹¹. The amplification was performed in 30 µl reaction volume as described earlier, with 0.1 mM of each dNTP and 0.5 µM of both forward and reverse primer. Eppendorf Master Cycler gradient was programmed for initial denaturation at 94°C for 4 min, and 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, the amplification was completed with a final extension at 72°C for 10 min. Electrophoresis and visualization of amplified bands was done as described above. A 100 bp DNA ladder served as the standard molecular weight marker.

The restriction enzyme digestion analyses were performed using 15 µl of the amplified PCR product. The following enzymes were used: *Taq*I, *Sau*3A, *Hae*III, *Alu*I, *Eco*RI, *Sma*I, *Bam*HI, *Hind*III, *Pst*I, and *Sac*I, as per the manufacturers' specifications. (Bangalore Genei). The restriction fragments were size separated by electrophoresis on 2.0% agarose gel and visualized as described above.

Only clear and reproducible bands were scored. The polymorphic RAPD markers were scored as binary digit code of 0 and 1-character states for the absence and presence of polymorphic RAPD band, respectively, each of which was treated as an independent character regardless of the fluorescence intensity. Data were used for similarity-based analysis using the programme NTSYS-PC (version 2.02)¹⁵. The SIMQUAL programme was used for calculating the similarity index. Similarity coefficients were

used for construction of UPGMA (Unweighted Pair Group Method with Arithmetic average) dendrogram¹⁵.

A total of 33 random primers were tested. Polymorphic bands were obtained with all the 33 primers used for amplification. The average numbers of polymorphic bands observed per primer were 4.70. The number of bands generated by each primer that produced a polymorphic banding pattern varied from 1 (OPK1, 3, 5, 6, 8, 12-15, OPD 3, 4, 16, 18, OPL 1, 20) to 10 (OPK3, 7, 11, OPD10, 20, OPN4). On an average, the approximate product size ranged from 2.5 kb to 500 base pairs. One representative RAPD profile using RAPD primers OPL19 and OPL20 is shown in Figure 1. A dendrogram based on UPGMA analysis indicated that the 11 isolates formed 3 major clusters A, B and C. (Figure 2). The similarity coefficients ranged from 0.55 to 0.88, indicating that no two isolates were 100% similar. Cluster A was further subdivided into 2 sub clusters which separated the isolate obtained from Nagpur from

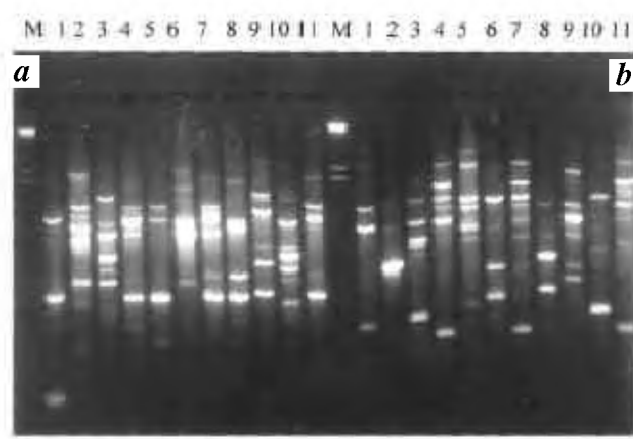


Figure 1. Random amplified DNA polymorphisms of *Cercospora canescens* isolates with random primers (a) OPL19 and (b) OPL20. Lanes 1–11 represent isolates CC(NU), B4-96, B6-20, B8-97, B2-95, B5-97, B9-96, B7-97, B12-98, B3-96, B10-97. M indicates the molecular weight marker lambda DNA digested with *Hind*III.

other 4 isolates (B8-97, B9-96, B2-95, and B10-97) obtained from Varanasi (Table 1). Out of these 4 isolates, 2 (B8-97 and B9-96) were similar with 88% similarity, although they were isolated from *V. sylvestris* and *V. unguiculata* (cowpea) respectively. These two isolates of *V. sylvestris* and cowpea also showed high similarity with the isolates of *V. mungo* (blackgram) (B10-97) and *V. radiata* (mungbean) (B2-95) (similarity coefficient 0.80 and 0.81 respectively). The isolates of mungbean (B2-95) and blackgram (B10-97), which were highly similar to isolates of *V. sylvestris* and cowpea, also were similar to each other with similarity coefficient of 0.75. Cluster B contained 3 isolates of which isolate B5-97 was from Gujarat and other 2 from Varanasi. These three isolates were from different hosts (Table 1). Cluster C contained 3 isolates, all of which were isolated from Varanasi and were from mungbean.

Although the isolates from different hosts obtained from the same geographical location showed considerable similarity, the isolates that were from mungbean (NU, B2-95, B5-97, B4-96, B6-20 and B3-96) showed high degree of genetic variation as they were distributed in all the three clusters. Similar genetic diversity studies have shown that local populations of plant pathogenic fungi are generally diverse, but may be dominated by one or few genotypes¹⁶⁻¹⁸. In a recent study a very high genetic variability was observed among isolates of *Colletotrichum graminicola* and among variants for a single lesion isolate in RAPD profile suggesting them to be hypervariable with distinct genetic variations¹⁹. Several factors were described earlier to explain the extent of genetic diversity. Factors like Founder effect, or random genetic drift followed by selection, can lead to reduction in diversity^{20,21}. Conversely, increase in genetic diversity can be explained on the basis of immigration from the environment. Selection over time

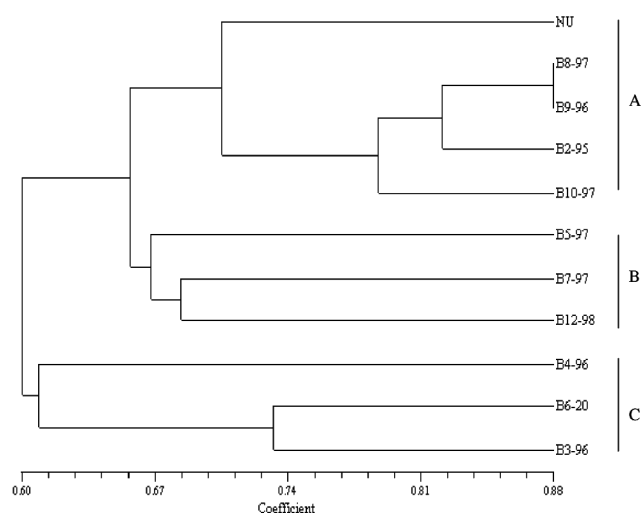


Figure 2. Dendrogram showing relationship among *C. canescens* isolates based on RAPD.

might either increase certain genotypes or decrease their presence. Host/pathogen co-evolution may contribute to the maintenance of genetic variability¹⁷.

The consensus primers ITS1 and ITS4 were used to amplify a region of the rRNA gene repeat unit, which includes two non-coding regions designated as ITS1 and ITS2 and the 5.8s rRNA gene. All the isolates amplified a single band of about 550 bp, except for the two isolates from mungbean B4-96 and B6-20. These two isolates showed a length variation in this region, in which isolate B4-96 showed a single band of higher molecular weight of around 600 bp, whereas isolate B6-20 showed two bands: one of 550 bp and other of 600 bp (Figure 3). Similar length variation in the ITS region has been observed for yeast strains belonging to different species⁹. The ITS region was digested with 10 different tetra (*TaqI*, *Sau3A*, *HaeIII*, *AluI*), and hexa (*EcoRI*, *SmaI*, *BamHI*, *HindIII*, *PstI*, *SacI*) base pair cutter restriction endonucleases. Of these 10 different enzymes tested, 5 had restriction sites on ITS region, namely *TaqI*, *Sau3A*, *HaeIII*, *AluI* and *EcoRI*. The five enzymes which had restriction sites in the ITS region revealed polymorphism in two isolates of mungbean B4-96 and B6-20, collected from Varanasi (data not shown). With the enzyme *AluI*, isolate B4-96 showed two digestion products of around 275 bp and 300 bp, but these two digested products were not present in isolate B6-20 (Figure 4b), although this isolate also has the 600 bp ITS region as isolate B4-96 (Figure 3). Difference in banding pattern was also observed when the ITS region of isolate B4-96 and B6-20 was digested with *HaeIII* (Figure 4a) with no common digestion products from the 600 bp ITS region which is present in both (Figure 3). These results indicate that the two 600 bp ITS region observed in B4-96 and B6-20 are different from each other.

AluI could also detect variation in the restriction site of another isolate of mungbean from Varanasi-B3-96, as one digested product of size around 400 bp was missing in this isolate (Figure 4b). The dendrogram (Figure 5) constructed based on similarity coefficients also indicates that the two isolates B4-96 and B6-20, which showed different banding patterns in ITS amplification and restriction digestion



Figure 3. Internal transcribed spacer region (ITS) of 11 isolates. Lanes 1–11 represent isolates CC(NU), B4-96, B6-20, B8-97, B2-95, B5-97, B7-97, B12-98, B3-96, B10-97. M indicates the molecular weight marker 100 bp ladder.

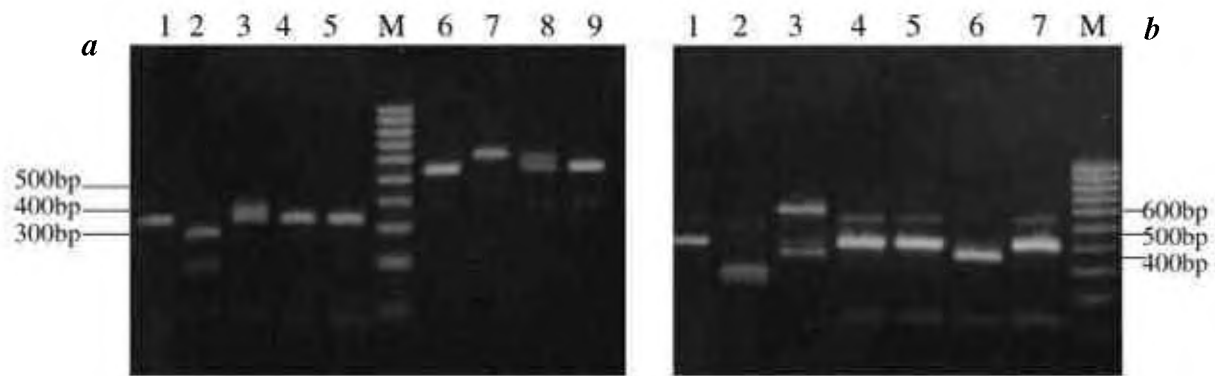


Figure 4. *a*, Restriction analysis of ITS region with *Hae*III. Lanes 1–5: Digested products of ITS region from isolates CC(NU), B4-96, B6-20, B3-96, B10-97. Lanes 6–9: Controls: undigested ITS region from isolates CC(NU), B4-96, B6-20, B3-96. M indicates the molecular weight marker 100 bp ladder; *b*, Restriction analysis of ITS region with *Alu*I. Lanes 1–7: Digested products of ITS region from isolates CC(NU), B4-96, B6-20, B8-97, B2-95, B3-96, B10-97. M represents molecular weight marker 100 bp ladder.

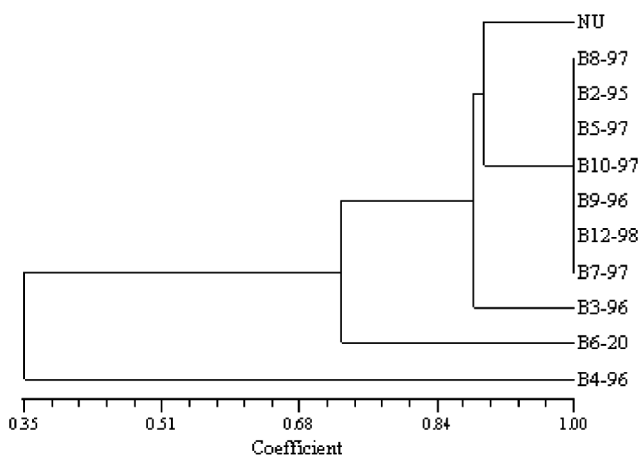


Figure 5. Dendrogram showing relationship among *C. canescens* isolates based on ITS restriction analysis data.

Patterns, are dissimilar from each other (similarity coefficient 0.31), although both of them are from the same host plant and same geographical location. A similar study on ectomycorrhizal fungi in Fennoscandia has shown intra-specific polymorphism in seven species. The polymorphisms were found to be due to length mutations, ranging from 5 to 15 bp in four of the seven polymorphic species and mutation in endonuclease restriction sites in six species²². Comparisons of ITS region analysis and RAPD profiling indicate that in the present study ITS amplification and restriction digestion of the amplified products were not as sensitive as RAPD, to distinguish between the 7 isolates (B8-97, B2-95, B5-97, B10-97, B9-96, B12-98, and B7-97) which could be divided into two clusters A and B using RAPD (Figure 2). This indicates that RAPD markers were well suited for determining the genetic diversity and differentiation present in *C. canescens* isolates.

The present study indicates a high degree of genetic diversity existing between the isolates of mungbean from

different geographical locations. This finding is significant in breeding work, as in order to test for varieties resistant to *Cercospora* leaf spot they need to be tested against different isolates prevalent in that particular region. Though there are reports on host-specific specialization (*formae speciales*) of *C. canescens* isolates from *V. mungo*⁶ and *V. radiata*⁷, the present study showed considerable genetic diversity at the molecular level among the isolates from the same host for the first time. Such genetic heterogeneity previously has been observed for other fungi like *Ascochyta rabiei*¹⁷ and *Rhynchosporium secalis*²³. In case of *A. rabiei*, population sampled from a single chickpea field contained a large amount of subtle genetic variation, with more than one *A. rabiei* haplotype being present on single host plant even within single lesion. At the same time, in the present study, similarity between isolates of different hosts from the same geographical location was observed. *C. canescens* is an aggregate species comprising many specific forms, infective to different species of *Vigna*, *Phaseolus* and others, both under natural and artificial inoculation conditions. Existence of *forma specialis* in *C. canescens* has clearly been demonstrated⁷. A recent study of different *Cercospora* species based on sequence of ITS regions has shown a close relationship among the species within the *Cercospora* cluster. It has been proposed based on the same studies that all *Cercospora* species share a common ancestor that acquired the ability to produce a phytotoxic metabolite called cercosporin. The ability to produce cercosporin allowed the ancestral *Cercospora* species to expand its host range. This would explain the occurrence of a large number of closely related species, some with identical ITS sequences, on widely divergent hosts⁵. Similar studies on *formae speciales* of *Fusarium oxysporum* have been done based on mitochondrial DNA RFLP, and the studies have indicated that in some cases isolates of different *formae speciales* were genetically more similar than isolates of same *forma specialis*²⁴. It has been hypothesized based on the same studies that the genetic

differences between the *formae speciales* were relatively small and the determinants for the host specificity could be combined or lost in individual strains.

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Cytochalasin B and taxol modulate cell surface ultrastructure in hydra

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Direct physical contacts between neighbouring cells in embryos, tissues and organs are often governed by changes in the cell surface architecture. Cytoskeleton is one of the cell organelles that regulate cell surface architecture. We have studied the role of microfilaments and microtubules in maintenance of cell surface architecture in diploblastic hydra by using drugs that specifically interact with individual cytoskeletal components. Adult hydra were exposed to 10 µM concentration of either the microfilament-disrupting agent cytochalasin B or the microtubule-stabilizing drug taxol for 1 h and cell surfaces were examined by scanning electron microscopy. It was found that changes in microfilaments and microtubules alter the cell surface in hydra although the effects of the two are quite different. The present results suggest the possibility that func-

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