



Research article

## Morphological and molecular characterization of *Colletotrichum capsici* causing leaf-spot of soybean

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**Abstract:** In the present investigation an attempt was made to carry out morphological and molecular characterization of *Colletotrichum* isolate (CI) associated with leaf spot disease of soybean. Pathogenicity test was carried out with the isolate and symptoms of leaf spot were found to appear after 7 days of inoculation. The pathogen was compared with 2 isolates of *Colletotrichum capsici* (CII and CIII) and one isolate of *Colletotrichum gloeosporioides* (CIV) from chilli. On the basis of morphological and cultural characteristics the pathogen was identified as *C. capsici*. For molecular identification random amplified polymorphic DNA (RAPD) primers and internal transcribed spacer (ITS2) primers were tested on the genome of *Colletotrichum* isolates from soybean and chilli plants and the data provided characteristic genetic evidence of 100% similarity within the CI isolate from soybean and *C. capsici* isolates from chilli (CII and CIII). To confirm the molecular identification of the pathogen species specific primers (CcapF/CcapR) were used and it was established that the pathogen is indeed *C. capsici*. Results of present investigation revealed the first report of *C. capsici* in soybean plant.

**Keywords:** Soybean - leaf-spot - *Colletotrichum* - RAPD - ITS.

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### INTRODUCTION

The genus *Colletotrichum* contains many morphologically similar taxa comprising endophytic, saprobic and plant pathogenic fungi (Photita *et al.* 2004, Damm *et al.* 2009). Anthracnose disease caused by the *Colletotrichum* species is a major problem worldwide. Among these species, *C. gloeosporioides* (Penz.) Penz. & Sacc. and *C. capsici* (Syd.) E.J. Butler & Bisby are most frequently cited as causal agents of anthracnose (Than *et al.* 2008, Gautam 2014, Ramdial & Rampersad 2015). *C. capsici* also has been reported to have a wide putative host range associated with symptoms of foliar blight and leaf spot diseases (Shenoy *et al.* 2007). Leaf spot disease caused by *Colletotrichum capsici* is the most important economic constraint which hamper, turmeric (*Curcuma longa* L.) production in major turmeric growing regions of the India, and often results in high yield losses (Adhipathi 2013, Uma Devi 2008).

The occurrence of different virulent strains of *Colletotrichum* species has been well documented in India (Sharma *et al.* 2005, 2013). Numerous cases have been reported in which several *Colletotrichum* species or biotypes are associated with a single host (Peres *et al.* 2002) making their identification by morphological and physiological methods more difficult. The use of molecular marker techniques has improved the accuracy and speed of identification of *Colletotrichum* spp. (Cai *et al.* 2009). Among these molecular techniques, RAPD technique has been extensively used to investigate relationships among isolates of *Colletotrichum* spp. (Madhavan *et al.* 2010, Sangdee *et al.* 2011). Similarly nucleotide sequence information for the 5.8S rDNA gene and the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) have been used to design species specific primers of members of *Colletotrichum* for diagnostic purposes and for phylogenetic analysis (Thalhinhas *et al.* 2002).

Soybean is an important global crop that is grown in tropical, subtropical and temperate climate. Anthracnose is a major fungal disease of soybean which is caused by *Colletotrichum capsici* and several related

species. Anthracnose of soybean can cause severe symptoms and yield loss in southern Taiwan. Strains of *C. truncatum* and *C. gloeosporioides* derived from diseased pods were noted as causal agents (Chen *et al.* 1986). Purkayastha & Banerjee (1990) reported anthracnose of soybean in India caused by *C. dematium*. Soybean foliage is also susceptible to a number of fungal and bacterial pathogens causing leaf spots and blights. Among leaf spot fungal diseases, brown spot disease caused by *Septoria glycines* and frog-eye disease caused by *Cercospora sojina* are most frequently encountered (Hershman 2013).

In the present investigation an attempt was made to carry out morphological and molecular characterization of *Colletotrichum* isolate associated with leaf spot disease of soybean plants.

## MATERIALS AND METHODS

### *Isolation and identification of pathogens from infected plants*

Fungal isolate (CI) was collected from leaves of infected soybean plants grown in the field of experimental garden. The diseased leaf parts were cut at the advanced margin of lesions into small pieces (5×5 mm) and then surface disinfected with 0.1% HgCl<sub>2</sub> for 1 min, followed by rinsing with sterile distilled water two times. The pieces were then transferred to potato dextrose agar (PDA) medium and incubated at 30±2°C for 2–3 days. Pure cultures of the fungi were obtained by single spore isolation method. The isolate was first identified to species by morphological observation under a compound microscope. Two other isolates of *C. capsici* (CII and CIII) and one isolate of *C. gloeosporioides* (CIV) from chilli were used for identification of *C. capsici*.

### *Pathogenicity test*

In order to prove Koch's postulate pathogenicity test was carried out. One month old soybean plants were sprayed with spore suspension of CI (10<sup>6</sup> conidia/ml) and covered with moist polythene bags for 48 hours under natural condition of light and temperature (20–30°C). Disease intensity was measured after 5, 7, 10, 14, 21 days after inoculation following the method of Purkayastha & Banerjee (1990). Disease symptom is necrotic spots (lesion) on the leaves of plant. Six leaves from each plant were selected randomly and the number and size of the lesions on each leaf were noted. On the basis of visual observation, the necrotic lesions were graded into small, medium and large groups and a value of 0.5, 1 and 2 were given to lesions denoting their diameter of 1, 2 and more than 2 mm respectively. The number of spots in each group was multiplied by value assigned to it. The sum total values taken together for all leaves divided by the number of test plants.

### *Cultural characters of Colletotrichum isolates*

Growth characteristics of fungal isolates were measured in potato dextrose agar (PDA) solid medium. The mycelial diameter and morphological nature of mycelia was recorded in solid medium. Shape and size of conidia of the fungal isolates were also observed.

### *Fungal DNA extraction and RAPD analysis*

For DNA extraction, each isolate was grown in 100 ml conical flasks, containing 30 ml of potato dextrose broth, for 5 days at room temperature (28±2°C). The mycelia were harvested by filtration and stored at -20°C for 2–3 days. Freeze-dried mycelium (0.5 g) was ground to a fine powder and DNA was extracted using CTAB (hexa-decyltrimethylammonium bromide) method described by Bhadra & Bandyopadhyay (2015). RAPD analysis was performed using 25 10-mer primers initially, among which 10 were chosen for final analysis due to high reproducibility of the banding pattern generated (Table 1). Amplification was performed in 25 µl reaction volume consisting of 100 ng template DNA, 100 pmole of primer, 10 millimole dNTPs (Applied Biosystems, Carlsbad, California, USA), 1.5 U *Taq* DNA polymerase and 10X reaction buffer containing 15 mM MgCl<sub>2</sub> (Merck Genei, Bangalore, India). PCR was performed using Veriti® 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, California, USA) with an initial denaturation step for 5 minutes at 94°C followed by 40 cycles of 1 minute at 94°C, 1 minute at 37°C and 2 minutes at 72°C with a final extension for 10 minutes at 72°C. 1.5 % (W/V) agarose gel was used to analyse the PCR product. Size of the amplified fragments was determined using 100bp DNA ladder (Applied Biosystems, Carlsbad, California, USA). The gel was photographed using E-Gel® Imager (Applied Biosystems, Carlsbad, California, USA).

The amplified bands thus obtained were scored as present (1) or absent (0) for data analysis. Regardless of the intensity of the amplified band, each was treated as an independent entity. The Rp (resolving power) of each primer was calculated using the formula  $Rp = 1/[2*(0.5-p)]$  (where p is the proportion of taxa containing the band) (Prevost & Wilkinson 1999). The scored binary matrix was then subjected to analysis using NTSYSpc

software, version 2.02i (Rohlf 1999). Jaccard's similarity co-efficient was calculated using SIMQUAL program using the formula  $N_{AB} / (N_{AB} + N_A + N_B)$  where  $N_{AB}$  is the number of amplified fragments shared by samples,  $N_A$  is the number of fragments in sample A, and,  $N_B$  denotes those in sample B (Souframanien & Gopalakrishna 2004).

#### *Amplification and Sequencing of ITS1-5.8S-ITS2 of rDNA*

The four *Colletotrichum* isolates were further characterized by nucleotide sequence analysis after amplification of the ITS1-5.8S-ITS2 regions using the universal primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White *et al.* 1990), and soybean isolate CI was used as positive control. PCR reactions were performed in reaction volumes of 50 µl containing 25 ng of genomic DNA, 1×PCR buffer (Merck Genei, Bangalore, India), 0.5 mM of each dNTP (Applied Biosystems, Carlsbad, California, USA), 1 µM primers, and 1U *Taq* polymerase (Merck Genei, Bangalore, India). DNA amplification was performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, California, USA), and the program consisted of an initial denaturing step at 95°C for 3 min, followed by 25 cycles of 30 s at 95°C, 30 s at 54°C, and 60 s at 72°C; and a final extension step of 10 min at 72°C. PCR products, approximately 600 bp, were separated by electrophoresis in 1.5% (W/V) agarose gels and visualized by ethidium bromide staining and were photographed using the gel documentation system. Specific bands were then cut off from gel and the amplified DNA was extracted using Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific Inc., Waltham, MA USA).

DNA sequencing was performed with two primers (ITS4 and ITS5) in both directions to ensure that there was no misreading. PCR products were sequenced by ABI 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). Alignment and edition were carried out with the CAP3 Sequence Assembly Program (Huang & Madan 1999) and visually corrected. Sequences were then compared using Mega 6.0 software program (Tamura *et al.* 2013).

#### *PCR using Species-Specific Primers*

*Colletotrichum capsici* specific primer pairs CcapF (5'-GTA GGC GTC CCC TAA AAA GG-3') and CcapR (5'-CCC AAT GCG AGA CGA AAT G-3') previously reported by Torres-Calzada *et al.* (2011) were used for rDNA amplification of all the samples. Amplification reactions were performed using protocol reported earlier. PCR products were visualized by electrophoresis in 1.5% (W/V) agarose gels, stained with ethidium bromide and documented using gel documentation system.

## RESULTS AND DISCUSSION

### *Identification of pathogen*

Pathogen associated with leaf spot disease of soybean was identified as *C. capsici* on the basis of morphological characteristics.

### *Pathogenicity test*

Healthy soybean plants were inoculated after one month of sowing and disease index was noted after 5, 7, 10, 14 and 21 days of inoculation. Symptoms were found to appear 7 days of inoculation and disease intensity was found to increase with the incubation period. Disease symptom was noted as necrotic spots (lesion) on the leaves of the plant. The results are presented in the table 1 and figures 1A & B.

**Table 1.** Pathogenicity test of *Colletotrichum capsici* (CI) on healthy soybean cultivar.

Incubation period (days) <sup>a</sup>	Disease index / plant <sup>b</sup>
5	0
7	3.5
10	5.75
14	7.25
21	8

**Note:** <sup>a</sup> - Days after inoculation; <sup>b</sup> - Average of 4 plants. Age of plant at the time of inoculation: 1 month; Inoculation potential: 10<sup>6</sup> conidia/ml

### *Cultural characteristics*

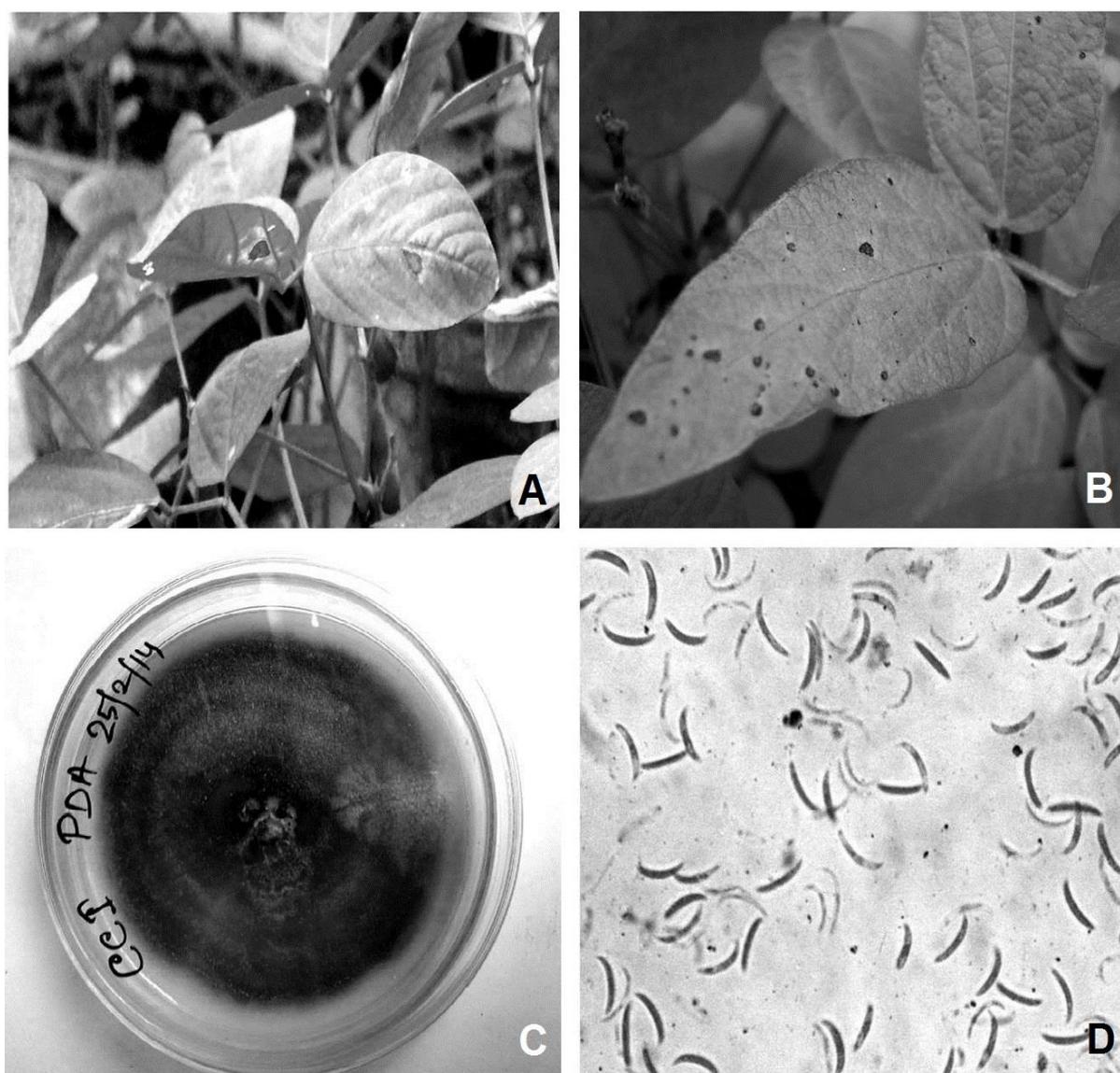
The mycelial growth of *Colletotrichum* isolates in solid PDA medium was measured after 12 days of incubation. The CI, CII and CIII isolates showed dense white to greyish brown mycelia with ring like zonations

in solid oat meal medium (Fig. 1C). *C. gloeosporoides* (CIV) produced pinkish white colony with cylindrical conidia (Table 2).

**Table 2.** Morphological and cultural characters of strains of *Colletotrichum capsici*.

Isolate	Cultural characters				Morphological characters	
	Colony colour	Margin	Topography	Zonation	Conidia shape	
CI	Grayish brown	smooth	Mycelium flat growth	Concentric zonation	7.13±0.19	Falcate, fusiform
CII	Grayish brown	smooth	Mycelium flat growth	Concentric zonation	8.4±0.21	Falcate, fusiform
CIII	Grayish brown	smooth	Mycelium flat growth	Concentric zonation	7.66±0.04	Falcate, fusiform
CIV	Pinkish white	smooth	Mycelium flat growth	Concentric zonation	7.85 ±0.16	cylindrical

**Note:** Incubation period: 12 days. <sup>a</sup> - Average of three replicates.



**Figure 1.** Symptoms of leaf spot disease on infected leaves of soybean and morphological and cultural characters of the pathogen.

#### Morphological characters

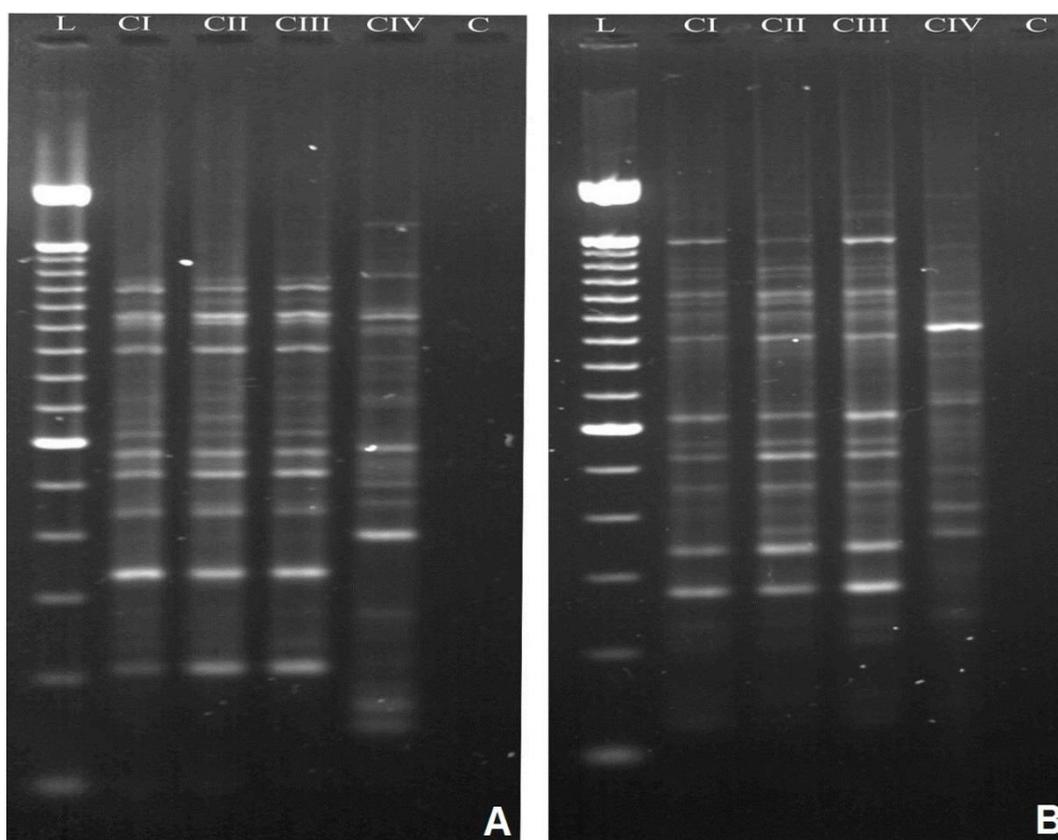
The conidia of *Colletotrichum* isolates from soybean and chilli (CI, CII and CIII) is falcate, fusiform with acute apices. (Fig. 1D) The size ranges from 19.65–21.00  $\mu\text{m}$  in length and 3.0–3.5  $\mu\text{m}$  in breadth. In *C. gloeosporoides* (CIV) conidia are hyaline, one celled, cylindrical. The size ranges from 8–12  $\mu\text{m}$  in length and 4–6  $\mu\text{m}$  in width.

*Molecular identification of the pathogen***i. RAPD-PCR analysis**

25 RAPD primers were tested on the genome of CI, CII, CIII and CIV. The generated DNA fingerprints were evaluated for assessing genetic similarity among the four isolates. Data generated by 10 primers were analyzed as they reliably and reproducibly detected polymorphism among the selected isolates (Table 3). Figure 2 shows typical profile and polymorphic bands generated after amplification with the primers.

**Table 3.** List of primers used, their sequences, and amplified products generated.

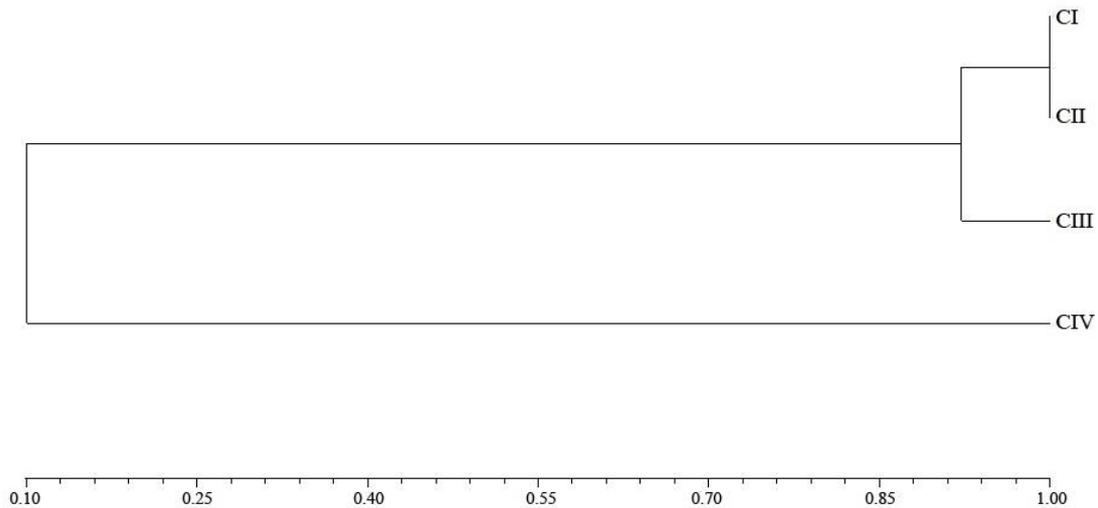
Primer	Sequence of primers	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	Percentage polymorphism	Resolving power (Rp)
OPA 04	5'-AATCGGGCTG-3'	35	31	4	88.6	35.5
OPA 11	5'-CAATCGCCGT-3'	29	24	5	82.8	33
OPB 01	5'-GTTTCGCTCC-3'	16	14	2	87.5	16.5
OPB 07	5'-GGTGACGCAG-3'	27	23	4	85.2	31.5
OPC 09	5'-CTCACCGTCC-3'	22	19	3	86.4	23.5
OPD 04	5'-TCTGGTGAGG-3'	15	14	1	93.3	15
OPE 03	5'-CCAGATGCAC-3'	37	36	1	97.3	35.5
OPG 08	5'-TCACGTCCAC-3'	26	23	3	88.5	29.5
OPK 17	5'-CCCAGCTGTG-3'	32	30	2	93.8	35.5
OPL 03	5'-CCAGCAGCTT-3'	37	36	1	97.3	40



**Figure 2.** RAPD amplification patterns of four *Colletotrichum* isolates using the primers OPC09 and OPA11. L: Ladder; CI, CII, CIII & CIV: *Colletotrichum* isolates; C: negative control.

The 10 RAPD primers produced a total of 276 amplified fragments with 250 polymorphic and 26 monomorphic bands. The average polymorphism of these primers varied from 82.8% to 97.30% with an average of 90.05%. The resolving power (Rp) of the primers varied from 15 to 40. Table 3 summarizes the above data.

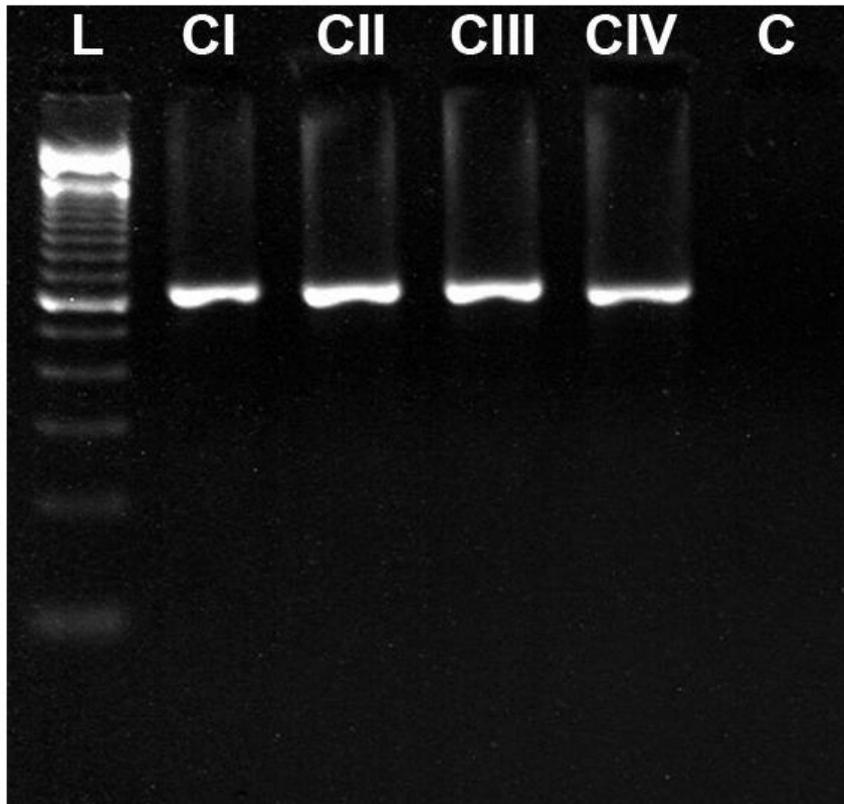
The dendrogram generated from the RAPD data using Jaccard's similarity coefficient showed a variation from 0.10 to 1.00 among the four isolates. While the three *Colletotrichum* isolates (CI, CII and CIII) separated out in a single cluster showing 0.85 similarity coefficient among them, *C. gloeosporoides* (CIV) showed similarity coefficient of 0.10 with them. Based on the high similarity value of 1.0 between CI and CII it may be assumed that both of them belong to the same species (Fig. 3).



**Figure 3.** Dendrogram generated using Jaccard's similarity distance obtained from RAPD primers. CI, CII, CIII & CIV: *Colletotrichum* isolates.

#### ii. Amplification and Sequencing of ITS1-5.8S-ITS2 of rDNA

To further confirm the result obtained using RAPD marker-based study, PCR amplification of the ITS region of all the studied samples were also done using universal primers ITS4 and ITS5. A single band of 620 bp was obtained in all cases (Fig. 4). No nonspecific banding was found. The amplified fragments were, thus, purified and sequenced.

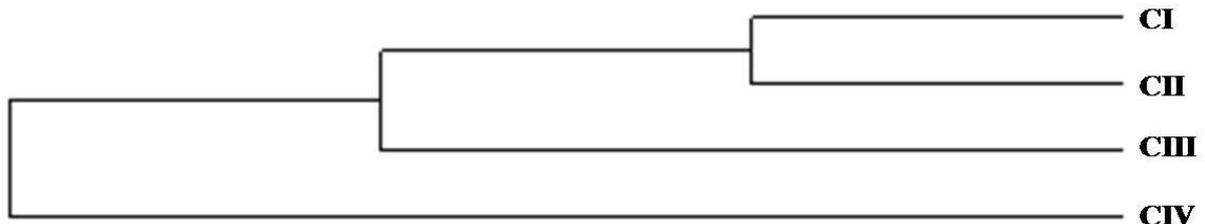


**Figure 4.** Products of PCR amplification of ITS region using primer set ITS 4and ITS 5. Lane L: Marker DNA ; CI,CII,CIII & CIV: *Colletotrichum* isolates; C: negative control.

The sequence data, thus obtained, were compared using Mega 6.06 software. The sequence analysis of the ITS region revealed 100% sequence similarity within the three isolates namely CI, CII and CIII. The ITS2 sequence of *C. gloeosporoides* (CIV), on the other hand, revealed significant difference with other three isolates (Fig. 5). The sequences were also used to generate a dendrogram revealing phylogenetic relationship among the studied taxa using UPGMA method (Fig. 6).

Contig S1	- T K K G G T C G C G G A G G W C A T T A C T G A G T T A C C G C T C A T C A A C C C T T T G T G A A C A T A C C T C A A C T G T T G C T T C G G C G G G T A G G C G T
Contig S2	- T K K G G T C G C G G A G G W C A T T A C T G A G T T A C C G C T C A T C A A C C C T T T G T G A A C A T A C C T C A A C T G T T G C T T C G G C G G G T A G G C G T
Contig S3	T T K T A G T C G C G G R G W C A T T A C T G A G T T A C C G C T C A T C A A C C C T T T G T G A A C A T A C C T C A A C T G T T G C T T C G G C G G G T A G G C G T
Contig S4	- T K G G T C G C G G A G G W C A T T A C C G A G T T A C A A C T C C C A A C C C C T T G T G A A C A T A C C - - A A T T G T T G C C T C G G C G G A T - - - - -
Contig S1	C C C C T A A A A A G G A C G T C T C C C G G C C C T C T C C C G T C G C G G G T G G G C G C C C G C C G G A G G A T A A C C A A A C T C T G A T T A A C G A C G
Contig S2	C C C C T A A A A A G G A C G T C T C C C G G C C C T C T C C C G T C C G C G G T G G G C G C C C G C C G G A G G A T A A C C A A A C T C T G A T T A A C G A C G
Contig S3	C C C C T A A A A A G G A C G T C T C C G C C C T C T C C G T C C G C G G T G G G C G C C C G C C G G A G G A T A A C C A A A C T C T G A T T A A C G A C G
Contig S4	- - - - - C A G C C C G C T C C C G G T A A A A C G - G G A C G G C C C G C C A G A G G A C C C C T A A A C T C T G T T C T A T - A T G
Contig S1	T T T C T T C T G A G T G A C A C A A G C A A T A A T C A A A A C T T T T A A C A A C G G A T C T T G G T T C T G G C A T C G A T G A A G A A C G C A G C G A A A
Contig S2	T T T C T T C T G A G T G A C A C A A G C A A T A A T C A A A A C T T T T A A C A A C G G A T C T T G G T T C T G G C A T C G A T G A A G A A C G C A G C G A A A
Contig S3	T T T C T T C T G A G T G A C A C A A G C A A T A A T C A A A A C T T T T A A C A A C G G A T C T T G G T T C T G G C A T C G A T G A A G A A C G C A G C G A A A
Contig S4	T A A C T T C T G A G T A A A A C C A T A A A T A A A T C A A A A C T T T C A A C A A C G G A T C T C T T G G T T C T G G C A T C G A T G A A G A A C G C A G C A A A A
Contig S1	T G C G A T A A G T A A T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C A T T G C G C C C G C C A G C A T T C T G G C G G G C A
Contig S2	T G C G A T A A G T A A T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C A T T G C G C C C G C C A G C A T T C T G G C G G G C A
Contig S3	T G C G A T A A G T A A T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C A T T G C G C C C G C C A G C A T T C T G G C G G G C A
Contig S4	T G C G A T A A G T A A T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C A T T G C G C C C G C C A G T A T T C T G G C G G G C A
Contig S1	T G C C T G T T C G A G C G T C A T T T C A A C C C T C A A G C T C T - - - G C T T G G T G T T G G G G C T C T A C G G - - - - T T G A C G T A - - - - G G C C C T T A
Contig S2	T G C C T G T T C G A G C G T C A T T T C A A C C C T C A A G C T C T - - - G C T T G G T G T T G G G G C T C T A C G G - - - - T T G A C G T A - - - - G G C C C T T A
Contig S3	T G C C T G T T C G A G C G T C A T T T C A A C C C T C A A G C T C T - - - G C T T G G T G T T G G G G C T C T A C G G - - - - T T G A C G T A - - - - G G C C C T T A
Contig S4	T G C C T G T T C G A G C G T C A T T T C A A C C C T C A A G C C C C G G G T T G G T G T T G G G G A T C G C G G A G C C C T T G C G G C A A G C C G G C C C G A
Contig S1	A A G G T A G T G G C G G A C C C T C T C G G A G C C T C C T T T G C G T A G T A A C A T T T C G T C T C G C A T T G G G A T T C G G A G G G A C T C T A G C C G T A A
Contig S2	A A G G T A G T G G C G G A C C C T C T C G G A G C C T C C T T T G C G T A G T A A C A T T T C G T C T C G C A T T G G G A T T C G G A G G G A C T C T A G C C G T A A
Contig S3	A A G G T A G T G G C G G A C C C T C T C G G A G C C T C C T T T G C G T A G T A A C A T T T C G T C T C G C A T T G G G A T T C G G A G G G A C T C T A G C C G T A A
Contig S4	A A T C T A G T G G C G G T C T C G C T - G C A G C T T C C A T T G C G T A G T A A A A C C C T C G C A A C T G G T A C G C G C G C G C G C - C A A G C C G T A
Contig S1	A A C C C C C A A T T T A C T A A G G T T G A C C T C G G A T C A G G T A G G A T A C C C G C T G A A C T T A A G C A T A T C A A T A A G C G G A G G A A
Contig S2	A A C C C C C A A T T T A C T A A G G T T G A C C T C G G A T C A G G T A G G A A T A C C C G T G A A C T T A A G C A T A T C A A T A A G C G G A G G A A
Contig S3	A A C C C C C A A T T T A C T A A G G T T G A C C T C G G A T C A G G T A G G A A T A C C C G T G A A C T T A A G C A T A T C A A T A A G C G G A G G A A
Contig S4	A A C C C C C A A C T T C - T G A A T G T T G A C C T C G G A T C A G G T A G G A A T A C C C G T G A A C T T A A G C A T A T C A A A A A G C G G A G G A A

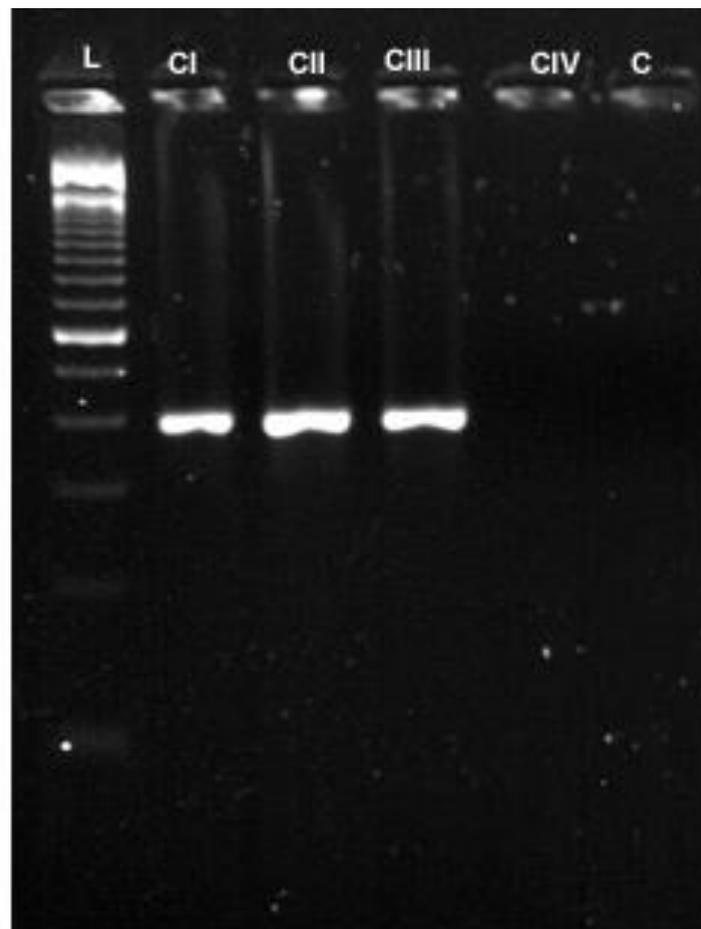
**Figure 5.** Alignment of ITS1-5.8S-ITS2 sequences of the rRNA gene region of all accessions used in the present investigation using ClustalW. Contig S1= CI; Contig S2= CII; Contig S3: CIII; Contig S4: *C. gloeosporoides*.



**Figure 6.** Dendrogram generated using the UPGMA method obtained from ITS sequences. CI, CII, CIII & CIV: *Colletotrichum* isolates.

### iii. PCR using Species-Specific Primers

When the primer pair CcapF/CcapR were used to amplify the genomic DNA in the present study, the two chilli isolates of *C. capsici* (CII and CIII) and the CI isolate from soybean tested positive with a single amplified fragment in each taxa. The size of this fragment was 394 bp in all of the *C. capsici* tested. The primers, however, used failed to amplify genomic DNA of *C. gloeosporoides* (Fig. 7).



**Figure 7.** Products of PCR amplification using *C. capsici* specific primer pairs Ccap F / Ccap R. Lane L: Marker DNA; CI, CII, CIII & CIV: *Colletotrichum* isolates; C: negative control.

## DISCUSSION

In the present investigation results of pathogenicity test indicated that *Colletotrichum* sp. (CI isolate) can establish leaf spot disease in susceptible soybean cultivar. Traditional identification and characterization of *Colletotrichum* species relies primarily on difference in morphological features such as colony, colour, size and shape of conidia and appressoria. In this study morphological characterization of *Colletotrichum* isolates from chilli and soybean plants were done. Results revealed that CI, CII and CIII isolates showed similar characteristics, whereas CIV is morphologically different from the other 3 isolates. This result is in agreement with a previous study by Sandgee *et al.* (2011) who found a morphometric overlap of conidial size among *Colletotrichum* species. Thangamani *et al.* (2011) performed morphological and physiological characterization of *Colletotrichum musae*, the causal organism of banana anthracnose. However morphological characterizations

are not always adequate for reliable differentiation among species of *Colletotrichum*. Thus molecular methods have been employed successfully to differentiate between populations of *Colletotrichum* from many hosts. In the present investigation, RAPD primers were tested on genome of four *Colletotrichum* isolates. Analysis of data generated using 10 RAPD primers exhibited polymorphism varying from 82.76% to 97.30% with an average of 90.05%. Cluster analysis using Jaccard's coefficient clearly separated the isolates of chilli and soybean from *C. gloeosporioides*. RAPD analysis was also performed by Ratancherdchai *et al.* (2007) on 18 isolates belonging to 2 species, *C. gloeosporioides* and *C. capsici* isolated from three varieties of chilli, *i.e.* chilli pepper (*Capsicum annum*), long cayenne pepper (*C. annum* var. *acuminatum*) and Birds eye chilli (*C. frutescens*). Their study showed that a clear difference could be established between *C. gloeosporioides* and *C. capsici*. The three *C. capsici* isolates analysed in the present study show similarity of 0.85–1.00 among themselves. The existence of molecular variability among isolates of *C. capsici* that differed in virulence was earlier established by Srinivasan *et al.* (2010) using RAPD markers. The result of RAPD in the present investigation was further confirmed by sequence analysis of the ITS region which revealed 100% sequence similarity between the two *C. capsici* isolates of chilli and CI isolate of soybean. *C. gloeosporioides*, on the other hand, revealed significant sequence difference with *C. capsici* isolates CI. Freeman (2008) used sequence analysis of ITS region to establish similarity between *Colletotrichum acutatum* isolates from almond and strawberry. Shi *et al.* (2008) had used ITS universal primer pair ITS1-F/ITS4 to characterize *Colletotrichum acutatum* and *C. gloeosporioides* isolates from flowering dogwood (*Cornus florida*). Katoch *et al.* (2016) carried out Metageographic population analysis of *Colletotrichum truncatum* associated with chili fruit rot and other hosts using ITS region nucleotide sequences. Molecular characterization of the soybean isolate was finally established by using species specific primer which revealed that this isolate and two other *C. capsici* isolates tested positive with a single amplified fragment in each taxa. *C. gloeosporioides*, however, failed to produce any amplified DNA fragment using these primers. The results of molecular characterization and morphological identification thus established that the CI isolate from soybean is *C. capsici*. *C. capsici* was not reported in soybean till date. Results of present investigation revealed the first report of *C. capsici* in soybean plant.

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#### REFERENCES

- Adhipathi P, Nakkeeran S & Chandrasekaran A (2013) Morphological characterization and molecular phylogeny of *Colletotrichum capsici* causing leaf spot disease of turmeric. *The Bioscan* 8: 331–337.
- Bhadra S & Bandyopadhyay M (2015) A fast and reliable method for DNA extraction from different plant parts of Zingiberaceae. *Journal of the Botanical Society of Bengal* 69(2): 91–98.
- Cai L, Hyde KD, Taylor PWJ, Weir BS, Waller JM, Abang MM, Zhang JZ, Yang YL, Phoulivong S, Liu ZY, Prihastuti H, Shivas RG, McKenzie EHC & Johnston PR (2009) A polyphasic approach for studying *Colletotrichum*. *Fungal Diversity* 39: 183–204.
- Chen LS, Chu C, Liu C, Chen RS & Tsay JG (2006) PCR-based detection and differentiation of Anthracnose pathogens, *Colletotrichum gloeosporioides* and *C. truncatum*, from vegetable soybean in Taiwan. *Journal of Phytopathology* 154: 654–662.
- Damm U, Woudenberg JHC, Cannon PF & Crous PW (2009) *Colletotrichum* species with curved conidia from herbaceous hosts. *Fungal Diversity* 39: 45–87.
- Freeman S (2008) Management, Survival Strategies, and Host Range of *Colletotrichum acutatum* on Strawberry. *Hortscience* 43: 66–68.
- Gautam AK (2014) The genera *Colletotrichum*: an incitant of numerous new plant diseases in India. *Journal on New Biological Reports* 3(1): 9–21.
- Hershman DE (2003) Soybean Foliar Spots and Blights. Available from: [http://www.ca.uky.edu/agcollege/plant\\_pathology/ext\\_files/PPFShtml/ppfsags19.pdf](http://www.ca.uky.edu/agcollege/plant_pathology/ext_files/PPFShtml/ppfsags19.pdf) (accessed: 10 Feb. 2016).
- Huang X & Madan A (1999) CAP3: A DNA sequence assembly program. *Genome Research* 9: 868–877.
- Katoch A, Prabhakar CS & Sharma PN (2016) Metageographic population analysis of *Colletotrichum truncatum* associated with chili fruit rot and other hosts using ITS region nucleotide sequences. *Journal of Plant Biochemistry and Biotechnology* 25: 64–72.

- Madhavan S, Paranidharan V & Velazhahan R (2010) RAPD and virulence analyses of *Colletotrichum capsici* isolates from chilli (*Capsicum annuum*). *Plant Diseases and Protection* 117: 253–257.
- Peres NR, Kuramae EE, Dias CM & De'souza N (2002) Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil. *Journal of Phytopathology* 150: 128–134.
- Photita W, Taylor PWJ, Ford R, Hyde KD & Lumyong S (2005) Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Diversity* 18: 117–133.
- Prevost A & Wilkinson MJ (1999) A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theoretical and Applied Genetics* 98: 107–112.
- Purkayastha RP & Banerjee R (1990) Immunoserological studies of cloxacillin-induced resistance of soybean against anthracnose. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 97: 349–359.
- Ramdial H & Rampersad SN (2015) Characterization of *Colletotrichum* spp. causing anthracnose of bell pepper (*Capsicum annuum* L.) in Trinidad. *Phytoparasitica* 43: 37–49.
- Ratanacherdchai K, Wang HK, Lin FC & Soyong K (2007) RAPD analysis of *Colletotrichum* species causing chilli anthracnose disease in Thailand. *Journal of Agricultural Technology* 3: 211–219.
- Rohlf FJ (1999) *NTSYS-pc Numerical Taxonomy and Multivariate Analysis System*. Version 2.02i. Exeter Software, Setauket, New York, US.
- Sangdee A, Sachan S & Khankhum S (2011) Morphological, pathological and molecular variability of *Colletotrichum capsici* causing anthracnose of chilli in the North-east of Thailand. *African Journal of Microbiology Research* 5: 4368–4372.
- Sharma G, Pinnaka AK & Shenoy BD (2013) ITS-based diversity of *Colletotrichum* from India. *Current Research in Environmental & Applied Mycology* 3: 194–220.
- Sharma PN, Kaur M, Sharma OP, Sharma P & Pathania A (2005) Morphological, pathological and molecular variability in *Colletotrichum capsici*, the cause of fruit rot of chillies in the subtropical region of north-western India. *Journal of Phytopathology* 153: 232–237.
- Shenoy BD, Jeewon R, Lam WH, Bhat DJ, Than PP, Taylor PWJ & Hyde KD (2007) Morpho-molecular characterisation and epitypification of *Colletotrichum capsici* (*Glomerallaceae*, *Sordariomycetes*) the causative agent of anthracnose in chilli. *Fungal Diversity* 27: 197–211.
- Shi ABC, Kantartzi SK, Mmbaga MT, Chen P, Mrema F & Nnodu E (2008) PCR-based markers for detection of *Colletotrichum acutatum* and *C. gloeosporioides* in flowering dogwood (*Cornus florida*). *Australasian Plant Pathology* 37: 65–68.
- Souframanien J & Gopalakrishna T (2004) A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. *Theoretical and Applied Genetics* 109: 1687–1693.
- Srinivasan M, Vaikuntavasan P & Rethinasamy V (2010) RAPD and virulence analyses of *Colletotrichum capsici* isolates from chilli (*Capsicum annuum*). *Journal of Plant Diseases and Protection* 117: 253–257.
- Talhinhas, P, Sreenivasaprasad S, Neves-Martins J & Oliveira H (2002) Genetic and morphological characterization of *Colletotrichum acutatum* causing anthracnose of lupins. *Phytopathology* 92: 986–996.
- Tamura K, Stecher G, Peterson D, Filipski A & Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725–2729.
- Than PP, Prihastuti H, Phoulivong S, Taylor PWJ & Hyde KD (2008) Chilli anthracnose disease caused by *Colletotrichum* species. *Journal of Zhejiang University Science Biology* 9: 764–778.
- Thangamani PR, Kuppusamy P, Peeran MF, Gandhi K & Thiruvendam R (2011) Morphological and physiological characterization of *Colletotrichum musae* the causal organism of banana anthracnose. *World Journal of Agricultural Sciences* 7: 743–754.
- Torres-Calzada C, Tapia-Tussell R, Quijano-Ramayo A, Martin-Mex R, Rojas-Herrera R, Higuera-Ciajara I & Perez-Brito D (2011) A Species-Specific Polymerase Chain Reaction Assay for Rapid and Sensitive Detection of *Colletotrichum capsici*. *Molecular Biotechnology* 49: 48–55.
- Uma Devi G (2008) Efficacy of fungicides against *Colletotrichum* leaf spot of turmeric. *Indian Journal of Plant Protection* 36: 112–113.
- White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH & Sninsky JJ (eds) *PCR Protocols: a guide to methods and applications*. Academic Press, San Diego, CA, pp. 315–322.