Plant Pathology & Quarantine 7(2): 146–152 (2017) ISSN 2229-2217
www.ppqjournal.org **Article**

Doi 10.5943/ppq/7/2/7
Copyright © Mushroom Research Foundation

Molecular and phylogenetic studies of *Colletotrichum truncatum* associated with soybean anthracnose in India

Marmat N[1,2], Ansari MM[1], Chand S[2] and Ratnaparkhe MB[1*]

[1] Indian Institute of Soybean Research, Indian Council of Agricultural Research, Indore (M.P.), India
[2] School of Life Sciences, Devi Ahilya University, Indore (M.P.), India

Marmat N, Ansari MM, Chand S, Ratnaparkhe MB 2017 – Molecular and phylogenetic studies of *Colletotrichum truncatum* associated with soybean anthracnose in India. Plant Pathology & Quarantine 7(2), 146–152, Doi 10.5943/ppq/7/2/7

Abstract
Colletotrichum species are destructive pathogens of soybean causing anthracnose disease in pre- and post-harvest stages. *Colletotrichum truncatum*, which is associated with soybean anthracnose, was isolated from four geographical locations of India. Isolates were identified and grouped on the basis of rDNA internal transcribed spacer (ITS1 and ITS2) sequences. Multiple sequence alignment showed that one isolate had nucleotide variation at two positions, indicating genetic variability within *C. truncatum*. A maximum likelihood phylogenetic tree constructed for all the isolates indicated high sequence similarity of *C. truncatum* isolates. This study identified new *C. truncatum* isolate associated with soybean anthracnose which is different from the previously reported *C. truncatum* isolates.

Key words – genetic variability – maximum-likelihood – phylogenetic – ribosomal ITS region – sequencing

Introduction

Colleotrichum truncatum causing anthracnose, is the most important seed-borne fungal pathogen of soybean (Sinclair & Backman 1989). The pathogen produces a significant reduction of seed germination, seed quality and yield loss in warm and humid subtropics. In India, anthracnose is also considered the most serious soybean disease (Khare & Chacko 1983), and up to 100% yield losses have been reported (Sinclair & Backman 1989, Ploper & Backman 1992, Manandhar & Hartman 1999). Disease severity disease and yield loss can vary from location to location. Stability of morphological traits and the existence of intermediate forms due to environmental influence are not always adequate for reliable differentiation among *Colletotrichum* species, hence making it difficult to classify species for taxonomical studies. Primarily, morphological classification of *Colletotrichum* species is supported by conidial and appressorial size and shape. Molecular techniques provide alternative methods in solving the problems of species delimitations (MacLean et al. 1993, Jayawardena et al. 2016, Diao et al. 2017). Evolutionary divergence within *Colletotrichum* species can be detected by using highly conserved rDNA genes that include 18S, 5.8S and 28S segments that code for rRNA. These genes are separated by two less conserved regions, the internal transcribed spacers 1 and 2 (ITS1 and ITS2). The sequence analysis of ITS

Submitted 22 August 2017, Accepted 4 November 2017, Published 21 November 2017
Corresponding Author: M.B. Ratnaparkhe – e-mail – milind.ratnaparkhe@gmail.com

regions has proved useful in studying phylogenetic relationships and is also accepted as a universal DNA barcode marker for fungi including *Colletotrichum* species. The ITS1 and ITS2 spacers show a high rate of evolution and have proved to be robust evolutionary markers for determining intra- and inter- specific relationships. Shi et al. (2008) used ITS universal primer pair ITS5/ITS4 to characterize *Colletotrichum acutatum* and *C. gloeosporioides* isolates from flowering dogwood (*Cornus florida*). Similarily, Katoch et al. (2016) carried out a metageographic population analysis of *Colletotrichum truncatum* associated with chili fruit rot and other hosts using ITS region nucleotide sequences. ITS fungal primers were used for detection of seed borne infection of soybean in Northern Karnataka (Sajeesh et al. 2014). A similar approach has been used to evaluate systematics of *Colletotrichum* spp. (Bailey et al. 1996, Sherrif et al. 1994, Sreenivasaprasad et al. 1996), and relatedness of anthracnose isolates causing diseases on crops (Johnston & Jones 1997, Ford et al. 2004). This study evaluates the genetic variability using rDNA-ITS sequences of *Colletotrichum truncatum* isolates from soybean cultivated in different geographic regions of India.

Table 1 Geographic origin of *Colletotrichum truncatum* isolates used in this study.

Isolate ID	GenBank Accession No.	Host	Geographical regions
CoT1	KY287672	*Glycine max*	Amravati, Maharashtra (Western India)
CoT2	KY287673	*Glycine max*	Palampur, H.P. (Northern India)
CoT3	KY287674	*Glycine max*	Dhar, M.P, (Central India)
CoT4	KY287675	*Glycine max*	Indore, M.P, (Central India)
CoT5	KY287676	*Glycine max*	Jabalpur, M.P, (Central India)
CoT6	KY287677	*Glycine max*	Umiam, Meghalaya (North-East region of India)

Materials & Methods

Collection and isolation of *Colletotrichum* species

Six soybean isolates of *Colletotrichum* species were collected from different regions of India (Table 1). The isolates were cultured on potato dextrose agar (PDA) medium for 10–14 days. For harvesting of mycelia, a small piece of fungal colony was transferred to potato dextrose broth containing streptomycin and incubated for two weeks at room temperature in aseptic conditions.

Genomic DNA extraction

The freshly grown mycelia in potato dextrose broth was filtered on Watman filter paper and rinsed with sterile water. The harvested mycelium, approximately 1 g, was crushed to powder in liquid nitrogen using a sterile mortar and pestle. The DNA extraction was done by modified CTAB method (Schafer & Wostmeyer 1992) comprising of 2% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl and 1% 2-mercaptoethanol extraction buffer. The quality of extracted DNA was determined on 0.8% agarose gel.

Polymerase chain reaction

ITS primers (ITS5/ITS4) were used to amplify the internal transcribed regions 1 and 2 of fungal isolates (White et al. 1990). A reaction mixture of 25 µl total volume was prepared containing 10X PCR buffer with 25 mM $MgCl_2$, 2 mM dNTPs mixture, 0.01 µmol of each primer, 5 units of Taq polymerase and 25 ng DNA template. Amplification was performed in a thermocycler programmed for initial step of denaturation at 94°C for 4 mins, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 52°C for ITS primers for 30 sec and an extension

at 72°C for 30 sec. After 35 cycles, an extra elongation at 72°C for 5 mins was done and a final hold at 4°C. The PCR products were analyzed in 0.8 % agarose in 10X TAE buffer and size of amplified products were determined.

Table 2 Geographical origin, host information and accession number of referral *Colletotrichum* ITS sequences used for genetic analysis

Isolate	GenBank ID	Length ITS (bp)	Host	Country
Colletotrichum truncatum	GU227880	522	*Capsicum annuum*	India
	GU227866	522	*Glycine max*	USA
	AF451899	606	*Lens culinaris*	Australia
	KJ677256	552	*Jatropha curcas*	Mexico
	JN390854	584	*Polianthes tuberosa*	India
	KX197395	604	*Hylocereus* sp.	Taiwan
	KX197396	604	*Hylocereus* sp.	Taiwan
	KX197397	604	*Hylocereus undatus*	Taiwan
Colletotrichum dematium	KJ425579	552	*Amorphophallus muelleri*	China
	JX487153	554	*Peucedanum praeruptorum*	China

Sequencing and phylogenetic analysis

The purified PCR products of 5.8S/ITS region were sequenced twice using forward and reverse primers. Sequences were deposited in GenBank and compared against those sequence already found in the databases using the Basic Local Alignment Search Tool (BLAST) of NCBI (Table 2). ITS 1 and ITS 2 including 5.8S sequences were aligned using multiple sequence alignment program CLUSTALW. Phylogenetic analyses were conducted in MEGA 6.0. by maximum likelihood (ML) method. The evolutionary distances were computed using the maximum composite likelihood method and all positions containing gaps and missing data were eliminated from the data sets. Clade stability was assessed in bootstrap analyses with 1000 replicates.

Demographic modelling

To determine whether a model of the population expansion was applicable to isolates we performed the neutrality test of Tajima's D statistics to estimate deviation from selective neutrality (Ramdeen et al. 2012). All the parameters were tested against the expected values under the hypothesis of a recent population expansion based on 1000 bootstrap replicates. Tajima's D was calculated to test for neutrality (Tajima 1989). Neutrality test was used as an indication of recent population expansion when the null hypothesis of neutrality was rejected due to significant negative values where Tajima's D statistic was significant at $P \leq 0.05$.

Results

Six pathogenic *Colletotrichum* strains (CoT1 to CoT6) were isolated from typical anthracnose lesions found on stems of soybean from four different locations of India (Table 1). When grown on PDA, the colour and appearance of the *Colletotrichum* isolates varied from cottony white to dark grey (Fig. 1).

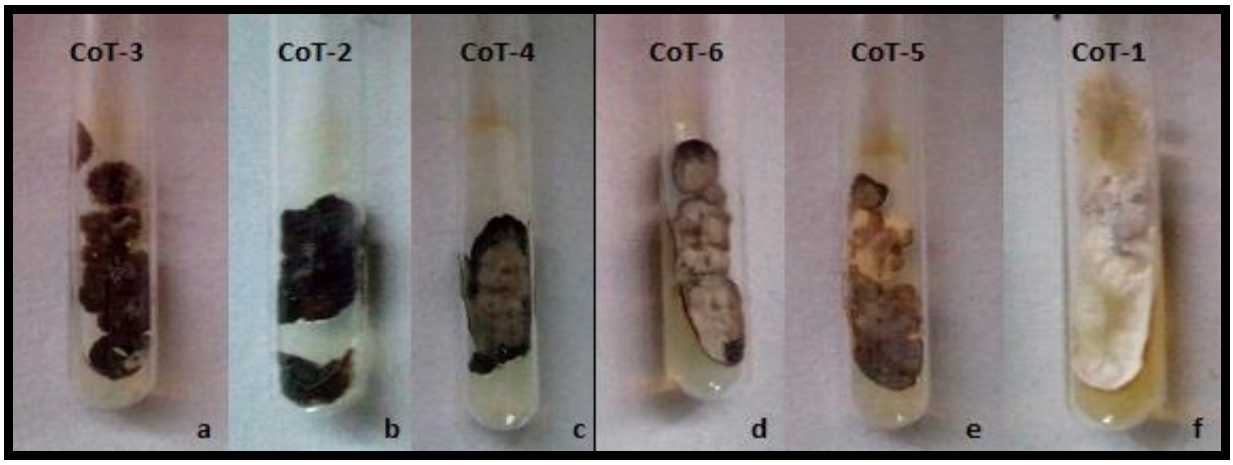

Fig 1 – Colony characters of *Colletotrichum truncatum* on potato dextrose agar medium. Isolates collected from different regions of India (a) Dhar, Madhya Pradesh, (b) Palampur, Himachal Pradesh, (c) Indore, Madhya Pradesh, (d) Umiam, Meghalaya, (e) Jabalpur, Madhya Pradesh, (f) Amravati, Maharashtra.

Sequence analysis of the ribosomal DNA spacer sequence (ITS)

PCR amplification and sequencing of 5.8S/ITS regions of the six isolates was conducted for genetic variability studies. The total size of the ITS 1 and ITS 2 regions, including the 5.8S rDNA region of the isolates studied was 580 base pairs. Multiple sequence alignment of all the six isolates along with the reference sequences of *C. truncatum* was carried out to identify the variations at nucleotide level. Multiple sequence alignment indicated that five Indian isolates (CoT1, CoT2, CoT3, CoT4, and CoT6 have 100% similarity with the 5.8S/ITS region of other *C. truncatum* isolates from GenBank. Isolate CoT5 differed in nucleotide A at position 74 and nucleotide G at position 97 (Fig. 2).

Fig 2 – Multiple sequence alignment of 5.8S/ITS regions of *Colletotrichum truncatum* isolates. Polymorphic residues are highlighted. Isolate CoT5 (GenBank KY287676) collected from central India showed polymorphism at two positions, indicating that it is different from all other *C. truncatum* isolates used in this study.

Phylogenetic and evolutionary analysis

We conducted phylogenetic analysis of 5.8S-ITS sequences from six isolates along with fourteen reference sequences representing *Colletotrichum truncatum, C. dematium* and *Fusarium* species. The phylogenetic tree derived from maximum likelihood parsimony analysis consisted of two major clades with 100% bootstrap support (Fig. 3). The phylogenetic tree clustered all the six isolates with other reference isolates of *C. truncatum* in a single clade irrespective of origin and

host of the isolate. A second sub-group was formed by *C. dematium* isolates (GenBank KJ425579 and JX487153); the *Fusarium* species grouped together in a separate clade. We conducted neutrality tests using ITS sequences of all the six isolated and reference sequences. Strongly negative and significant values suggest recent population expansion or selection. Tajima's D values were negative (-2.776362, *P*=0.020109) for ITS region in all the isolates and reference sequences.

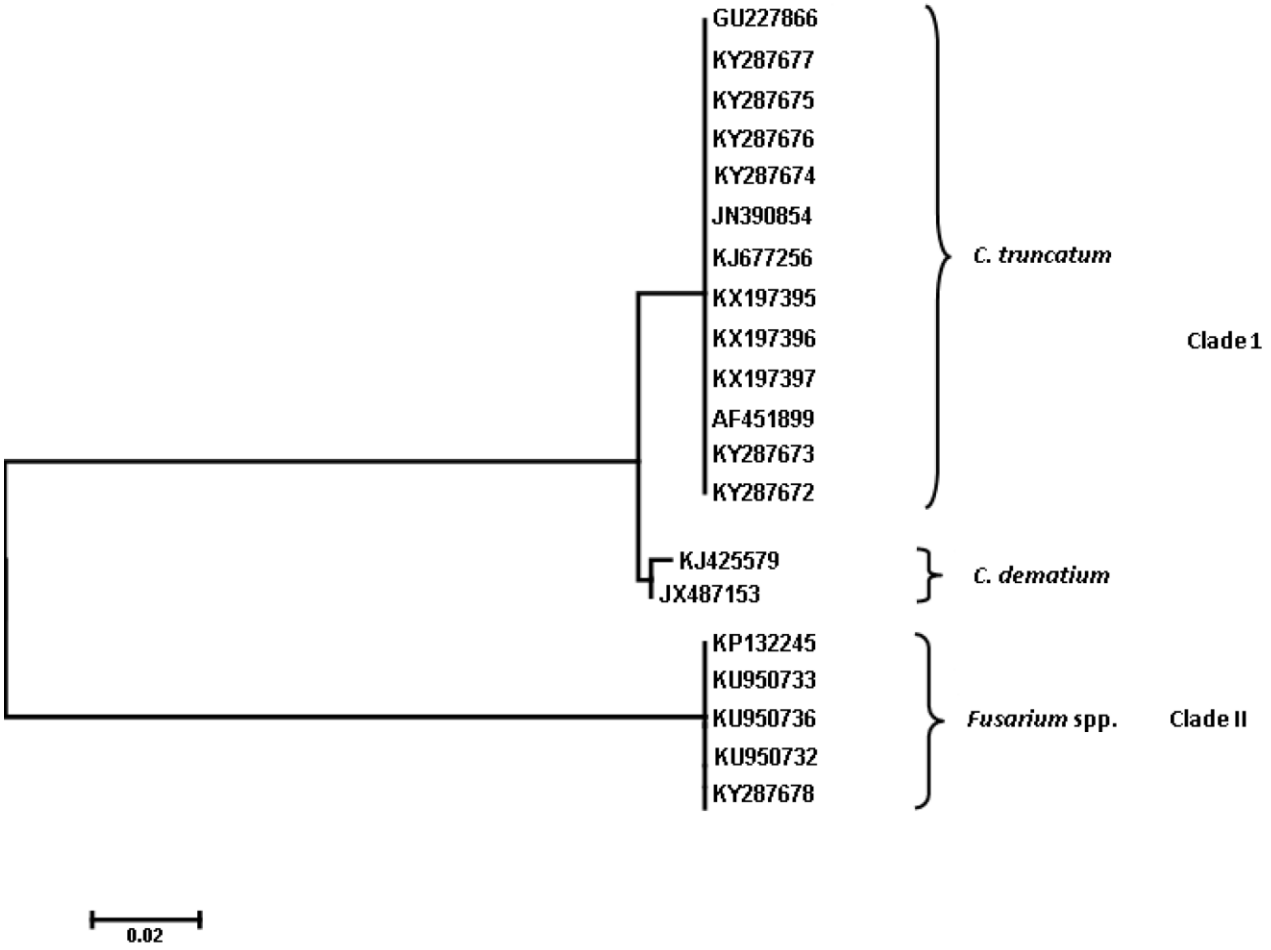

Fig 3 – Phylogenetic analysis of *Colletotrichum* and *Fusarium* species based on 5.8S/ITS sequence. All six *Colletotrichum truncatum* isolates grouped together in clade I.

Discussion

To our knowledge this is the first report of ITS based genetic variability studies of *C. truncatum* infecting soybean in different geographic region of India. ITS sequences revealed that five *C. truncatum* isolates collected from different geographic regions of India exhibited high similarity with previously reported isolates, but one isolate (CoT5) from Central India was found to be polymorphic at two positions that separated it from all the other *C. truncatum* isolates. Isolate CoT5 shares the nucleotide A at position 74 with another previously reported *C. truncatum* isolate (GenBank GU227880) infecting *Capsicum annuum* in India (Damm 2009). This indicates that *C. truncatum* infecting *Capsicum annuum* can also be associated with soybean anthracnose. Two nucleotide changes of isolate CoT5 indicates that this isolate is quite different from all the other *C. truncatum* isolates. Significantly these differences may not necessarily influence the function of the isolates or strains, especially parasitism and pathogenicity on plants. Maximum likelihood tree placed *C. truncatum* together while differentiating it from *C. dematium* and *Fusarium* sp. Ford et al. (2004) showed 99.8% identity among ITS-rDNA sequences of *C. truncatum* that were isolated

from soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and alfalfa (*Medicago sativa*). This indicated near compete conservation in ITS sequence between isolates from different host species. Based on the 5.8S-ITS information, all the soybean isolates tested in the current study were designated as *C. truncatum.* The differentiation of various isolates of *C. truncatum* based on ITS sequences supports a previous report that *C. truncatum* isolates from different hosts can be identified using ITS based molecular techniques (Ford et al. 2004). Our results will help in understanding the pathogen diversity and in the management of soybean anthracnose.

Acknowledgements

We thank the Director, ICAR-Indian Institute of Soybean Research, Indore for encouragement and infrastructure facilities.

References

Bailey JA, Nash C, Morgan, LW, O'Connell RJ, TeBeest DO. 1996 – Molecular taxonomy of *Colletotrichum* species causing anthracnose on the Malvaceae. Phytopathology 86, 1076–1083.

Damm U, Woudenberg JHC, Cannon PF, Crous PW. 2009 – *Colletotrichum* species with curved conidia from herbaceous hosts. Fungal Diversity 39, 45–87.

Diao YZ, Zhang C, Liu F, Wang WZ, Cai L, Liu XL. 2017 – *Colletotrichum* species causing anthracnose disease of chili in China. Persoonia 38, 20–37.

Ford R, Banniza S, Photita W, Taylor PWJ. 2004 – Morphological and molecular discrimination of *Colletotrichum truncatum* causing anthracnose on lentil in Canada. Australasian Plant Pathology 33, 559–569.

Jayawardena RS, Hyde KD, Damm U, Cai L et al. 2016 – Notes on currently accepted species of *Colletotrichum*. Mycosphere 7, 1192–1260.

Johnston PR, Jones D. 1997 – Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. Mycologia 89, 420–430.

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.

Khare MN, Chacko S. 1983 – Factors affecting seed infection and transmission of *Colletotrichum dematium* f. sp. *truncata* in soybean. Seed Science & Technology 11, 853–858.

MacLean DJ, Braithwaite KS, Manners JM, Irwing JAG. 1993 – How do we identify and classify fungal pathogens in the era of DNA analysis? Advances in Plant Pathology 10, 207–244.

Manandhar JB, Hartman GL. 1999 – Anthracnose. In: Hartman GL, Sinclair JB, Rupe JC (eds.), Compendium of Soybean Diseases, pp 13–14, 4th edition. St. Paul, APS Press, USA.

Ploper LD, Backman PA. 1992 – Nature and management of fungal diseases affecting soybean stems, pods and seeds. In: Copping LG, Green MB, Rees RT. (eds.), Pest Management in Soybean, pp 174–184. Elsevier Applied Science, London and New York.

Ramdeen S, Rampersad SN. 2012 – Intraspecific differentiation of *Colletotrichum gloeosporioides* sensu lato based on in-silico multilocus PCR-RFLP fingerprinting. Molecular Biotechnology 53, 170–181.

Sajeesh PK, Rao MSL, Jahagirdar S. 2014 – Molecular detection, transmission and histopathological studies of seed-borne fungal infection of soybean. The Bioscan 9(1), 247–251.

Schafer C, Wostmeyer J. 1992 – Random primer dependent PCR differentiates aggressive and non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*). Journal of Phytopathology 136, 124–136.

Sherriff C, Whelan MJ, Arnold GM, Lafay JF, Brygoo Y, Bailey JA. 1994 – Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. Experimental Mycology 18, 121–138.

Shi A, 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.

Sinclair JB, Backman PA. 1989 – Compendium of Soybean Diseases. (3rd edition). American Phytopathological Society, St. Paul, Minnesota, USA, p. 106.

Sreenivasaprasad S, Mills PR, Meehan BM, Brown AE. 1996 – Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome 39, 499–511.

Tajima F. 1989 – Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

White TJ, Bruns T, Lee S, Taylor JW. 1990 – Amplication and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ. (eds.), PCR Protocols: A Guide to Methods and Application. Academic Press, San Diego, 315-322.