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Studies on genetic variability in *Ceratocystis fimbriata* Ell. and Halst. incitant of Pomegranate wilt in Karnataka using random amplified polymorphic DNA (RAPD) markers

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Abstract
Pomegranate wilt disease incited by *Ceratocystis fimbriata* Ell. and Halst. is one of the important diseases of pomegranate adversely affecting crop cultivation in all major growing regions of India. Nine isolates of the fungus selected from different geographical regions of Karnataka were analysed through four RAPD primers *viz*., A3, A9, A13 and B10 for genetic variability. The primers showed amplification of different isolates with polymorphism between the isolates. Out of the 28 marker loci amplified, 13 were found to be specific for five species and only two loci were common in all nine samples indicating 83.20 per cent polymorphism. Dendrogram obtained from RAPD analysis indicated three major clusters, which separated Cf-R from rest of the isolates, The isolates from Cf-Ct, Cf-H, Cf -K, Cf-T and Cf-B were grouped in second cluster. While, third cluster includes Cf-Bg, Cf-C and Cf-V isolates.

Keywords: *Ceratocystis fimbriata,* RAPD markers, polymorphism, variability

Introduction
Pomegranate (*Punica granatum*) an important fruit crop of India, is commercially cultivated in the states of Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Rajasthan and Tamil Nadu. Wilt disease is one of the important diseases of pomegranate adversely affecting crop cultivation in all major growing regions of India. Pomegranate wilt was first reported in India from Nashik district in Maharashtra in 1978 and subsequently from Kaladgi and Kanamadi areas of Karnataka in 1988 and Cadapa, Andhra Pradesh in 2002 and disease was attributed to *Ceratocystis fimbriata* Ell. and Halst. (Somasekhara 1999, 2006) [10, 11]. The disease is prevalent in parts of a Maharashtra, Karnataka, Andhra Pradesh, Gujarat and Tamil Nadu states in India (Somasekhara 1999 Jadhav and Sharma 2009) [10, 5]. Pomegranate wilt results in complete wilting of plant and is characterized by the initial symptoms as yellowing and wilting of leaves on one to several branches (Somasekhara 1999) [10]. Since, wilt disease is prevalent in different agro-climatic regions with varying degrees of severity in Karnataka, it was found essential to examine the genetic variability among the different isolates of *Ceratocystis fimbriata* and to assess geographical differences between isolates using RAPD markers.

Material and Methods
Isolation, purification and morphological studies of *C. fimbriata*
The infected tissues from diseased roots were cut into small strips of about 1 cm were baited for *Ceratocystis fimbriata* by placing these between two carrot slices (5 mm thick) that had first been treated with streptomycin sulphate (100 mg/l) and incubated at 25 °C for 8-10 days with humidity to induce fungal sporulation (De Vay & Moller 1968) [7]. Ascospore mass that developed at the apices of ascomata on carrot slices were transferred to potato dextrose agar in Petri plates. These cultures were incubated at 25 °C and observed periodically for fungal growth and sporulation. Fungal structures developed from the bits were mounted on glass slides in lactic acid and examined under microscope. After identification isolates were transferred to new agar in Petri plates for purification. The pure culture of the fungus was sub cultured on potato dextrose agar slants and kept in laboratory at 25±2 °C and used for further studies. In order to confirm the identity of the fungus, the ascospores and perithecia were observed under microscope.

Colony and morphological characterization

Morphological characteristics of the *C. fimbriata* isolates collected from different places were compared for their variabilities. Morphological observations like colony diameter, colony colour, colony margin, ascomatal length and sporulation of different isolates were made using structures produced on PDA incubated for 12 days at 25 °C. Isolates were prepared by mounting fungal structures on glass slides in lactic acid for observing these under a light microscope. For each isolate, 20 measurements were taken for the lengths and widths of the ascomatal base, neck, ascospores as well as for conidia and chlamydospores with ocular and stage micrometers. Rates of growth of different isolates were measured for each representative isolate grown on PDA and incubated at 25 °C.

Pathogenicity tests

Pathogenicity tests were performed on three months old seedlings of pomegranate cv. Bhagva maintained under glasshouse conditions. An approximate of 3 cm deep downward slanting cut was made from the outer bark to the inner wood with a sterile knife from the base of the plant. A volume of 0.2 ml of the inoculum (2.5×10^6 spores/ml) was applied into the wound and at the inoculation site, a 5 mm mycelium plug was inserted inside and the wound was covered with parafilm. Inoculation was done using the method of Harrington *et al.* (2005) [3]. The control plants were wounded and treated with the same volume of sterile distilled water. Plants were monitored daily and 25 days after inoculation, the wilting symptoms were observed. Parafilm was removed and the infected tissue was placed between two slices of carrot and incubated under humid conditions which resulted in growth of *C. fimbriata.* The pathogen was confirmed by transferring the ascospore mass developed on carrot slices to potato dextrose agar in Petri plates (De Vay & Moller & 1968) [7]. This was incubated at 25 °C and observed periodically for fungal growth and sporulation. Thus the symptoms produced by the pathogen were similar to those occurring in the original infection.

Molecular characterization of isolates

Genomic DNA isolation

Nine different *Ceratocystis fimbriata* isolates (Cf-B, Cf-Bg, Cf-C, Cf-Ch, Cf-H, Cf-K, Cf-R, Cf-T and Cf-V) from different districts of Karnataka state were used for DNA sequence comparisons. For DNA extraction, cultures were grown on 2per cent potato dextrose agar for two weeks. Mycelial masses were scraped off, placed in an Eppendorf tubes and freeze dried overnight. DNA extraction was performed as described by Van Wyk *et al*. (2006) [12]. The Internal Transcribed Spacer regions including the 5.8S rDNA operon were amplified using the polymerase chain reaction (PCR) and using primers ITS1 and ITS4 (White *et al*., 1990) [13]. Five ng of the DNA template was added to a 25 µl polymerase chain reaction (PCR) mixture containing 0.2 mM of each dNTP, 0.4 µM of each primer, 1 X buffer containing 1.5 mM MgCl2 and *Taq* enzyme (Genei Bangalore),

The PCR amplification consisted of an initial denaturation step at 95 °C for 90 seconds. This was followed by 35 cycles denaturation at 95 °C for 10 s, annealing for 10 s at 35°C and an elongation step for 30 s at 72 °C. Subsequently, 30 cycles consisting of 94ºC for 20 s, 55 ºC for 40 s with a 5 s extension step, after each cycle and 72 ºC for 45 s were performed with a final step of 10 min at 72 ºC. PCR products were visualized using UV light after separation on a 2 per cent agarose gel containing ethidium bromide.

Purification and quantification

The products were then purified using 6 per cent Sepha-dex G-50 columns (Steinheim, Germany). PCR products were sequenced using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems) and the same primers as used in the PCR reactions. Sequencing reactions were run on an ABI Prism 3100 DNA sequencer. Sequences for the *Ceratocystis fimbriata* from different districts were compared with those of closely related *Ceratocystis fimbriata* spp. obtained from the GenBank (National Centre for Biotechnology Information) nucleotide database (http://www.ncbi.nlm. nih.gov/) are published in previous studies (Barnes *et al*., 2003a; Marin et *al*., 2003; Van Wyk *et al*., 2006; Johnson *et al*., 2005) [1, 6, 12].

The molecular size of the amplified products was estimated using the ladder. Banding profiles for each primers were scored for the presence of similarities and pleomorphism among different isolates. The gene regions selected for sequencing were the Internal Transcribed Spacer region (ITS) one and four including the 5.8s rDNA operon. The primers selected for the PCR and sequencing reactions were ITS1 and ITS4 developed by White *et al.* (1990) [13]. For sequencing two separate reactions were used for the forward and reverse primers respectively.

RAPD primers

10 mm Tris-HCl; 50 mm KCl; 2´5 mm MgCl2; 0´2 mm each of dATP, dCTP, GTP and dTTP (Polymed), 0´2 mm primer (Genei, Bangalore), 1 U Taq polymerase (Polymed); 20 ng genomic DNA. The primers used were B10 (50-CTGCCTGGGAC-30), A3 (50-AGTCAGCCCAC-30), A9 (50-GGGTAACGCC-30) and A13 (50-CAGCACCCAC- 30) were selected. RAPD banding profiles for each primer were scored for the presence (1) or absence (0) of bands (Santini and Capretti 2000) [9]. The data matrix thus generated was used to calculate similarity coefficient for pair wise comparison. The cluster analysis was carried out using Minitab 16.0 software by a single linkage method for drawing distance matrix table as well as genetic distance cluster.

Results

Polymorphic markers

Results of this study provide clear evidence among the four primers tested *viz*., A3, A9, A13 and B10, all the primers showed amplification of the different isolates. The primer A3 and A9 showed better polymorphism between the isolates when compared to primers B10 and A13. The amplification of bands confirmed the identity of *C. fimbriata.* Out of the 28 marker loci amplified, 13 were found to be specific for 5 species and only two loci were common in all 10 isolates (Primers A3 and B10) indicating 83.20 per cent polymorphism. Genetic variation observed in the study was maximum where the primer A3 amplified a specific marker for Cf-Bg and Cf-K isolates separately. Similarly, the primer A13 amplified specific markers for the isolate from Cf-B. The primer B10 amplified specific markers for isolates from Cf-5 and Cf-K and the primer A9 was specific to the isolate from Cf-K. Finally, the primer ITS was specific to the isolate from Cf-H. Overall, maximum of 4 loci were specific to the isolate from Cf-H indicating the possibility of using these markers as DNA fingerprints for respective locations. Apart from these, three primers amplified common bands specifically in only two isolates *viz*., A3 in Cf-K / Cf-B and Cf-V and Cf-H; A13

in Cf-T / Cf-Bg, Cf-B and Cf-Bg / Cf-C ITS in Cf-T and Cf-K isolates.

Dendrogram

The cluster analysis was carried out using Minitab 16.0 software by a single linkage method for drawing distance matrix table as well as genetic distance cluster. The dendrogram obtained from RAPD analysis of nine different isolates of *C. fimbriata* indicated that there were three major clusters with the isolate from Cf-R grouped separately from others and hence, it was quite different from others with different alleles indicating high genetic variation. The isolates from Cf-C, Cf-H, Cf-K, Cf-T and Cf-B were grouped in one group forming the second cluster. While, the third cluster had a grouping of isolate from Cf-Bg, Cf-Ch and Cf-V (Fig.1)

In RAPD analysis, the average of polymorphism percentage ranged from 33.33 to 83.20 per cent. The lowest polymorphism percentage was recorded for primers B10 and A13. The highest polymorphism percentage was recorded for primers A3 and A9. The RAPD profiles showed a high level of genetic variability among the isolates of *C. fimbriata.* Hence, by this study it is proved that genetic variations prevail among the different isolates of *C. fimbriata* collected from different parts of Karnataka.

Genetic variations observed in the study was maximum between the isolates from Cf-R and Cf-H (83.20 per cent) closely followed by isolates Cf-R and Cf-Ch. Isolates Cf-Ch and Cf-H were genetically closer, followed by Cf-Bg and Cf-C than any other isolates. Genetic similarity of 65.0 per cent between Cf-B and Cf-T isolates were closely behind them. Isolates Cf-Bg was genetically closely related with Cf-C isolate (82.50 per cent). It was closely observed that isolates Cf-Bg and Cf-C and Cf-Ch and Cf-H which had a common geographical origin were genetically closer. The isolates Cf-B and Cf-T have high genetic similarity. However, isolate Cf-R was equidistant from rest of the isolates used in the study.

Characterization of different isolates using universal primers

Nine different isolates were further characterized using ITS gene technology and after 35 cycles of PCR amplification universal primers were able to successfully amplify the entire ITS region and produced an amplicon of size 400 bp. The ITS region of rDNA was sequenced using the procedures of Baker and Harrington (2005) [3]. The analysis pertaining to the sequences obtained was carried out using various BLASTn analysis available online. Analysis of ITS revealed its homology with various other ITS gene sequences. Characterization of different isolates on basis of the ITS gene coding genes revealed that it showed maximum similarity with *Ceratocystis fimbriata* species deposited in Gen-bank around the world. The sequences of nine different isolates were deposited in the Gen-bank and the accession numbers obtained for the same are listed in table 2.

Discussion

Analysis of ITS revealed its homology with various other ITS gene sequences for different isolates revealed that it showed maximum similarity with *Ceratocystis fimbriata* species deposited in Gen-bank around the world. The sequences of different isolates were deposited in the Gen-bank and the accession numbers were obtained for the same. The present results is in agreement with the findings of Engelbrecht and Harrington (2005) [3] who reported that ITS region had 488 nucleotide base pairs and it was closely related (99%) to the type strain of *Ceratocystis paradoxa* (GenBank Accession Number JQ418543.1) by Blast analysis. The phylogenetic analysis revealed that 525 bps sequence of ITS region of *Hypocrea virens* MAPSC2 was similar (100 %) to the existing species of *Hypocrea virens* (GenBank Accession Number JQ418544.1).

The cluster analysis carried out to know the genetic variability indicated that there were three major clusters with the isolate (Cf-R) grouped separately from others and hence, it was quite different from others with different alleles indicating high genetic variation. The isolates from Cf-C, Cf-H, Cf-K, Cf-T and Cf-B were grouped in one group forming the second cluster. While, the third cluster had a grouping of sample from Cf-Bg, Cf-Ch and Cf-V. The present findings are supported with the results of Santini and Capretti (1998, 2000) [8, 9] who reported the genetic variability of *C. fimbriata platani* in Italian populations using RAPD and mini satellite markers and compared with a number of *C. fimbriata* isolates from various hosts growing in different parts of the world. A high level of homogeneity was revealed in the Italian population, whereas, certain variability was recognized in isolates from others hosts. The Italian population appears to be clonal, with the same genotype occurring throughout the country.

Maximum genetic variation was observed between Cf-R and Cf-H closely followed by Cf-R and Cf-Ch. Isolates Cf-Ch and Cf-H were genetically closer, followed by Cf-Bg and Cf-C than any other isolates. Isolates Cf-Bg was genetically closely related with Cf-C isolate. It was closely observed that isolates Cf-Bg and Cf-C and Cf-Ch and Cf-H which had a common geographical origin were genetically closer. The isolates Cf-B and Cf-T have high genetic similarity. However, Cf-R was equidistant from rest of the isolates. The present findings are in accordance with the findings of Huang *et al*., (2003) [4] who reported that analyses of 35 isolates with 14 microsatellite markers revealed that the Yunnan population was nearly uniform, consisting of only 19 alleles and seven closely related genotypes, suggesting that the population was not natural and was the result of an introduction. As in comparisons of sequences of ITS rDNA and mating type genes, the microsatellite alleles of the Yunnan isolates were most similar to those of eucalyptus isolates from Minas Gerais and Bahia, Brazil.

Table 1: Place of collection and designation given to individual isolates of *C. fimbriata* (Cf)

Sl. No.	Location	Designation to isolates
1	Bellari	Cf-B
2	Bagalkot	Cf-Bg
3	Chickmagalur	Cf-C
4	Chitradurga	Cf-Ch
5	Hassan	Cf-H
6	Koppal	Cf-K
7	Raichur	Cf-R
8	Tumakuru	Cf-T
9	Vijayapura	Cf-V

Table 2: Accession numbers obtained for different isolates of *C. fimbriata* deposited in Gen-bank (NCBI)

Sl. No.	Designation of isolates	Accession numbers
1	Cf-B	KY450792
2	Cf-Bg	KY451453
3	Cf-C	KY451620
4	Cf-Ch	KY451726
5	Cf-H	KY465500
6	Cf-K	KY465501
7	Cf-R	KY465502
8	Cf-T	KY465503
9	Cf-V	KY465504

Table 3: Similarity of different isolates of *C. fimbriata* and those reported in the database (NCBI) of computer program "BLAST"

Isolate	*C. fimbriata* closest match	Per cent similarity	Accession number
Cf-B	*Cf* strain PP02, C1839, C1534, C144, CMW 5312 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	90 %	KJ 499362 HQ157547
Cf-Bg	*C f* YMY061, PF02, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence Strains UHS *Cf*-24, 23,12	99 % 99 %	AM712446 K1469362 KU877208 KU877207
Cf-C	*Cf* YMY061 internaltranscribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete equence Strains UHS *Cf*-24, 23 and12	99 % 99%	AM712446 KU877208, KU877207
f-Ch	NRCP-*Cf* 22, *Cf* 24, *Cf* 19 internal transcribed spacer 1, partial sequence; 5.8Sribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence Strains UHS *Cf* 20, 19, 18, 16,	93 % 93 %	KU877210, KU877211, KU877203 KU877202
Cf-H	*C f* isolate HF-31, 29, 28, 22 internal transcribed spacer 1, partial sequence; 5.8Sribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	97%	KU204826 KU963165 KT963163,
Cf-K	*C f* Win(m) 921 isolate PB67 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence Strains UHS-*Cf*-6	98 % 94 %	DQ318204 KU877190
Cf-R	*Cf* YMY061 internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence Strain PP02 Strain UHS *Cf* 24, 23 12	99 % 99 %	AM712446 KJ469362 KU877208, KU877207
Cf-T	Internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence *Cf* NRCP- *Cf* 22, 24,19 UHS *Cf*- 20,19,18,16,3 and 24	95% 95%	KU877210 KU877211 KU877212 KU877204,
Cf-V	*Cf* NRCP Cf26 internal transcribed spacer 1, partial sequence; 5.8Sribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence UHS strains Cf - 17,19,18,16,3 and24	91% 91 %	KU877212 KU877201 KU877204 KU877203, KU877202,

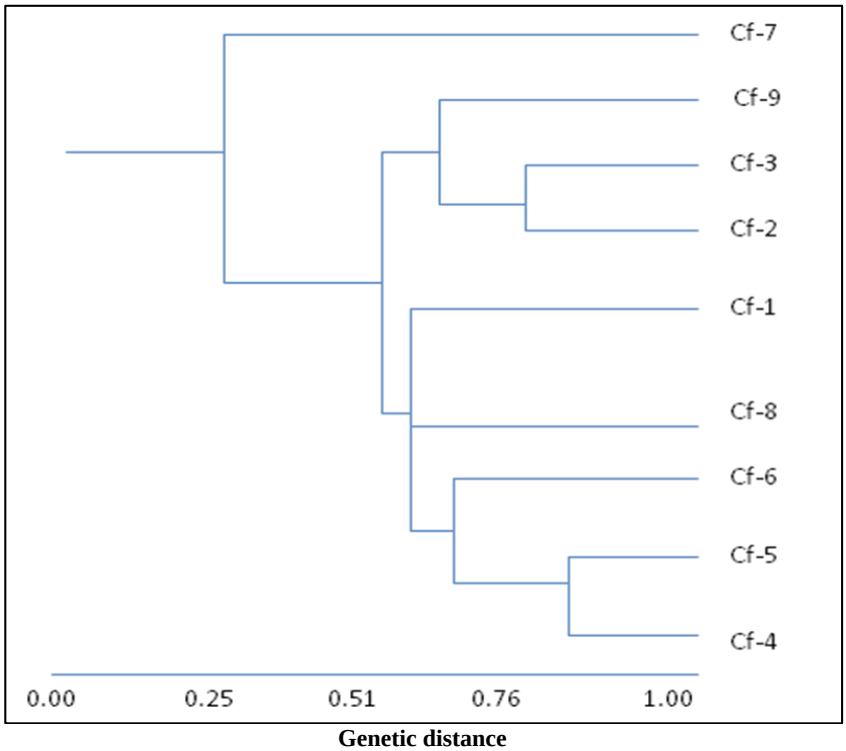

Fig 1: Dendrogram showing relationship between different isolates of *C. fimbriata* based on RAPD analysis

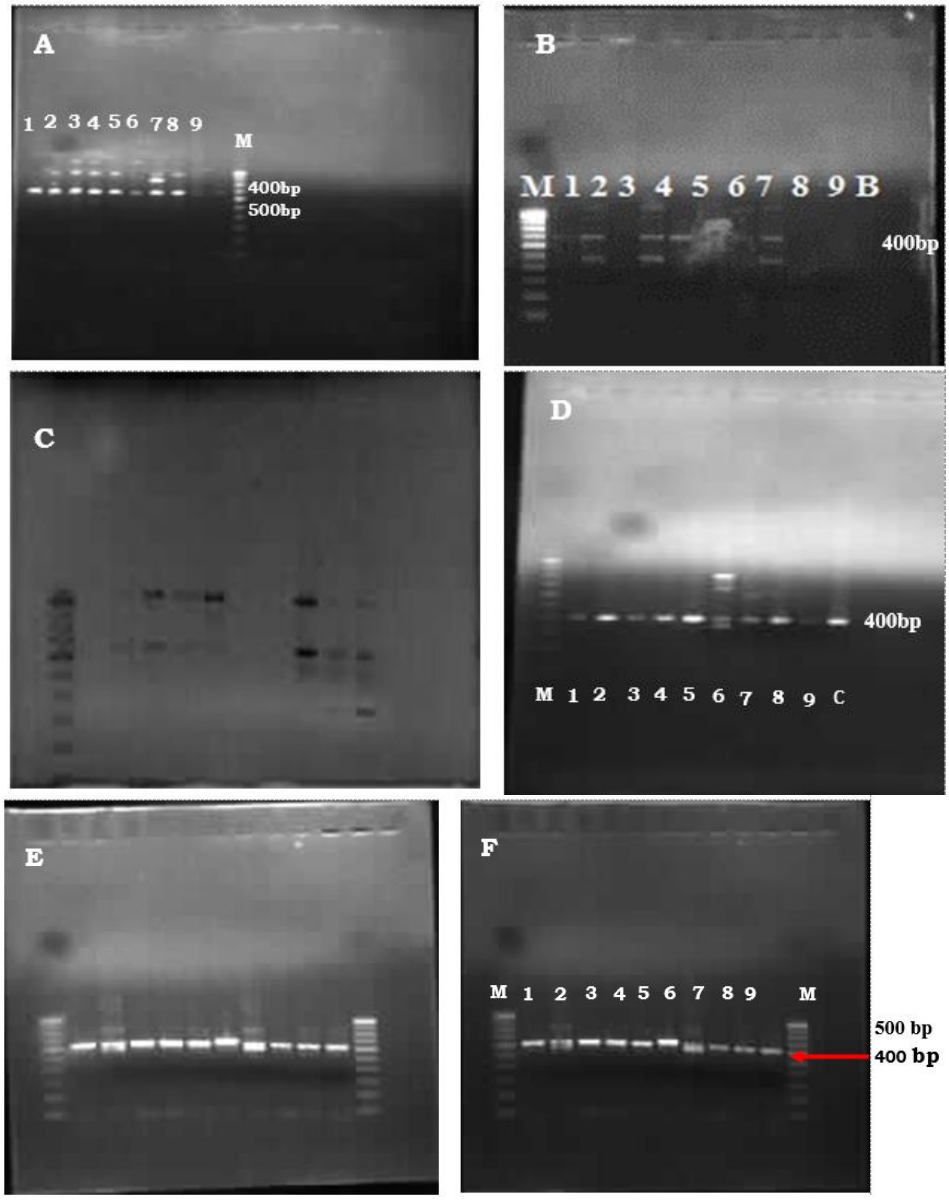

Fig 2: Polymorphism between the isolates of *C. fimbriata* by RAPD primers A) A3 primer B) A9 primer C&D) B10 primer E&F) ITS primer

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