GENETICAL VARIABILITY AMONG *CERATOCYSTIS FIMBRIATA* ISOLATES CAUSING WILT DISEASE OF POMEGRANATE

Abstract: Pomegranate (*Punica granatum* L.) is an important fruit crop farmed all over the world, notably in the tropical and subtropical zones. It is afflicted by a number of diseases, the most serious of which is *Ceratocystis fimbriata*. Very little work is done on the genetical variability of *C. fimbriata* associated with pomegranate wilt in Karnataka and the morphological structures defining this species are reasonably defined, these characteristics are insufficient to recognize emergent grouping within *C. fimbriata*. The aim of this study is to confirm the identity of *C. fimbriata* isolates from pomegranate in Karnataka and to compare the DNA sequence of the internal transcribed spacer (ITS-1 and 4 regions) variability is the property of an organism to change its characters from one generation to the other. there is a need to study *C. fimbriata* based on molecular variability. Fifty isolates of *C. fimbriata* were amplified with a range of 600-650 bp length, twelve isolates were sequenced and deposited in the GenBank Maryland, USA database under the accession number KY038512- KY038523. All isolates from different districts of Karnataka show specific patterns of similarity according to geographical region.

Key words: Pomegranate; *Ceratocystis fimbriata*; molecular variability; isolates; accession number

1. INTRODUCTION

"Pomegranate (*Punica granatum* L.) is an ancient fruit, that belongs to the family lythraceae. Pomegranate is native to Iran, where it was first cultivated in about 2000 BC and spread to the Mediterranean countries. It is cultivated in India, Iran, China, Turkey, USA, Spain, Azerbaijan, Armenia, Afghanistan, Uzbekistan, the Middle East, Pakistan, Tunisia, Israel, dry regions of Southeast Asia, Peninsular Malaysia, the East Indies, and tropical Africa". [8] Pomegranate cultivation is expanding globally because to its hardiness, adaptability, drought tolerance, increased output levels with outstanding keeping quality, and remunerative prices in both domestic and export markets. It thrives in the dry tropics and subtropics and grows well on low fertility soils as well as saltwater soils. India is the world's biggest producer of pomegranates. **It is one of the most adaptable subtropical fruit crops. In India it is regarded as a "vital cash crop", extensively grown in Maharashtra, Karnataka, Andra Pradesh, Telangana, and Gujarat, and is picking up fast in Himachal**

Pradesh, Rajasthan, and Madhya Pradesh. Small areas are under cultivation in Tamil Nadu, Mizoram, Odisha, Nagaland, Lakshadweep, Jharkhand and Jammu Kashmir. total area under pomegranate in India is 1,80,640 ha out of which 1,28,650 ha is in Maharashtra only. The total production in India is 17,89,310 metric tons and 11,97,710 metric tons in Maharashtra. In Karnataka, the total area is 23,230 ha with production of 2,61,820 metric tonnes (http://nhb.gov.in).

"In Karnataka, the crop has spread across different districts *viz.*, Vijayapura, Bagalkot, Koppal, Yadgir, Raichur, Ballari, Chitradurga, Tumakuru, and Hassan. The most popular varieties suitable for processing and table use are Ganesh, Mridula, Arakta, Bhagwa (Kesar), G-137 and Khandar. Successful cultivation of pomegranates in recent years has been threatened by different pests and diseases. Bacterial blight, wilt, anthracnose, leaf spot, and root-knot nematode are important diseases. Among them, wilt caused by *Ceratocystis fimbriata* Ell. and Halst. is an emerging threat. At present the crop is severely affected by wilt pathogen and day by day the wilting severity is increasing at a faster rate. It was first noticed in some areas of Vijayapur districts of India during 1990. By 1993, the rapid spread of this disease was observed in the entire Vijayapura district. The cause was not identified until 1995; however, in 1996 the fungus *C. fimbriata* was isolated from discolored stem, root, and branch tissues on wilting plants. Disease is characterized by initial symptoms of yellowing and wilting of leaves on one to several branches leading to the death of affected plants in a few weeks. Cross sections of diseased plants revealed brown discoloration in the outer xylem from the roots to the main trunk" [8].

"The disease is prevalent in parts of Maharashtra, Karnataka, Telangana, Gujarat, and Tamil Nadu states" [3]. "Despite many factors conducive to the high severity, seedlings' selection for planting, soil-borne nature, and also an association with shot hole borer and plant parasitic nematodes is noticed. This might be the reason for the current rampant spread of the disease in south Indian states. Several agents are known to cause wilt in pomegranate, but *C. fimbriata* is the major cause, hence, emphasis should be on *C. Fimbriata"*. [6, 7] Very little work has been done on the characterization of *C. fimbriata* associated with pomegranate wilt in Karnataka. Although the morphological structures defining this species are reasonably defined, these characteristics are insufficient to recognize emergent grouping within *C. fimbriata*. In recent years, several orchards of farmers have been severely infected by wilt and were removed in Karnataka state. This may be due to changes in the pathogenic

characteristics of the fungus. Moreover, variability is the property of an organism to change its character from one generation to the other. The aim of this study is to confirm the identity of the *C. fimbriata* pathogenic isolates in the major pomegranates-growing areas of Karnataka, to explore their genetic diversity to compare the DNA sequence of the internal transcribed spacer (ITS-1 and 4 regions), and to provide the eco-friendly management methods of *C. fimbriata* infections in pomegranate.

2. MATERIAL AND METHODS

2.1 General laboratory procedure

2.1.1 Cleaning of glassware

Borosil and Corning glassware were used for all the laboratory experimental studies. They were kept for a day in a cleaning solution, prepared by dissolving 60 g of potassium dichromate ($K_2Cr_2O_7$), and 60 ml of concentrated sulphuric acid (H_2SO_4) in one liter of water. Each of these chemicals dissolved separately in 500 ml of water and finally mixed. Then glassware was cleaned by washing it with a detergent solution followed by tap water and finally rinsing in distilled water.

2.1.2 Sterilization

All the glassware used in the study wrapped were sterilized in an autoclave at 15 p.s.i pressured for 20 minutes and kept for drying in a hot air oven at 60^{o} C for two hours. Both solid and liquid media were sterilized at 15 p.s.i pressure for 15 minutes.

2.2.1 Isolation of the pathogen

"*Ceratocystis fimbriata*, associated with wilt was isolated from the infected roots of the pomegranate plant which were collected from Ganjalli field. The sliced pieces of collected stem portions with characteristic symptoms of vascular staining were surface sterilized with 1 per cent $NaHCO_3$ (sodium hypochlorite) for about 2 minutes and washed in alcohol (70%) and twice with sterile water to remove traces of $NaHCO_3$. Pathogen isolation was made using the carrot bait technique in which, stems were placed in between the carrot disks kept in a humid chamber, and incubated at 25 ± 2 °C under 12-hour photoperiod" [4]. "After perithecium formation, a portion of the fungi was transferred to freshly prepared PDA and oat meal agar media to allow the full development of fungi. In order to confirm the identity of the fungus, the ascospores, aleroconidia, endoconidia, and perithecia were

observed under the high power (40x) microscope from Raichur isolates the pure culture. The identification of studies of pathogens has been done" as explained by Sharma *et al*. [7].

2.2.2 Hyphal tip isolation

"This method was followed to maintain pure culture. Hyphal tip isolation was done on water plates. Dilute spore suspension of the pathogen was prepared in sterilized distilled water containing eight to ten spores per ml from a 15-day-old culture. One ml of such suspension was spread uniformly on two per cent solidified water agar plates and observed for spores under the microscope. A single spore was marked with a marker on the backside of the Petri plate and it was allowed to germinate. Such plates were periodically observed for spore germination under the microscope. The hyphae growing from each cell of the single spore were traced and marked with a marker. The tip of the hyphae was cut carefully transferred to PDA plates and incubated at 25 ± 2 ^{o}C for 15 days. Later, mycelial bits of the fungus were transferred to the center of Petri plates containing PDA and incubated at 25 ± 2 ^{o}C for 15 days. Saltation or sectoring was observed in the culture to confirm the pure culture of the fungus". [7]

2.2.3 Maintenance of the Culture

The hyphal tip cultures of the fungus were sub-cultured on potato dextrose agar slants and kept in the laboratory at 25 ± 2 ^{o}C for 15 days. Such mother culture slants were preserved at 5 ^{o}C in the refrigerator. Further, these cultures were sub-cultured once a month and used for future studies.

2.3.1 Pathogenicity

Pathogenicity tests were conducted on six-month-old seedlings of pomegranate *cv*. Kesar was raised in plastic pots (30 x 45 cm). The potting mixture was sand: red soil: FYM (1:2:1) and it was tyndallized in an autoclave at 1.1 kg/cm^2 (121 ^{0}C) pressure for 30 min. successively for two days. Wounds of 1 mm depth x 0.5 mm width were made on the epidermis of the roots with a sterilized razor blade. The wounded area in each plant was inserted with *C. fimbriata* culture utilizing a disinfected needle and wrapped with cotton material (saturated with sterile refined water) and plastic film. The method was replicated thrice with inoculation on the other two plants under glasshouse conditions. Plants that were inoculated with distilled water served as control. The inoculated plants were kept in a glass house (average temperature of 27 °C) for further observation. When plants were inoculated

with the pathogen and started to express symptoms, symptoms plants were collected for re-isolation on PDA culture for Koch's postulates prove.

2.4.1 Studies on molecular-level confirmation of *C. fimbriata*

Molecular level confirmation of fifty isolates of *C. fimbriata* was studied by ITS rDNA conserved region. Fifty samples were collected from nine pomegranate-growing districts of Karnataka during the survey. The isolates were obtained by tissue isolation using the carrot bait technique followed by inoculation on oatmeal agar. Fifty isolates were obtained from such samples and designated as Cf-1 to Cf-50 for variability studies (Table 1). GenElute plasmid miniprep kit method was followed to isolate fifty samples of genomic DNA of each isolate of *C. fimbriata*, with minor modifications (Sigma, USA). All the steps were carried out at room temperature as per the protocol described below [9].

Table 1. Designation of *C. fimbriata* isolates of pomegranate wilt collected from different districts of Karnataka

Sl. No.	Name of the Place		Designation of the isolate
	District	Village	
1	Viajaypura	Kumtagi	Cf-1
2		Babaleshwar	Cf-2
3		Hittinhalli	Cf-3
4		Jumnal	Cf-4
5		Kannollo-1	Cf-5
6		Devara hippargi-1	Cf-6
7		Bandal	Cf-7
8	Bagalkot	Devanal	Cf-8
9		Govindkoppa	Cf-9
10		Kaladgi-1	Cf-10
11		Lokapur-1	Cf-11
12		Mahalingapur-1	Cf-12
13	Koppal	Kalkbandi	Cf-13
14		Kamanur	Cf-14
15		Kustgi	Cf-15
16		Maladgatti-1	Cf-16
17		Kodkera	Cf-17

18	Yadgir	Gogi K	Cf-18
19		Wandurga-1	Cf-19
20		Tumkur	Cf-20
21		Heggandoddi-1	Cf-21
22		Chincholi-1	Cf-22
23	Raichur	Yatgal	Cf-23
24		Chandrabanda	Cf-24
25		Karekal	Cf-25
26		Ganjhalli-1	Cf-26
27		Kurkihalli	Cf-27
28		Benkal	Cf-28
29		Arkera-1	Cf-29

Contd….

Sl. No.	**Name of the Place**		**Designation of the isolate**
	District	**Village**	
30	Ballari	Kampli	Cf-30
31		Lakshmipura	Cf-31
32		Khondanhalli	Cf-32
33		Thambrahalli	Cf-33
34		Basarkodu	Cf-34
35	Chitradurga	Sirana hatti-1	Cf-35
36		Ramajjanahalli	Cf-36
37		Nagayana hatti-1	Cf-37
38		Maskal-1	Cf-38
39		Seerana katte-1	Cf-39
40		Shraranagar	Cf-40
41	Tumakur	Madana kunte-1	Cf-41
42		Karekyatana halli	Cf-42
43		Chikka halikute-1	Cf-43
44		Thogargunte-1	Cf-44
45		Hosahali	Cf-45
46	Hassan	Mylanahalli-1	Cf-46
47		Nadakihalli	Cf-47

48		Chika bidane-1	Cf-48
49		Haranhalli-1	Cf-49
50		Goran koppal-1	Cf-50

2.5.1. Disrupting cells

- Grinding fungal culture into a fine powder in liquid nitrogen using a mortar and pestle
- Transferring up to 100 mg of the powder to a microcentrifuge tube
- Keeping the sample on ice for immediate use or freeze at -20 ^{0}C for overnight or 18-20 hrs

2.5.2. Lysis cell

- Adding 350 µl of lysis solution (RNase A Solution) and 50 µl of lysis solution (Lysis solution B) to the tube
- Thoroughly mixing by vortexing and inverting. A white precipitate will form upon the addition of lysis solution (Lysis solution B)
- Incubating the mixture at 65 ^{0}C for 30 min with occasional inversion to dissolve the precipitate

2.5.3. Precipitating debris

- Adding 130 µl of precipitation solution to the mixture mix completely by inversion and place the sample on ice for 5 minutes
- Centrifuging the sample at maximum speed (12,00-16,000 rpm) for 5 minutes to pellet the cellular debris, proteins, and polysaccharides

2.5.4. Filtrating

- Carefully pipetting the supernatant from step 3 onto a gen elute filtration column (blue insert with 2 ml collection tube)
- Centrifuging at maximum speed for 2 minutes removes any cellular debris which is not removed in step 3
- Discarding the filtration column but retaining the collection tube

2.5.5. Preparation for binding

- Add 700 µl of binding solution directly to the flow-through liquid from step 4 and mix thoroughly by inversion

2.5.6. Prepare binding column

- Insert a genelut mini prep binding column (with a red o-ring) into a provided microcentrifuge tube if not already assembled. Later, add 500 µl of the column preparation solution at 12,000 rpm for 30 seconds to 2 minutes and discard the flow-through liquid. It is to be noted that the column preparation solution maximizes the binding of DNA to the membrane resulting in more consistent yields.

2.5.7. Loading lysate

- Carefully pipetting 700 µl of the mixture from step 5 onto the column prepared in step 6 and centrifuge at maximum speed for 2 minutes
- Discarding the flow-through liquid, retain the collection tube
- Returning the column to the collection tube
- Appling the remaining lysate from step 5 onto the column, repeating the centrifugation as above, and discarding the flow-through liquid and collection tube

2.5.8. First column wash

- Prior to first-time use, we have to be sure to add ethanol to the wash solution concentrate. Placing the binding column into a fresh 2 ml collection tube and applying 500 µl of the diluted wash solution to the column
- Centrifuging at maximum speed or 2 minutes
- Discarding the flow-through liquid but retaining the collection tube

2.5.9. Second column wash

- Appling another 500 µl of diluted wash solution to the column and centrifuging at maximum speed for 3 minutes to dry the column

- We should not allow the flow-through liquid to contact the column, wipe off any fluid that adheres to the outside of the column
- Emptying wash for 1 minute

2.5.10. Elute DNA

- Transferring the binding column to a fresh 2 ml collection tube
- Applying 100 µl / 40 µl of pre-warmed (65 ^{o}C) elution solution to the column and centrifuging at maximum speed for 1 minute
- Repeating the elution and we should not allow the flow-through liquid to contact the column
- Eluates are to be collected in the same collection tube
- The eluates contain pure genomic DNA for short-term storage of DNA, 2-8 ^{o}C is recommended for long-term storage of DNA kept at -20 ^{o}C.

Quantification of DNA was performed by measuring the absorbance at 260 nm and the purity was analyzed by A_{260}/A_{280} ratio by Nanodrop (Thermo Scientific).

2.5.11Primers

"The rDNA gene cluster, consisting of ITS-1, the 5.8 S rDNA, and ITS-4, was amplified with primers homologous to conserved sequences within the small subunit (SSU) rDNA gene. The ITS primers used were ITS-1 (TCCGTAGGTGAACCTGCGG) as the forward primer and ITS-4 (TCCTCCGCTTATTGATATGC) as the reverse primer" [10].

PCR was performed in a total volume of 25 µl containing 2 µl of 10 X 3 PCR buffer (100 mM, Tris-HCl, pH 8.3, 250 mM KCl), 15 mM $MgCl_2$ 1µl, 1U Taq DNA polymerase 0.33 µl (Bangalore Genei, India), 160 µM dNTP mixture 2 µl, 20 pmol of each ITS-1 1µl and ITS-4 1µl primers, and 50 ng genomic DNA 2 µl in sterile dH2O 15.67 µl. The PCR amplifications were performed by using a thermal cycler (Mastercycler) programmed for initial DNA denaturation at 94 ^{0}C for 4 min 1 cycle, denaturation at 94^{0} C for 30 seconds 35 cycles, annealing at 52^{o} C for 45 seconds 35 cycles, and extension at 72 ^{0}C for 1 min 35 cycles, with a final extension step at 72 ^{0}C for 5 min.

2.5.12. Analysis of genomic DNA by agarose gel electrophoresis

The genomic DNA was analyzed by agarose gel (0.8%) electrophoresis as described below.

2.5.12.1. Materials and solutions

1. Agarose (Himedia, Mumbai, India).
2. TAE 50 X buffer: Tris base- 24.2 g; glacial acetic acid- 5.71 ml; 0.5 M EDTA (pH 8.0) 10 ml, distilled water-vol to 80 ml. The pH was adjusted to 8.0 and the final volume was brought to 100 ml with distilled water. The buffer was filtered and sterilized by autoclaving and stored at room temperature.
3. Gel casting boat
4. Mini gel apparatus and power supply (Bangalore Genie, India).
5. Ethidium bromide stock solution (10 mg ml^{-1}):10 mg of ethidium bromide (Sigma, USA) was dissolved in 1 ml of distilled water. The solution was stored in a microcentrifuge tube wrapped in aluminium foil at 4 $^{\circ}$C.

2.5.12.2. Methodology

The boat was sealed with adhesive tape and the comb was placed for the wells. For a gel, 0.8 g of agarose was added to 100 ml of 1 X TAE buffer. The mixture was heated on a hot plate to allow the agarose to dissolve. 1 µl of EtBr stock (10 mg mL^{-1}) was added to the gel matrix when the matrix was approximately 60°C, mixed, and poured into the sealed boat. The gel was allowed to polymerize. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank with a sufficient volume of 1 X TAE buffer to cover the surface of the gel. The PCR reaction sample and the standard DNA size marker were loaded into the wells. Electrophoresis was carried out at 70 volts till the dye reached $3/4^{th}$ of the gel. The gel was examined on a UV transilluminator and documented using the Gel Documentation system (Carestream Gel Logic 212 PRO, Molecular imaging, USA) and photographs were taken by using the gel documentation system.

2.6.1 Sequencing of ITS region

Twelve isolates out of fifty isolates of *C. fimbriata* were selected based on the representation of geographic regions sequencing of ITS region. Sequencing

was carried out using the Sanger sequencing method (Eurofins Genomics India Pvt. Ltd., Bangalore, India). The resulting ITS sequences were analyzed for homologies in NCBI BLAST database. Based on previously published database sequences, sequences were deposited in the GenBank to get accession numbers. Online software UPGMA was used to construct the phylogenic tree using the maximum likelihood method (http://upgmasoftware.net).

3. RESULT

3.1 Molecular-level confirmation of *C. fimbriata* isolates

The molecular characterization and variability of fifty isolates were carried out as mentioned in the material and methods. All fifty isolates were characterized using ITS gene technology and after 35 cycles of PCR amplification, universal primers (ITS1 and ITS 4) were able to successfully amplify the entire ITS region and produced an amplification of size 600-650 bp length in all the fifty isolates indicating that all the isolates belong to genus *Ceratocystis* thus confirming the identity of all isolates (Plate 1).

Twelve isolates of *C. fimbriata* were selected out of fifty isolates based on the representation of geographic regions. Further, the sequencing of isolates was done at Eurofins Genomics India Pvt. Ltd., Bengaluru. The analysis pertaining to the sequences, so obtained, was carried out using various NCBI BLAST analyses available online. Analysis of ITS revealed its homology with various other ITS gene sequences. Characterization of the twelve isolates on the basis of the ITS gene coding genes revealed maximum similarity (99%) with *Ceratocystis* species. The resemblance of obtained ITS sequences with the analogs available in the database of the computer program "BLAST" is presented in Table 2. The sequences of twelve isolates are given below.

Cf-1

GTGGTCACCGAGTTAATGTACTCTATAACCATGTGTGAACGTACCTATCTTGTAGTGAGATG
AATGCTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTAGTCT
TCGCCACTGTAAACTCTTTTTATTATTTTCTAGATTTTTCATTGCTGAGTGGCATAACTATA
AAAAAAGTTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCTTT
TGTTCTTGGCGTTGGAGGTCCTGTTCTCCCCTGAACAGGGCGCCGAAATGTATCGGCTGTTA

TACTTGCCAACTCCCCTGTGTAATATAAAATTTCTAATTTTTACACTTTGAAGTTCTGGTGT
AACACCCCGCTAAACCCGCTCAACTTTTGTTGAAGTTTTCTCACAGTTGGGCTCTGAGGAGG
TAGGAATACCCGCTGAGATCCCCCATATCATACCAGGGAAGAGGAAAACCTATTAT

Cf-9

ATGTCCTGACTTTGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAATGC
TGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTAGTCTTCGCC
ACTGTAAACTCTTTTTATTATTTTCTAGATTTTTCATTGCTGAGTGGCATAACTATAAAAAA
AGTTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCT
GGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCTTTTGTTC
TTGGCGTTGGAGGTCCTGTTCTCCCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACTT
GCCAACTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAACAC
GCCGCTAAACCCGCTCAACTTTTGTTGAACTTTTCACAAGGTTGACCTCGGATCAGGTAGGA
ATACCCGCTGAACTTAAGCATATCATAAGCCGGGAAAGGAAAAATATTCTCCCTA

Cf-10

GTGCCCAGACTCTGTACTCTATAACCATGTGTGAACGTACCTATCTTGTAGTGAGATGAATG
CTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTAGTCTTCGC
CACTGTAAACTCTTTTTATTATTTTCTAGATTTTTCATTGCTGAGTGGCATAACTATAAAAA
AAGTTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
TGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCTTTTGTT
CTTGGCGTTGGAGGTCCTGTTCTCCCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACT
TGCCAACTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAACA
CGCCGCTAAACCCGCTCAACTTTTGTTGAACTTTTCACAAGGTTGACCTCGGATCAGGTAGG
AATACCCGCTGAACTTAACCATATCATCAACCCGGGAAAAGGAAAACTTATGAGTTT

Cf-14

TTGTCACCTATTACTGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAAT
GCTGTTTTGGTGGTAGGGCCCTTCTGAAGGGAGGGCACCGCTGCCAGCAGTATAGTCTCACC
ACTATGAACTCTTTTTATTATTTTCTAGATATTCATTGCTGAGTGGCATAACTATAAAAAAG
TTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCACCGAAATGCGA
TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCTGG
CAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCCTTTGTTCTT
GGCGTTGGAGGTCCTGTTCTACCCTGAACAGGCCGCCCAAATGTATCGGCTGTTATACTTGC

CAACTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAACACGC
CCCTCAACCCTCACCTTTTGTTGAACTTTCTCAAGGTTGACCTCGGATCAAGTAGGAATACC
CACTGAACTTACTCATATCATACCCGGGAGAAAAAAAAAAATAGGGGTTTGTC

Cf-20

GTGGCCCGACTTTTGTACTCTATAACCATGTGTGAACGTACCTATCTTGTAGTGAGATGAAT
GCTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTAGTCTTCG
CCACTGTAAACTCTTTTTATTATTTTCTAGATTTTTCATTGCTGAGTGGCATAACTATAAAA
AAAGTTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGAAAT
GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC
CTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCTTTTGT
TCTTGGCGTTGGAGGTCCTGTTCTCCCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATAC
TTGCCAACTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAAC
ACGCCGCTAAACCCGCTCAACTTTTGTTGAACTTTTCACAAGGTTGACCTCGGATCAGGTAG
GAATACCCGCTGAACTTCCCCATATCATAGCCCGGGAGGGGAAAACGTTCGGGTTTG

Cf-23

GTGGTCCGACTTTGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAATGC
TGTTTTGGTGGTAGGGCCCTTCTGAAGGGAGGGCACCGCTGCCAGCAGTATAGTCTCACCAC
TATAAACTCTTTTTATTATTTTCTAGATATTCATTGCTGAGTGGCATAACTATAAAAAAGTT
AAAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCTGGCA
GTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCCTTTGTTCTTGG
CGTTGGAGGTCCTGTTCTACCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACTTGCCA
ACTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAACACGCCG
CTAAACCCTCAACTTTTGTTGAACTTTCACAAGGTTGACCTCGGATCAGGTAGGAATACCCG
CTGAACTTAAGCATATCACAAACCCGGGGAGAAAGAAATCATTACTGAGTTTTGTACTCTAA

Cf-26

GGGCACGATTCTGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAATGCT
GTTTTGGTGGTAGGGCCCTTCTGAAGGGAGGGCACCGCTGCCAGCAGTATAGTCTCACCACT
ATAAACTCTTTTTATTATTTTCTAGATATTCATTGCTGAGTGGCATAACTATAAAAAAGTTA
AAACTTTCAACAACGGATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCTGGCAG
TATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACTCCTTTGTTCTTGGC
GTTGGAGGTCCTGTTCTACCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACTTGCCAA
CTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAACACGCCGC

TAAACCCTCAACTTTTGTTGAACTTTCACAAGGTTGACCTCGGATCAGGTAGGAATACCCGC
TGAACTTACGCATATCATAACCCGGGGAGAGAGAAAATCGTACTGAGTTTTGTAC

Cf-31

GTGGCCCTGGTTCTGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAATG
CTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTATTCCTCCC
CACTGAAAACTCTTTTTATTATTTTCTATATTTTTCGTTGCTGAGTGGCATAACTATAAAAA
AAGTTAAAACTTTCAACAACGGATCTCTTGGCTCTACCATCAATAAAGAACCCAACGAAATG
CAATAAGGAGTGTGAATTGCATAATTCAATGAATCAACCAATCTTTGAACGCTGATTGCGCC
TGGATTTATTCTGGCAGGCATGCCTGTCCGAGCGTTATTTCACCACTAAACACTCTTTTGTT
CTTGGCGATGGAGGTCCTGTTCTCCCCTGAACAGGCCACCTAAATGTACCGGCTGTTATACT
TGCCCCCCCCCCTGTGTAATAAAAAATTTCTTTTTTTTACTCTTTTAAGTTCTTGTGTAACA
CCCCACTAAACCCCCTTTTTTTTTGTTTTTTTTTTCAGATGGCCGACCACGAAGGAAGAAAA
ACTCCCCGCTTTTCTCCCATTTATTCAAAAAAAAAAAAAAAAAACGATATCTTTGC

Cf-33

TGTCCTGATTTTGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAATGCT
GTTTTGGTGGTAGGGCCCTTCTGAAGGGAGGGCACCGCTGCCAGCAGTATAATCTCTCCACT
ATGAACTCTTTTTATTATTTTCTAGATATTCATTGCTGAGTGGCGTAACTATAAAAAAGTTA
AAACTTTCTACAACGGATCTCTTGGCTCTCTCATCCATCAATAAAGCACCGAAATGCGAGAA
ATAATGTGAATTGCTTAATTCATTGAATCATCCAATCTTTGAACGCACATTGCGCCTGGCAG
TATTCTGCCAGGCATGCCTGTCCGACCGTCATTTCACCACTCAAGACTCCTTTGTTCTTGGC
GTTGGAGGTCCTGTTCTACCCTGAACAGGCCGCCCAAATGTATCGGCTGTTATACTTGCCTA
CTCCCCCGTGTGTTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAACACACCCC
TCAACCCTCCCCTTTTTTTTAAATTTCTTTCCGTTGACCGCCGACCAAGTAGGAAGAACCAC
TGACCTTATTCATATCACAATCCGAAGGAAGAAAAAAAATATGGCTTTCTCTCCTTGC

Cf-38

GGGTCCCTGACTTATGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAAT
GCTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTATTCTTCG
CCACTGAAAACTCTTTTTATTATTTTCTATATTTTTTGTTGATGAGTGGCACAACTATAAAA
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GCAATAATGAGTGTTAATTGCATAATTGAATGAATCAACCAATCTTTGAACGCTGATTGCGC
CTGGATTTATTCTGGCAGGCCTGTCTGACCGAGCGTTCTTTCACCACTAAACACTTTTTTGT
TCTTGGCGATGGAGGTCTTGTTCCCCCCTGAACAGGCCACCTAAATGTACCGTCTGTTATAC
TTGCCCCCCCCCCTGTGTAATAAAAAATTTCTTTTTTTTTACTCTTTTAAGTTCGTGTGTAAC
CCCCCACTCAACCCCCTTTTTTTTTTATTTTTTTTTTCAGATGGCCGACCACGGAGGAAGAAA
AACTCCCCGCTTTTCTCCATTTTTAAAAGGAAAAAAAAAAAAATGTTCGTT

Cf-42

GTGTCAATGATTACGTACTCTATAACCATGTGTGACGTACCTATCTTGTAGTGAGATGAATG
CTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTATCCCTCCC
CTCTGAAAACTCTTTTTTATATTTTCTATATTTTTTGTTGATTGGTGTAACAATAAAAAAAT
TAAAAAATTCTTCAAACGACCTATCGGTTGTCTCATCCATCAATAAAGAACCCAACTGCGAG
CAATAATGTGAGTTGCTTAAATAATTGAATCAACCAACCTTTCAATGCACATTGCTCCCGCC
AGTATTCTGTCTGGCATGCCTGTCCGACCGACCTTTCTTTCCTCACTAAACACTTGTTTTTG
CCGTTGGAGGTCCTGTTCGTTCCCCCCCGAACCGCCCACATGTATCTGCCGTCAGTTATACT
TACTCCCCCCCCTGTTAAAAAAATTCTTTCTTTTTCCCCCTCAATTTATTGTGTGACACACC
CCCCACCCCCCCCCCTTTTTTTTTATATTTTTTTCCAGAGGGCCCACCAAGAAGGAAGAAAA
ACTCCCGTTTTTCCCCCCACTATTAGGGGGGAAGAAAGAAAAATACTGTGGTGTT

Cf-48

ATAAAGCCCTGGTCTGTACTCTATAACCATGTGTGAACGTACCTATCTTGTAGTGAGATGAA
TGCTGTTTTGGTGGTAGGGCCCTTCTGAAGAGAGGGCACCGCTGCCAGCAGTATTATCCCTC
CCCTCTGAAAACTCTTTTTAATATTTTCTATATTTTTTGCTGATTGGTGGCACAACTAAAAA
AAAAAAAAATTCTTTCAACGACCTATCGGTTGGCTCTACCATCAATAAAGAACCCAACGGAA
AGCAATAATGAGAGTTGATTAAATAATTGAATGAACCAACCTTTCATTGCACATTGATTGCG
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TGGCTGTGGATGGACGGTCTGTTCCCCCCCGAGCCGGCCACATAAATGTATCGTCTGTTATA
CTTGCTCCCCCCCCTGTTAAAAAAAATATTTCTTTTATTCACTCAATTAAGTGCGTGACACA
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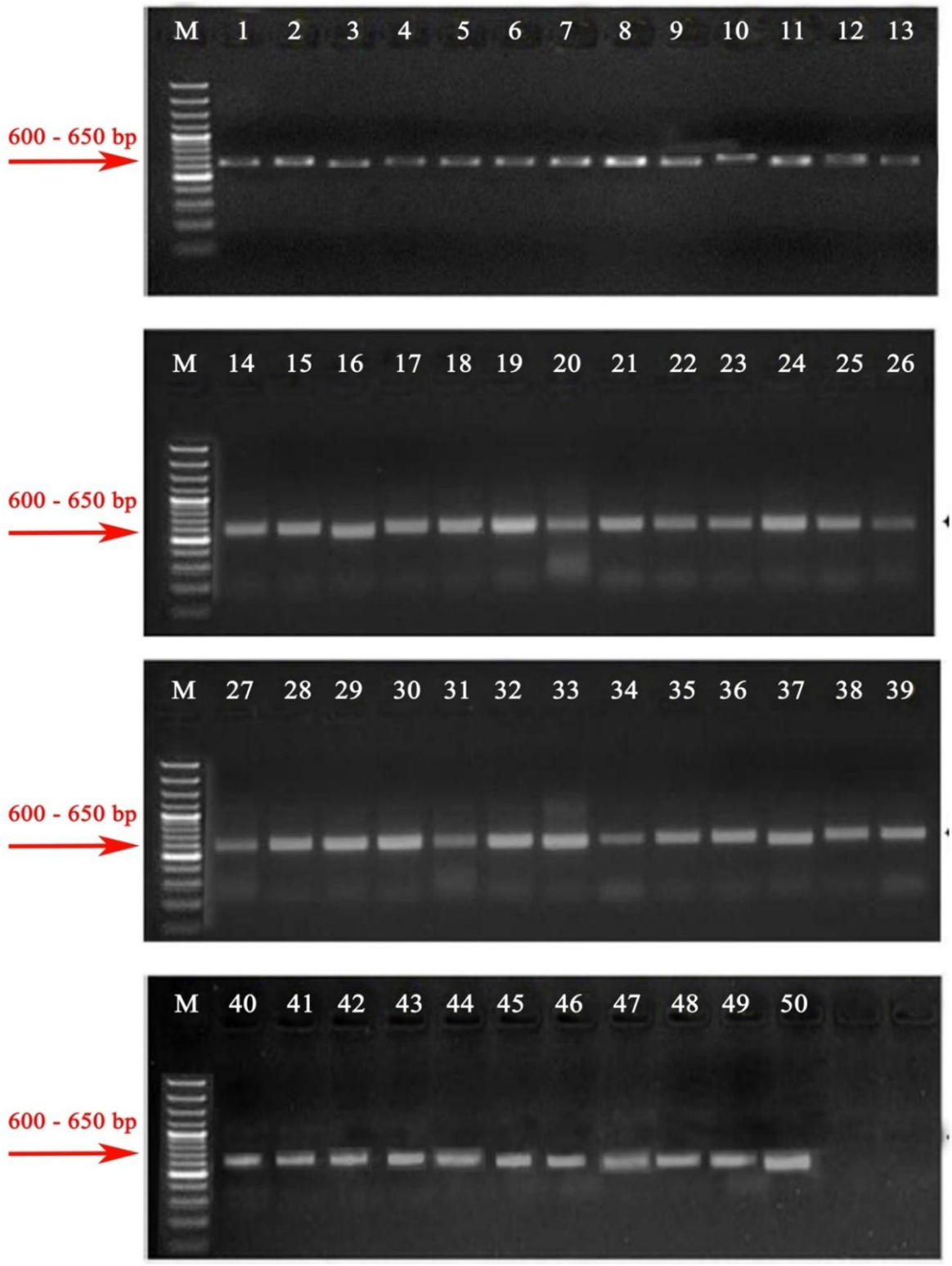

Plate 1. Amplification product of Internal Transcribed Spacer (ITS) with ITS-1 and ITS-4 ribosomal DNA primers

1=Cf-1, 2=Cf-2, 3=Cf-3, 4=Cf-4, 5=Cf-5, 6=Cf-6, 7=Cf-7, 8=Cf-8, 9=Cf-9, 10=Cf-10, 11=Cf-11, 12=Cf-12, 13=Cf-13, 14=Cf-14, 15=Cf-15, 16=Cf-16, 17=Cf-17, 18=Cf-18, 19=Cf-19, 20=Cf-20, 21=Cf-21, 22=Cf-22, 23=Cf-23, 24=Cf-24, 25=Cf-25, 26=Cf-26, 27=Cf-27, 28=Cf-28, 29=Cf-29, 30=Cf-30, 31=Cf-31, 32=Cf-32, 33=Cf-33, 34=Cf-34, 35=Cf-35, 36=Cf-36, 37=Cf-37, 38=Cf-38, 39=Cf-39, 40=Cf-40, 41=Cf-41, 42=Cf-42, 43=Cf-43, 44=Cf-44, 45=Cf-45, 46=Cf-46, 47=Cf-47, 48=Cf-48, 49=Cf-49, 50=Cf-50

All the sequences of *Ceratocystis fimbriata* isolates of pomegranate were deposited in NCBI GenBank, Maryland, USA along with the location of the isolates. Accession numbers obtained are: KY038512, KY038513, KY038514, KY038515, KY038516, KY038517, KY038518, KY038519, KY038520, KY038521, KY038522 and KY038523 and all isolates are identified as *Ceratocystis fimbriata* (Table 2).

Comparison and identity of representative twelve *C. fimbriata* isolates of pomegranate was done. Cf-1 isolate accession number is KY038512 and its resemblance of 91 per cent with accession no KU877212 its host is *Punica granatum.* Similarly, Cf-9 accession no is KY038513 resemblance 79 per cent with the accession no KC261853 host is *Mangifera india*, Cf-10 KY038514 resembles of 91 per cent with the accession no KX703025 host is *Punica granatum*, Cf-14 accession no KY038515 resemblance 91 per cent with the accession no KX703025 host is *Punica granatum*, Cf-20 accession no KY038516 resemblance 99 per cent with the accession no AM712447 host is *Punica granatum*, Cf-23 accession no KY038517 resemblance 98 per cent with the accession no KU877212 host is *Punica granatum*, Cf-26 accession no KY038518 resemblance 99 per cent with the accession no KU877201 host is *Punica granatum*, Cf-31 accession no KY038519 resemblance 99 per cent with the accession no AM712448 host is *Colocasia esculenta*, Cf-33 accession no KY038520 resemblance 99 per cent with the accession no KU877193 host is *Punica granatum*, Cf-38 accession no KY038521 resemblance 99 per cent with the accession no KX703025 host is *Punica granatum*, Cf-42 accession no KY038522 resemblance 97 per cent with the accession no KX703025 host is *Punica granatum* and Cf-48 accession no KY038523 resemblance 81 per cent with the accession no KC261853 host is *Mangifera indica* respectively (Table 2).

The phylogenetic tree of *C. fimbriata* constructed using UPGMA online software indicated variation among isolates from different districts as well as within a district. Based on the results obtained, the phylogenetic analysis revealed that all 12 isolates fall into four major clusters, The first cluster consisted of the isolates, Cf-38, Cf-42, Cf-48, and Cf-14, the second cluster consisted of the isolates Cf-20, Cf-23, and Cf-26, the third major cluster consisted of the isolates Cf-1, Cf-9 and Cf-10 and Cf-33 and Cf-31 are in the fourth cluster (Fig. 1). Chitradurga district (Cf-38 Markal-1), Tumakur district (Cf-42 Karekyatanahalli-1) Hassan district Cf-48 Chikabidane-1) and Koppal district (Cf-14 Kamanur) come under one group, Yadgir district (Cf-20 Tumkur) and Raichur district (Cf-23 Yatgal and Cf-26 Ganjhalli-1) have another group, Vijaypura (Cf-1 Kumtagi) and Bagalkot district (Cf-9 Govindkoppa

and Cf-10 Kaladgi-1) under different group and Ballari district (Cf-33 Thambrahalli and Cf-31 Lakshmipura) under a separate group.

4. DISCUSSION

The amplification of isolated DNA from the fifty pathogenic cultures using ITS primers (ITS-1 & ITS-4) showed 600 to 650 bp size. The results indicated all the fifty isolates belonged to the same species and were represented as *C. fimbriata*. Among fifty, twelve isolates were selected based on the representation of geographic regions, and cultural and morphological categorizations. Such isolates were amplified and 5.8 S rDNA was sequenced. The NCBI - BLAST was carried out and the conformity of the isolates was obtained. The twelve rDNA sequences were deposited in the GenBank database under the accession numbers serially from KY038512 to KY038523.

The cluster first comprised the isolates of districts such as Chitradurga (Cf-38), Tumakur (Cf-42), Hassan (Cf-48) and Koppal (Cf-14) which are from South Karnataka except Koppal (Fig. 1). The cluster 2nd comprised two districts as Yadgir (Cf-20) and Raichur (Cf-23 and Cf-26). Cluster 3rd comprised Vijaypura (Cf-1) and Bagalkot (Cf-9 and Cf-10) and 4th cluster comprised the isolates from Ballari district (Cf-33 and Cf-31) which indicated that isolates from different districts of Karnataka showing specific patterns of similarity according to geographical region. It might be due to similar climatic conditions, the same type of soil, the and use of cuttings from the same infected fields. Uniformity in each location suggests that the strains may have been moved from site to site by humans and the tapping panels may have been infected via contaminated tools rather than infection from natural soil-borne inoculums. “Members of the *C. fimbriata* complex are homothallic due to unidirectional mating-type switching and insect dispersal of ascospores is generally of minor importance in the epidemiology of *Ceratocystis* wilt” [2]. Similarly, Christine *et al*. [1] reported that “genetic studies in populations of the fungus in Costa Rica, Colombia, and Bahia may have been introduced on cacao cuttings; whereas populations in Rondonia, Brazil, and Western Ecuador appear to be native. The fungal genotype present in Bahia is similar to those found in Rondonia and may have been introduced on propagative material with witches' broom resistance”. [5] “UPGMA dendrogram for genetic variation among the isolates of *C. fimbriata* showed that all the isolates fell into two major clusters, and the isolates from different districts did not show a specific pattern of similarity according to geographical

region. The dissimilarity coefficient ranged from 0.00 to 0.20 among the pathogenic isolates. Isolates Cf-1, Cf-3, Cf-4, Cf-5 Cf-6, Cf-7, Cf-8, Cf-9, Cf-11, Cf-12, and Cf-15 were found to be highly similar to each other as their dissimilarity coefficient was zero, and maximum dissimilarity (0.20) was found between isolate Cf-10 and all other isolates, which were seen to be genetically distinct from each other".

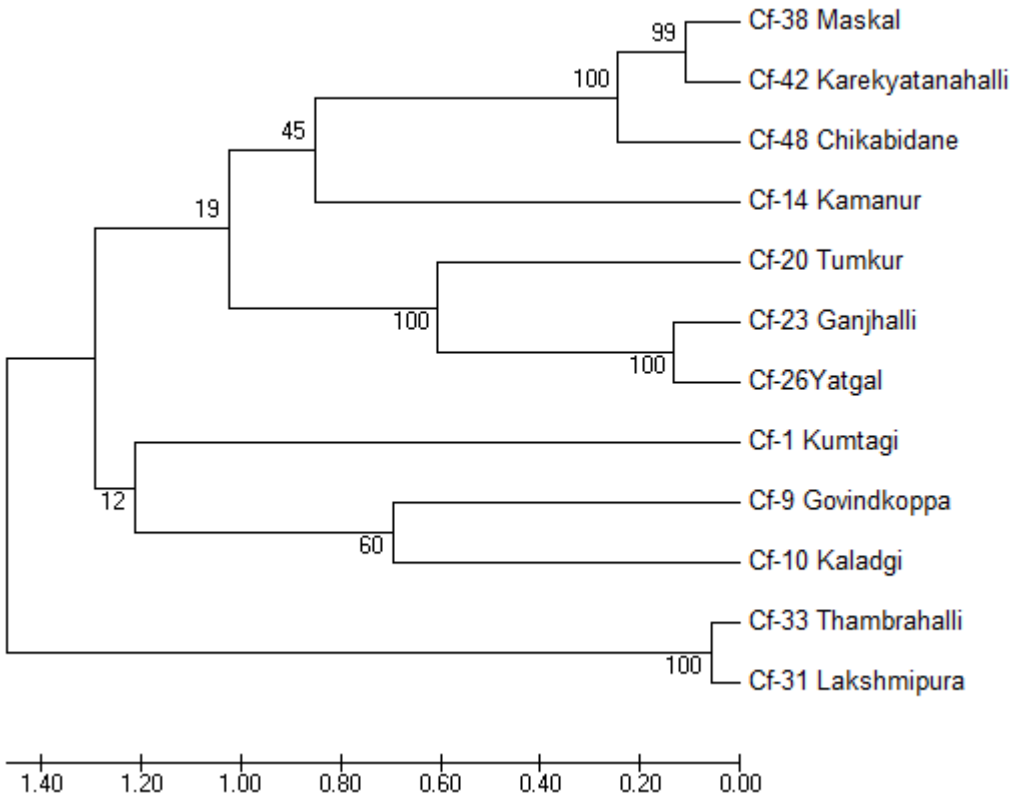

Fig. 1: Dendrogram based on UPGMA cluster analysis obtained from multiple sequences alignment tools for *C. fimbriata* isolated from pomegranate

Table 2. Comparison and identity of representative *C. fimbriata* isolates of pomegranate sequenced and deposited in GenBank, Mary land, USA

Sl. No.	Isolate	GenBank Accession No.	Identified as	Referenced Strain, Accession No. and Host			% Homology
				Strain	Accession No.	Host	
1	Cf-1	KY038512	*C. fimbriata*	NRCP-CF26	KU877212	*Punica granatum*	91
2	Cf-9	KY038513	*C. fimbriata*	CMW 13582	KC261853	*Mangifera indica*	79
3	Cf-10	KY038514	*C. fimbriata*	KP10032	KX703025	*Punica granatum*	91
4	Cf-14	KY038515	*C. fimbriata*	KP10032	KX703025	*Punica granatum*	88
5	Cf-20	KY038516	*C. fimbriata*	YM062	AM712447	*Punica granatum*	99
6	Cf-23	KY038517	*C. fimbriata*	NRCP-CF26	KU877212	*Punica granatum*	98
7	Cf-26	KY038518	*C. fimbriata*	UHS-CF17	KU877201	*Punica granatum*	99
8	Cf-31	KY038519	*C. fimbriata*	YMY062	AM712448	*Colocasia esculenta*	99
9	Cf-33	KY038520	*C. fimbriata*	UHS-CF9	KU877193	*Punica granatum*	99
10	Cf-38	KY038521	*C. fimbriata*	KP10032	KX703025	*Punica granatum*	99
11	Cf-42	KY038522	*C. fimbriata*	KP10032	KX703025	*Punica granatum*	97
12	Cf-48	KY038523	*C. fimbriata*	CMW 13582	KC261853	*Mangifera indica*	81

5. CONCLUSIONS

The Phylogenetic tree of *C. fimbriata* constructed using UPGMA online software revealed that all 12 isolates from different districts of Karnataka showed specific patterns of similarity according to the geographical region This study can provide useful information for effective pomegranate wilt disease management

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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