

## Identification and characterization of *Fusarium mangiferae* as pathogen of mango malformation in India

**Pradeep Kumar<sup>1\*</sup>; Madhu Kamle<sup>2</sup>; Asok Kumar Misra<sup>3</sup>; Anthonia O'Donovan<sup>4</sup>; Marcela Pagano<sup>5</sup>; Dinesh Raj Modi<sup>6</sup>.**

<sup>1</sup>Yeungnam University, School of Biotechnology, Gyeongsan, Korea; <sup>2</sup>Ben-Gurion University of the Negev, Dryland Agriculture and Biotechnology, Beer-Sheva, Israel; <sup>3</sup>Central Institute for Subtropical Horticulture Lucknow, Uttar Pradesh, India; <sup>4</sup>National University of Ireland, Discipline of Biochemistry, Galway, Ireland; <sup>5</sup>Lunds Universitet, Department of Biology, Lund, Sweden; <sup>6</sup>Babasaheb Bhimrao Ambedkar University, Department of Biotechnology, Lucknow, Uttar Pradesh, India;

### ABSTRACT

*Fusarium mangiferae* (= *F. subglutinans*) isolates collect from malformed samples from major mango-growing area of North India. Molecular identification and characterization of eleven most virulent isolates of *F. mangiferae*, based on pathogenicity tests used for the present study. Species-specific, genus specific ITS-PCR and PCR-RFLP performed for the accurate and easy detection of *F. mangiferae*. The rDNA-ITS 28S region sequences used for phylogenetic analysis of *Fusarium* isolates from India and other countries for homology search between them. The phylogenetic tree divided the isolates into three clades (i.e., American, Asian and African) and showed the high level of sequence based similarity (69-99%) among all *Fusarium* sequences from Asia. Thus, claimed *Fusarium mangiferae* as dominant pathogen of mango malformation. Furthermore, we conclude that exploiting the nested PCR coupled with PCR-RFLP will help in rapid and accurate detection of *F. mangiferae* pathogen of mango malformation.

**Key words:** *Fusarium mangiferae*, detection, phylogenetic analysis, vegetative malformation, floral malformation.

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<sup>1</sup>Authors for correspondence: pkbiotech@gmail.com

## INTRODUCTION

The Mango 'King of Fruits' is the most delicious fruit commonly grown in Southern Asia. Mango malformation emerge as most threatening constraint that limits Mango production and cause severe economic losses (10-90%) to stakeholders of tropical and subtropical countries [1-2]. The most visible symptoms of mango malformation were malformed flower and bunchy appearance of vegetative tissues. Panicles affected become small, do not bear fruits and aborted prematurely. The variability of malformation pathogen influenced by environmental conditions and agro-climatic regions. Disease spread severely in new areas by malformation of mature trees and infected seedlings [3]. In spite of several decades of never-ending research, the etiology of the disease becomes a contentious issue and no effective control measure yet known [4]. However, various fungicides assessed for their ability to control the pathogen under laboratory, greenhouse and field conditions [5], but still unable to cure completely in the field conditions. Several biotic and abiotic factors reported to affect mango malformation disease. Fungi is the most counted factor reported [6]. Summanwar [7] first reported the malformation caused by *F. moniliforme* Shield and proved its pathogenicity in India. *F. mangiferae* Britz, Wingfield & Marasas is universal spread pathogen of mango and isolated from Egypt, Florida (USA) and Israel. *F. mangiferae* (earlier reported=*F. subglutinans*) isolated from floral and vegetative malformed tissues [8] as evidenced by cultural and morphological data. Histopathological studies of floral and vegetative tissues confirmed the association of *F. mangiferae* [9]. According to recent classification, *F. subglutinans* now registered as *F. mangiferae* causing mango malformation disease [6, 10-13].

Disease management strategies required for detection and identification of damaging pathogens but also understand the pathogen threshold density, interaction between the pathogens and environments [14]. In associate with continuous monitoring and basic pathological techniques that are tedious, it is complementary to perform molecular characterization at genus and species level for significant management of disease incidence. Internal transcribed spacer (ITS) regions are highly conserved within fungal species (with intra-specific similarities >99%) but are variable between species and accepted by fungal

taxonomists [15-16]. The rDNA ITS sequences have been successfully used in resolving species-level and phylogenetic relationships in *Fusarium* [17-18]. The ITS along with 5.8S rDNA region amplified using ITS primer-pairs [19] which discern genetic variation in the population studied. Earlier, molecular characterization confirmed the association of *F. mangiferae* with mango malformation [20-21]. In this study, we developed species-specific and  $\beta$ -tubulin gene specific markers for identification of *F. mangiferae* isolates from mango malformed samples and their sequence based phylogenetic analysis to understand the diversity relationship among the Indian isolates compared with other countries geographical location.

## MATERIAL AND METHODS

### Isolation of Pathogen and Pathogenicity Test

The vegetative and floral malformed samples collected from different orchards of mango (Table 1), cultured on Potato Dextrose Agar (PDA, HiMedia) and incubate at  $28 \pm 2^\circ\text{C}$  for 6 days. Cultural and microscopic studies confirmed pathogens as *F. mangiferae* [22]. Pure culture using single spore and hyphal tip isolation techniques by following Koch's postulates [23]. Pathogenicity tests for all isolates performed on the mango seedlings of Dashehari cultivar (sensitive to disease) under field conditions as well as in greenhouse conditions with controlled humidity at 85% and temperature 10-25  $^\circ\text{C}$ . Pure cultures from each representative isolate used for inoculation in November to the host tissue covered with sterile moist cotton. Prior to inoculation, buds treated with 20% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) which detoxified *mangiferin* (the host defense anti *Fusarium* compound) as described by Chand and Chakrabarti [24].

### DNA Isolation

Fresh mycelium from all 11 representative isolates collected for genomic DNA extraction according to the protocol of Abd-Elsalam et al [25]. 100 mg mycelium homogenized using 300  $\mu\text{l}$  of extraction buffer containing 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5 % of sodium dodecyl sulphate and centrifuged at maximum speed for 5 min. Add 150  $\mu\text{l}$  sodium acetate (pH 5.2) to the samples and chilled in ice for 10 min. Centrifuged at 13,000 rpm for 5 min and collect supernatant in new tube. Add equal volume of isopropanol and mix by inverting the tubes gently.

Centrifuge samples at 14,000 rpm for 20 min at 4°C and collect DNA pellet, washed pellet twice with 70% ethanol to remove excess salt. Finally, the isolated DNA dissolved in a minimum quantity of Tris-EDTA (10 mM) buffer and stored at -20°C until use. Quantification and purity of DNA recorded using standard spectroscopic methods at an absorbance wavelength of 260 nm <sup>[26]</sup>.

### ITS-PCR

ITS-PCR for isolates performed by adopting the protocol described by Abd- Elsalam et al. <sup>[25]</sup>. PCR reactions for amplification of ITS regions were performed using the ITS1 5'TCCGTAGGTGAACCTGCGG 3' and ITS4 3' TCCTCCGCTTATTGATATGC 5' primer pair <sup>[19]</sup> in reaction volumes of 50 µl containing 25 ng of genomic DNA, 1X PCR buffer (Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM MgCl<sub>2</sub>, 1 µM primers and 1U Taq polymerase (Invitrogen). DNA amplification was performed in a Thermal Cycler (Eppendorf) and the program consisted of an initial denaturing step at 94°C for 1 min, followed by 30 cycles of 60 s at 94°C, 2 min at 58°C and 60 s at 72°C and a final extension step of 5 min at 72°C.

### Genus and β -Tubulin Gene-Specific PCR

PCR for *Fusarium* isolates using primers ITS-Fu-F and ITS-Fu-R for genus-specific PCR performed as given description by Kamel et al <sup>[27]</sup>. The primer pair specific to *Fusarium* spp. ITS-Fu-F 5' CAACTCCCAAACCCCTGTGA 3' and ITS-Fu-R 5' GCGACGATTACCAGTAACGA 3'. PCR reaction conditions described using ITS1 and ITS4 followed. *Fusarium* species reported to characterize at the molecular level using β-tubulin gene specific primers. Highly specific β-tub F 5'TGCTTCGGCGGGTAGGGTC 3' and β-tub R 5' ACGCAAAGGAGGCTCCGGGA 3' primers used. PCR reactions for gene specific amplification were performed in reaction volumes of 50 µl containing 25 ng of genomic DNA, 1X PCR buffer (Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM MgCl<sub>2</sub>, 1 µM primers, and 1U Taq polymerase (Invitrogen). PCR amplification was performed in a Thermal Cycler (Eppendorf) and the program consisted of an initial denaturing step at 94°C for 1 min, followed by 30 cycles of 60 s at 94°C, 2 min at 58°C and 60 s at 72°C; and a final extension step of 5 min at 72°C. PCR products were analyzed using 2.0% Agarose gel electrophoresis.

### PCR-RFLP

PCR-RFLP performed as per the protocol described by Bogale et al. <sup>[28]</sup> with minor modifications and the reaction conditions were standardized. For ITS-RFLP, restriction digestion of PCR products of all isolates carried out by a single digestion using restriction enzymes, viz., *Alu* I, *Msp* I, *Rsa* I and *Taq* I (Fermantas). The digestion reaction conditions include 1X buffer, 10U/ µl of enzyme and 100 µg of PCR product for a total reaction volume of 25 µl. The restriction digestion reactions carried out by incubation at 37°C for 2-3 hrs. The digested samples analyzed on 2.5% Agarose gel electrophoresis with control (undigested PCR product) and molecular size standard markers (100 bp DNA ladder).

### Sequencing and Phylogenetic Analyses

The PCR products of 11 isolates purified using a QIA quick PCR purification Kit (Qiagen GmbH, Germany). Representative *Fusarium* isolates sequenced in both directions with primers ITS1 and ITS4. Reactions were performed on an ABI PRISM 377 automated DNA sequencer with an ABI PRISM Dye Terminator Cycle ready reaction Kit (Perkin-Elmer, Warrington, UK). Sequence-based comparative analyses of *Fusarium* performed using reference sequences retrieved from NCBI-Genbank and search algorithm BLAST <sup>[29]</sup>. Multiple sequence alignments performed using ClustalW algorithm <sup>[30]</sup>. For phylogenetic analyses, ITS-rDNA sequences aligned manually by inserting gaps and checked for accuracy. *Fusarium* and *Giberrella* isolates selected from GenBank were at least 90% identical, determined using the molecular evolutionary genetics analysis (MEGA) version 5.05 software <sup>[31]</sup>. One thousand Bootstrap replicates employed to determine confidence in the branches order.

## RESULTS

### Cultural and Pathogenicity Bioassays

The morphological and cultural characterization of the 11 isolates grow on PDA medium studied and compared with those mentioned by Booth <sup>[22]</sup>. The mycelium show high-level variability in character with colony and metabolite color as described (Table 1). The size and shape of the different types of micro and macro conidia produced by the isolates draw and measured by Leica LEITZ DMRBE Microscope (Germany). Micro-conidia

had aseptate hyaline, mostly oval and typically borne on polyphialides and the size range of 7.8 X 2.8µm. Macroconidia thin walled, falcate with mostly 2-3 septate and the size range of 25 X 42 µm. All isolates showed positive pathogenicity test in three replicates performed on seedlings of Dashehari in controlled greenhouse conditions and in field. Bud swelling and scale like leaf disease

symptoms appeared approximately 2-3 months after the inoculation. Complete symptom appearance was achieved after 6-7 months and recovery of the pathogen performed to prove the 'Koch's Postulates'. However, no malformation symptoms noticed in the control sets.

**Table 1.** Cultural and morphological characters of 11 *F. mangiferae* isolates collected from different agro-ecological regions of India.

Sample No.	Place of sample collection	Isolates name	Symptom	Culture colour	Metabolite colour	Spore
1	Sabour	F1	FM	Light pink	Pink	+++
2	Sabour	F2	FM	Cream	pink	++
3	Dholi	F3	VM	Light Pink	Light brown	+++
4	Ranchi	F4	FM	Light Pink	Dark red	++
5	Lucknow	F5	VM	Yellow	Pink	++
6	Lucknow	F6	VM	Pink	Purple	+++
7	Lucknow	F7	VM	Pink	Light pink	++
8	Chittour	F8	VM	purple	Violet	++
9	Bangalore	F9	FM	Red	Light brown	+++
10	Shaharanpur	F10	FM	Light pink	Purple	+++
11	Shaharanpur	F11	FM	Purple pink	Purple	+++

VM: Vegetative malformation; FM: Floral malformation; ++ Moderate spore; +++ High Spores

### Molecular Characterization

Genomic DNA of all 11 isolates of *F. mangiferae* and control sample successfully amplified using ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers [19] obtained 570 bp band (Fig. 1a). *Fusarium* genus-specific primer ITS-Fu-f and ITS-Fu-r [27] specifically amplified a 398 bp band among all the isolates (Fig. 1b). No cross-reaction amplification detected in negative control *Colletotrichum gloeosporioides* tested for non-specific amplification. The PCR-RFLP restriction

digestions of *Fusarium* isolates performed using *AluI*, *HindIII* and *RsaI* restriction enzymes. *Hind III* and *Rsa I* show no restriction sites. However, *AluI* restriction gives two clear restriction bands of 410 bp and 120 bp. The gene specific PCR using  $\beta$ -tubulin primers resulted in a single band of 235 bp and no amplification in negative control. Hence,  $\beta$ -tubulin primer-pair can potentially employed as marker to discriminate *F. mangiferae* (Fig. 1c & d).

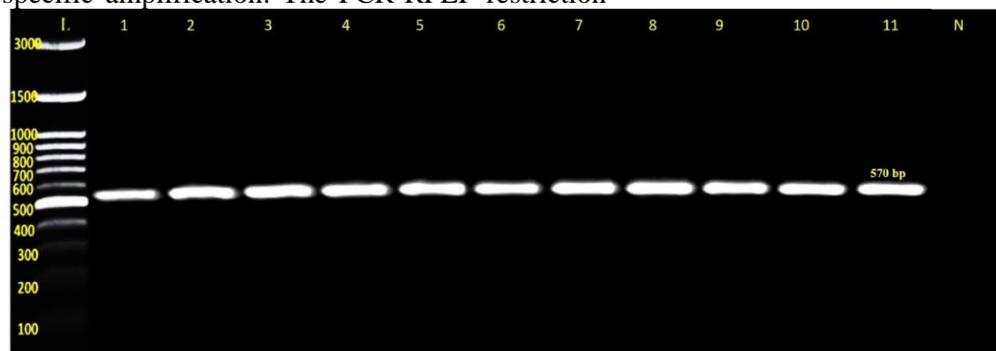


Fig 1 (a) ITS-PCR of 11 representative isolates of *F.mangiferae* using ITS1-4 primer (White et al.1990) amplify a common 570 bp band among all isolates.

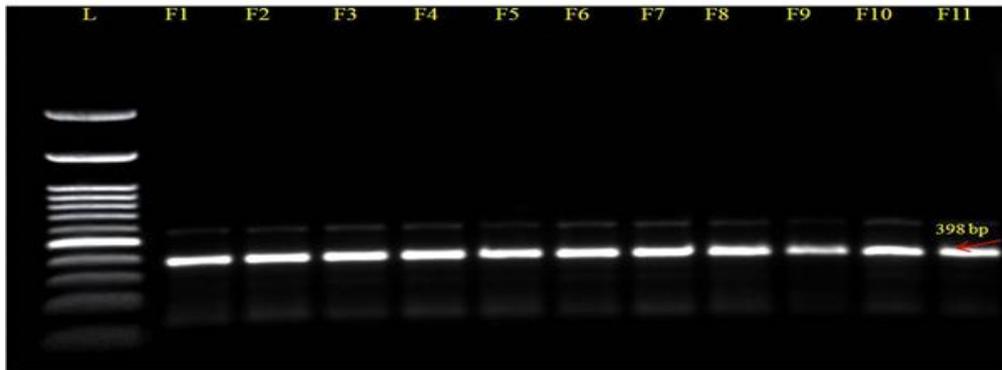


Fig1 (b): PCR amplification using Genus-specific primer of *F. mangiferae* isolates using ITS-Fu F&R primers (Abd-Elsalam, 2003).

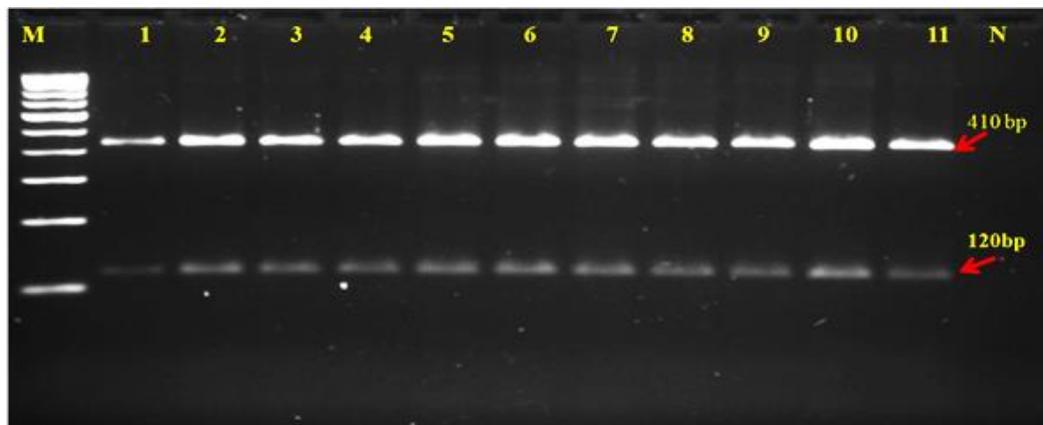


Fig1(c): PCR-Restriction digestion with *AluI*. (Lane 1-11 *F. mangiferae*; N- *F. oxysporum* (Negative control)).

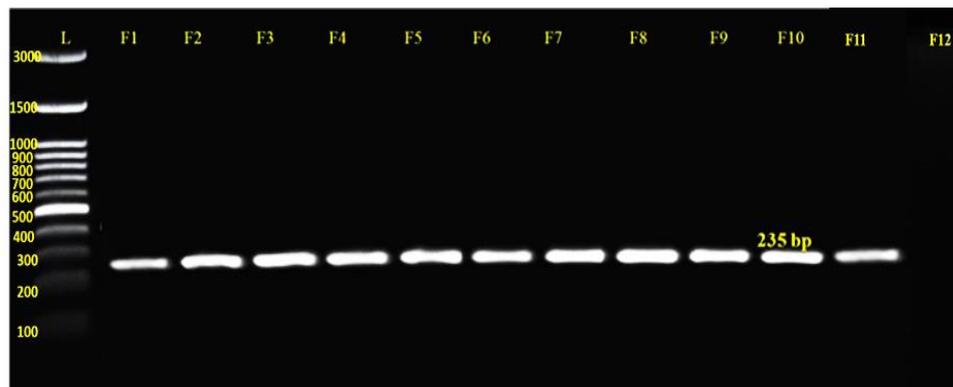


Fig 1(d). Nested-PCR using beta-tubulin ( $\beta$ -*tub*) gene-specific primer (Lane 1-11) *F. mangiferae* and lane 12- Negative control (*C. gloeosporioides*).

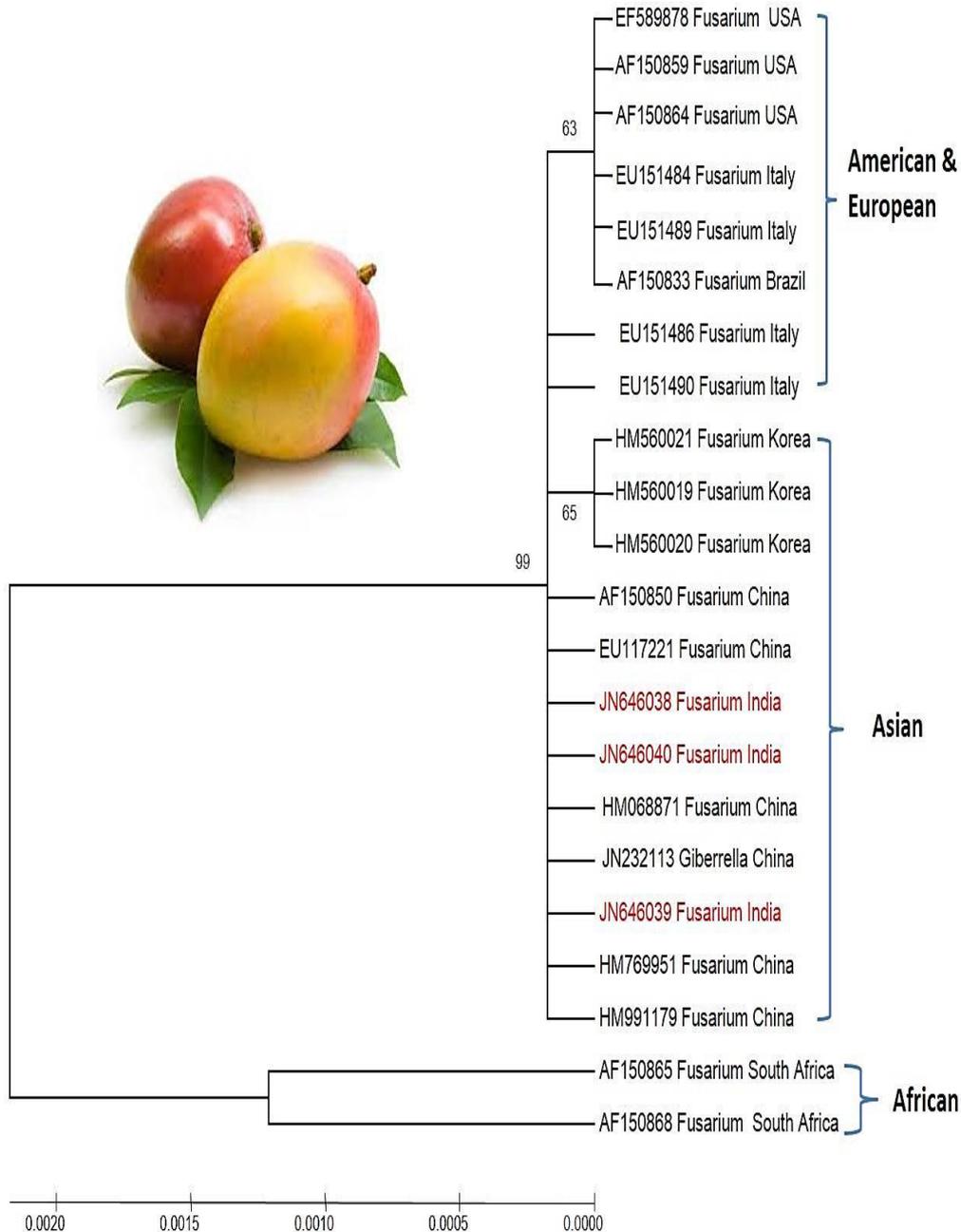
### Phylogenetic Analysis

Twenty-two sequences of *Fusarium* spp. reported to cause mango malformation from different countries (China, Italy, Brazil, Korea, South Africa and USA) selected and retrieved from NCBI. Three *Fusarium* isolates from present study submitted to NCBI (Accession id: JN646038, JN646039,

JN646040) with less than 90% sequence homology with the available *Fusarium* spp. reference sequences for phylogenetic analysis. All the sequences nBLAST-NCBI for sequence homology and multiple sequence alignment performed using ClustalW algorithm and phylogenetic tree constructed using MEGA 5.05<sup>[31]</sup>. The comparative

phylogenetic tree constructed using 22 *Fusarium* spp. isolates from other countries along with the three representative isolates from the present study showed more than 90% nucleotide sequence similarity and grouped the isolates into two major clusters I and II. Cluster I further divided into two major subgroups covering the present study

*Fusarium* isolates (India) and isolates from Brazil, USA, Italy, China and Korea. However, cluster II divided into two sub clades covering the two *Fusarium* spp. of South Africa and depicts genetic variability at the species level (Fig. 2).



**Fig 2.** Phylogenetic tree constructed using MEGA 5.05 version with ITS region sequences retrieved randomly from the GenBank along with the present study isolates (in red).

## DISCUSSION

Normal apical growth observed on the apex of healthy seedlings while seedlings inoculated with *F. mangiferae* showed vegetative malformed growth followed by excessive proliferation of leaves. The gathering of stubby leaves into close folds similar to incipient bunchy top met under natural normal conditions, which is in accordance with previous workers<sup>[9, 32]</sup> on pathogenicity. Early and easy disease detection of *F. mangiferae* is necessary for effective and timely management of mango malformation. PCR based molecular disease diagnostic techniques provide a rapid, cost-effective specific and sensitive detection system in comparison to conventional methods<sup>[33]</sup>. Globally, three *Fusarium* species under the *Giberrella fuzikoroi* species complex reported as the causal agents of mango malformation disease. The species are *F. mangiferae* from Egypt<sup>[3]</sup>, Israel<sup>[34]</sup>, South Africa<sup>[10]</sup>, Pakistan<sup>[35]</sup>, *F. proliferatum* from Malaysia<sup>[36]</sup>, and *F. subglutinans* reported from South Africa, India and USA<sup>[37]</sup>. PCR products of 570 bp, 398 bp and 235 bp amplified in *F. mangiferae* using *Fusarium* species-specific<sup>[25]</sup> ITS primers and  $\beta$ -tubulin gene-specific primers, respectively. By contrast, the nested PCR approach presented here produced consistent and reproducible results using  $\beta$ -tubulin gene-specific designed primers. Nested PCR increased the sensitivity of *F. mangiferae* (= *F. subglutinans*)  $\beta$ -tubulin gene-specific primers by 1000 fold<sup>[38]</sup>. The nested PCR assay potentially employed as a diagnostic tool for effective detection of *F. mangiferae* in mango malformation affected tissues. Moreover, nested PCR permits rapid identification of the fragmented samples and does not require sequencing. This technique is advantageous as it is rapid, reliable and robust. However ITS-PCR-RFLP using *Alu* I restriction enzyme proficiently identifies the pathogen by giving two clear bands of 410 and 120 bp sizes and making it an effective marker for *F. mangiferae*. The phylogenetic tree constructed using ITS-gene sequences comparing *Fusarium* spp. associated with mango malformation disease from different countries (Brazil, China, USA, Korea, Italy and South Africa) indicate a wide diversity among *F. mangiferae* isolates. This range is consistent with the previous description of isolates reported to cause malformation by *F. subglutinans* belongs to the same species within the *G. fuzikoroi* complex

<sup>[39]</sup>. Twenty-two *Fusarium* species associated with mango malformation in the *G. fuzikoroi* complex along with the present study isolates (JN646038, JN646039, and JN646040) divided into three distinct clades Asian clade, American clade and African clade. The phylogenetic tree showed 65-99% as overall genetic similarity between the isolates in two clusters. Cluster I is a major cluster and is further divided into group A comprising 8 *Fusarium* species (American and European clade) and group B comprising 12 *Fusarium* species (Asian clade). Cluster II is a minor cluster and is divide into two groups (South African clade). Based on sequence similarity of *Fusarium* spp. we concluded that the *F. mangiferae* is a dominant pathogen of mango malformation disease in India. Our findings are consistent and supports the findings of several research groups that identified and reported *F. mangiferae* as a pathogen causing mango malformation disease in Asia and America<sup>[37,40]</sup>. Similarly, American isolates sequences also supports that *F. mangiferae* is a mango malformation pathogen. However, the South African *Fusarium* species showed some level of distinctness as the clade represented by two *Fusarium* spp. viz. *F. sterilihyphosum* and *F. subglutinans*. Therefore, in the present study attempted, we develop a nested PCR based detection assay and sequence based phylogenetic analysis thus, claimed *F. mangiferae* as the dominant pathogen of mango malformation in India.

## CONCLUSION

The ITS PCR based species-specific and gene-specific detection and identification of *Fusarium* species isolates will be helpful in understanding the pathogenic relationship among the isolates from other geographical location. Identification and confirmation of *F. mangiferae* as the dominant pathogen of mango malformation disease in India. This PCR based technique would be a valuable component in large-scale pathogen diagnosis and confirmation. This study further explored beside effective crop management strategies.

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