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Full Length Research Paper

Genetic characterization of *Fusarium oxysporum* isolated from guava in northern India

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***Fusarium oxysporum* f. sp. *psidii* is a causal agent of wilt disease of guava in subtropical regions globally. The production of guava fruit is affected by wilt disease thus, there is a need to develop molecular markers for accurate, rapid, specific and sensitive diagnosis of *F. oxysporum.* A set of species-specific primers were developed for amplification of the internal transcribed spacer region. Subsequently, polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) of 18S-28S rRNA was used to analyse the genetic diversity and phylogenetic relationships of *F. oxysporum* which was isolated from northern agro-ecological regions of India. These finding provide an insight for earlier molecular diagnostic of *F. oxysporum* for better management of guava wilt disease in India.**

Key words: Diagnostic, *Fusarium oxysporum* f. sp. *psidii,* management, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), Wilt.

INTRODUCTION

Guava (*Psidium guajava* L.) is an important fruit crop of tropical and subtropical countries that include Mexico (Central America) Africa, South Asia, Southeast Asia, the Caribbean, subtropical regions of North America, New Zealand, Australia and India. The main cultivation of guava in India is northern agro-ecological regions. During the last decade, fruit production was adversely affected by a decline problem. Besides the other factors, diseases contibute significantly in lowering the productivity of fruit. A wide range of microbial pathogens such as fungi, bacteria, algae, nematodes and epiphyte affect guava production (Misra, 2006). However, wilt is a prominent disease among the other guava diseases for example fruit canker (*Pestalotia psidii* Pat.), fruit rot (*Gloeosporium psidii, Pestalotia psidii, Phytophthora nicotianae, Macrophoma allahabadensis, Phomosis psidii* etc.), anthracnose (*Gloeosporium psidii*) and grey leaf spot (*Cephaleuros virescens*); is caused by fungus *Fusarium oxysporum* f. sp. *psidii,* a major pathogen which affects guava fruit production in India (Prasad et al., 1952; Chattopadhyaya and Bhattachariya, 1968; Misra and Pandey, 2000; Misra, 2006).

Fusarium affects several parts of guava, which involves altering of the development process by premature shedding of leaves, maturation of fruits, entire/whole tree that becomes defoliated and resulting in plant death (Misra, 2006; Mishra et al., 2013a). Pathogens affects many economically important crops that have been traditionally categorized into host-specific formae speciales. Specifically, *F. oxysporum* is a ubiquitous, soil borne fungi which comprises of numerous saprophytic and plant pathogenic forms (Gordon and Martyn, 1997). Morphological characterizations of *Fusarium* species by using microscopic examination and cultural characteristics are

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not conclusive (Edel et al., 1995; Leong et al., 2009).

A number of PCR assays have been developed for accurate and rapid diagnostic of *F. oxysporum.* The genetic variation among isolates within or between formae speciales of *F. oxysporum* have been demonstrated (McDonald, 1997). Especially random amplified of polymorphic DNA (RAPD), RFLP, simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) have been increasingly used to demonstrate variability in pathogenic populations of *F. oxysporum* (Jimnez and Jimnez, 2003). While ribosomal RNA has been widely accepted for assessing genetic diversity, genome mapping, and molecular diagnostics of many fungal species (Annamalai et al., 1995), internal transcribed spacer (ITS) region is widely used for molecular identification of pathogen. The aim of present study was to develop molecular marker for ITS that is informative for both diagnostic assays as well as genetic variability analysis based on RFLP data of *F. oxysporum* f. sp. *psidii* isolated from different agro-ecological regions of India. Additionally, to our knowledge, we have established the ITS1/5.8S/ITS2 RFLP fingerprints and ITS+5.8S sequence based analysis of *F. oxysporum* f. sp. *psidii*.

MATERIALS AND METHODS

Isolation and characterization of *F. oxysporum*

The wilt diseased root and soil of guava were collected (Table 1). The different presumptive isolates of *F. oxysporum* were isolated from agro-ecological regions of northern India that includes Kanpur (Uttar Pradesh), Shamsabad (Uttar Pradesh), Unno (Uttar Pradesh), Rewa (Madhya Pradesh) and Farukhabad (Uttar Pradesh). The samples were serially diluted and spread on Potato Dextrose Agar (PDA), and incubated at 28±2°C for 5 days. Cultures were phenotypically characterized by microscopic analysis, colour, and metabolite production (colour production by isolates in the invert side of the Petri plates). The established and well grown fungi were stored on PDA at 4°C in the Molecular Diagnostics Laboratory, CISH, Lucknow for further molecular analysis.

Fungal genomic DNA extraction

The fungal mycellium was plated on PDA and incubated at 28±2°C for 4-5 days. Total genomic DNA was extracted using phenol: chloroform: Isoamyl alcohol protocol (Sambrook and Russell, 2001). The pellets were air dried and resuspended in 100 µl of 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The isolated genomic DNA was kept at -20°C for further molecular study.

Primer designing and PCR amplification

PRIMER3 (Koressaar and Remm, 2007) software was used for designing a set of new primers based on existing sequence in NCBI GenBank (Accession no. HM102501). Primers BKP1 (Mishra et al., 2013b) and 1029R (5'-CGGTGTCCAGACCTACCCTA-3') were designed using ITS 1-5.8S-ITS 2 region for specific amplification of 410 bp fragment of *F. oxysporum.* The ITS primer pair for *Fusarium*, ITS1F (5'-CCAGAGGACCCCCTAACTCT-3') and ITS1R (5'-GCCTGAGGGTTGTAATGACG-3') (Mishra et al., 2013a) was used to amplify PCR products of 230 bp of *F. oxysporum* f. sp. *psidii*. The standard PCR reaction was performed in 25 µl of reaction mixture. Each PCR reaction mixture consisted of 20 ng genomic DNA, 0.5 µM primers, a 0.5 mM of each dNTPs, 2.5 µl 10X PCR buffer, 2.5 mM $MgCl_2$, and 1.25 U Taq DNA polymerase (Fermentas). The thermal conditions was set up as initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annelling temperature of primer at 52.5°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min was used and 10 µl of PCR product were analysed by 2.5% agarose gel electrophoresis with ethidium bromide at 8 v/cm and gel was visualised under gel doc UViPro transilluminator.

Sequencing and analysis of ITS region

For sequencing of the partial region of ITS+5.8S, internal primer ITS 1 was used. Amplified product on agarose gel was purified with using QIAquick gel extraction kit (Qiagen, India) and sequenced in single direction (Forward) using the ITS1 primer and was carried out by Xcelris Bio-lab Hyderabad, India. The homology of their ITS sequences were analyzed in the Basic Local Alignment Search Tool (Altschul et al., 1990) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of 570 bp of ITS+ 5.8S rDNA of each *F. oxysporum* f. sp. *psidii* (Fop) isolates were also submitted to NCBI GenBank under accession numbers KC357561-KC357565 and KC292502 (Table 1). The ITS sequences were aligned using CLUSTALX (Thompson et al., 1997) and using the passion correction implemented in the MEGA4.0.2 (Tamura et al., 2007) program for construction of phylogenetic tree by neighbor-joining method. The topology of the phylogenetic tree was evaluated by bootstrap resampling method with 5.00 replicates.

PCR amplification and restriction analysis of ITS region

F. oxysporum specific 18S and 28S primers NS1: 5'-GTAGTCATATGCTTGTCTC-3') and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990) were used to amplify the 18S rRNA gene and the entire ITS1/5.8S/ITS2 segment of the rRNA operon (Bruns et al., 1991). The PCR reactions were performed in an Eppendorf thermal cycler (Eppendorf) as follows: 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 45 s at 52°C, 3 min at 72°C, with a final extension of 10 mins at 72°C. The PCR amplicons were subjected to electrophoresis in 2.0% agarose gels (1X TAE buffer), the 2.6 kb bands were excised and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen). Total 10 µl of purified PCR products were digested using two restriction enzymes, namely *Hae*III, *Msp*I (Fermentas) according to the manufacturer's instructions. The restriction fragments were separated by electrophoresis in 2.0 % agarose gel using 1X TAE as running buffer.

RESULTS

Development and assessment of species–specific primers for ITS region

All these six fungal isolates tested were amplified using ITS universal primers ITS1-ITS4 (Figure 1). The specificity of the primers was validated against *F. oxysporum* isolates. The primer pair ITS1F/ITS1R *Fusarium* species specific primer was able to amplify a unique DNA fragment

Table 1. List of *F. oxysporum* f. sp. *psidii* isolates used in this study.

Culture number	Geographic origin	Color of mycelium on PDA	Wilt symptom (%)	Source of isolation	Culture accession number	Genebank accession number
Fop-84	Kanpur (U.P.)	Light brown	80	Soil	(NAIMCC-F-00815)	KC357561
Fop-51	Shamsabad (U.P.)	Dark yellow	50	Root	(NAIMCC-F-02000)	KC357562
Fop-30	Unnao (U.P.)	Light brown	30-50	Soil	(NAIMCC-F-01998)	KC357563
Fop-164	Rewa (M.P.)	Creamy	100	Soil	(NAIMCC-F-00816)	KC357564
Fop-44	Farukhabad (U.P.)	Light pink	50	Root	(NAIMCC-F-02084)	KC292502
Fop-48	Farukhabad (U.P.)	Pink	70	Root	(NAIMCC-F-00813)	KC357565

of approximately 230 bp (Figure 2) of all *F. oxysporum* isolates from different agro-ecological regions of northern India. Similarly, primer pair (BKP1-1029R) was designed in such a way for rapid, reliable and specific detection of guava wilt pathogen *F. oxysporum* f. sp. *psidii* and all the isolates amplified a common amplicon of 410 bp. No amplification was obtained in other fungal species and control sample (Figure 3).

Sequencing and data analysis

To confirm the genetic profile of each isolates, we sequenced the partial region amplicon of ITS1 and ITS4 using ITS1 primer. Data obtained by sequencing (570 bp of ITS+ 5.8s rDNA of each isolates) was used for homology search analysis and result showed that 96-98% sequences were similar with isolates which were previously reported from other host plant and a phylogenetic tree was constructed based on these sequences using MEGA 4.0.2, and showed two major clades (Figure 4).

PCR amplification and RFLP analysis

For *F. oxysporum* f. sp. *psidii* isolates, DNA fragments approximately 2.6 kb were amplified using NS1 and ITS4 primer pair. The ITS of all the Fop isolates could be digested using *Msp*I and *Hae*III (Fermentas) indicating that the ITS regions of the isolates contain recognition sites for these enzymes. Figure 5A and B shows the restriction patterns of *F. oxysporum* isolates with *Msp*I and *Hae*III, respectively. Estimated sizes of the restriction fragments were generated using *Msp*I and was 120-900 bp while that by *Hae*III was found to be 90-500 bp. Restriction analysis revealed genetic variation among the *F. oxysporum* isolates. In RFLP gel pattern, there were two common bands of 300 and 900 bp produced by *Msp*I, which were present in all the isolates of Fop while band sizes of 500, 300, 250, 180 and 90 bp fragments were produced by *Hae*III (Table 2). A number of polymorphic bands were also amplified and used for generating the phylogenetic relationship on the basis of results. A dendrogram (Figure 6) was derived from the distance matrix by using UPGMA (Rohlf, 1998).

DISCUSSION

Guava wilt is a serious disease which affects the different parts of plant such as stems, twigs, leaves, roots and fruits. The causal organism has been identified as *F. oxysporum* f. sp. p*sidii*. In the present study, ITS1F and ITS1R pair of primer was sensitive enough to detect infection of *Fusarium* species. Specificity and sensitivity of the primer pair were investigated using *Fusarium* isolates from the soil samples (Mishra et al., 2013a) and we found 230 bp amplicon in all the Fop isolates tested (Figure 2). PCR with specific primer set generated amplified products from samples collected from infected root and soil of wilted guava crops. The marker was validated against *F. oxysporum* f. sp. *psidii* isolates that was able to amplify 410 bp size of amplicon although no cross reactivity was obtained in *Fusarium solani.* The primer set BKP1 and 1029R not only distinguish *F. oxysporum* f. sp. *psidii*, from other pathogens but could also accurately diagnose and survey the pathogen from diverse areas of guava growers. Moreover, the two primer sets had no cross-reactions from among a considerable number of different fungi.

In the present study, 2.6 kb band of 18S rRNA gene and the complete ITS1/5.8S/ITS2 region of the rRNA was amplified using NS1 and ITS4 primer pair that has been previously reported (Płaza et al., 2004). On the basis of restriction sites isolate, they were grouped into the two major clades (Figure 6). Fop-44, Fop-48 and Fop-84 had 100% similar homology that was found to be close with

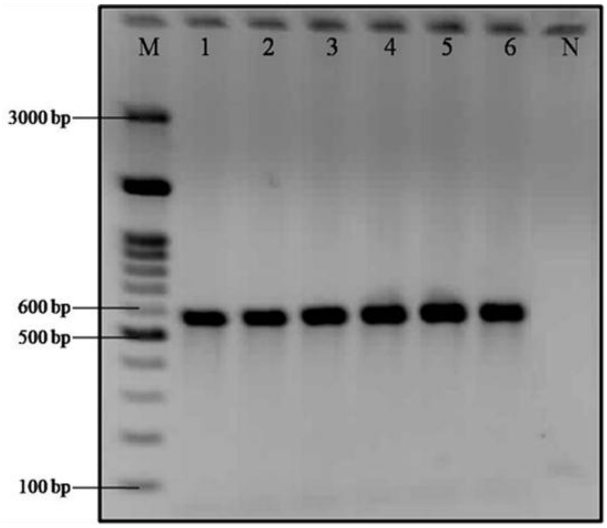

Figure 1. Agarose gel electrophoresis of PCR-amplified products using the primers ITS1/ITS4. Lane M, 100-bp DNA ladder marker; Lane 1-6, *F. oxysporum* f. sp. *psidii* isolates which corresponds to Table 1; lane N, negative control (dH_2O).

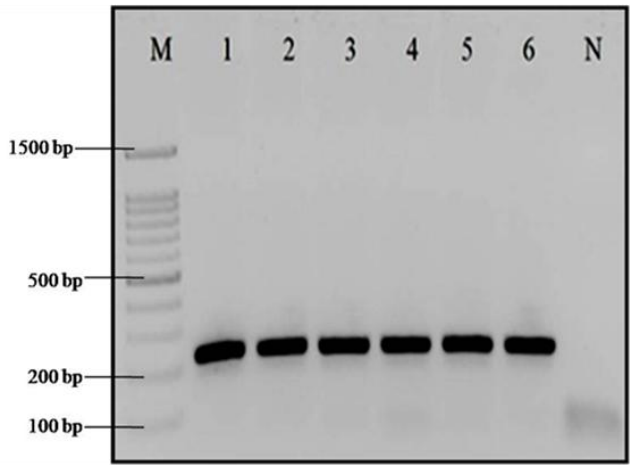

Figure 2 Agarose gel electrophoresis of PCR-amplified products using the primers ITS1F/ITS1R. Lane M, 100-bp DNA ladder marke; Lane 1-6, *F. oxysporum* f. sp. *psidii* isolates which corresponds to Table 1. Lane N, dH_2O.

Fop-30 with 77% homology. In other group, Fop-51 and Fop-164 had 84% similariry but both were 57% similar with other isolates. Similar approaches have been previously applied on ITS1 and ITS2 that was more variable thus, it has been used for study of the species-level relationships (Bruns et al., 1991; Samuels and Seifert, 1995). Sixteen (16) species of wood decaying fungi (Jasalavich et al., 2000) and several *Fusarium* species have been discriminated by RFLPs of rDNA (Paavanen-Huhtala et al., 1999, Lee et al., 2000).

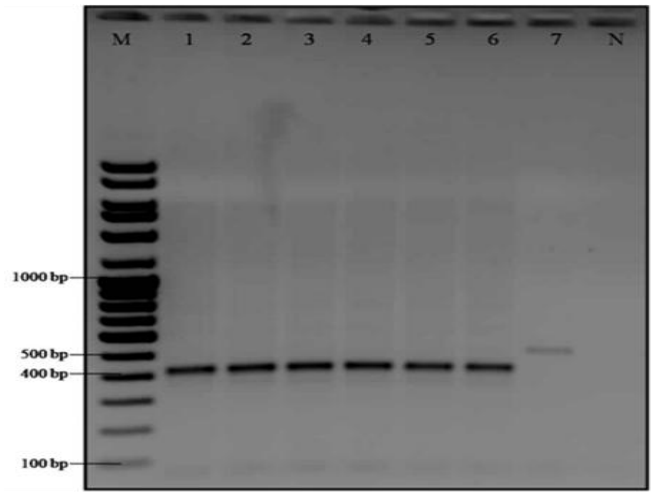

Figure 3. Agarose gel electrophoresis of PCR-amplified products using the primers BKP1/ 1029R. Lane M, 100-bp DNA ladder marker; Lane 1-6, *F. oxysporum* f. sp. *psidii* isolates which corresponds to Table 1; Lane 7, *F. solani*; Lane N: dH_2O.

Due to the high number of taxa studied and consequently the high variability encountered in ITS1 size, only the 5.8S gene and the ITS2 were used. In the result of partial ITS2 sequencing, strains were placed into group matching with those determined by phylogenetic analysis of ITS-RFLP. The isolates were grouped along with agro-ecological regions. Isolates from Uttar Pradesh Fop-30 (Unnao), Fop-44 (Farukhabad), Fop-48 (Farukhabad), Fop-51 (Shamsabad) and Fop-84 (Kanpur) grouped in a clade have similar soil type and agro-ecological conditions. In the case of Fop-164 isolated from Rewa (M.P.), it had different soil type and agro-ecological conditions. Two clades were identified in both reconstructions though Fop51 was placed with Fop164 based on the RFLP data and Fop-51 was placed in the other clade based on the sequence data. Isolate Fop-164 (Rewa, Madhaya Pradesh, India) was isolated from a region with a different soil type and agro-ecological region than the other isolates Fop-51 (Shamsabad, Uttar Pradesh, India). Rewa and Shamsabad both geographical locations are closed while the other four locations were closely established in the geographical regions. Due to the intermediate soil type and geographical condition, Fop-51 came close to the Fop-164. The sequence data provides a number of useful functionalities for exploring the statistical attributes of the data and also for selecting data subsets.

In this study, the use of specific primers for screening a large number of individuals might be useful to detect markers for wilt. We used molecular methods for the study of genetic diversity and specific detection of *F. oxysporum* f. sp. *psidii* isolated from different agro-climatic region. Thus, it was identified that methods are efficient molecular diagnostic tools complimenting mor-

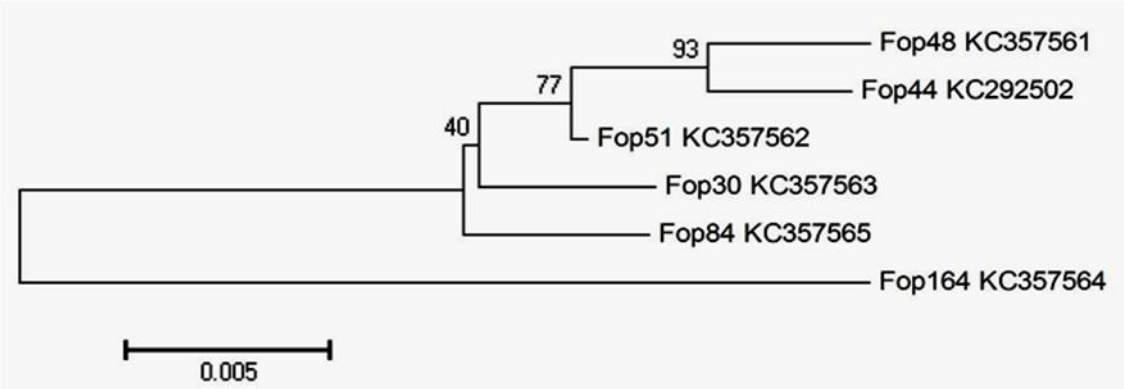

Figure 4. Neighbour joining phylogenetic tree based on the nucleotide sequence of the ITS region in the *F. oxysporum* f. sp. *psidii* isolates.

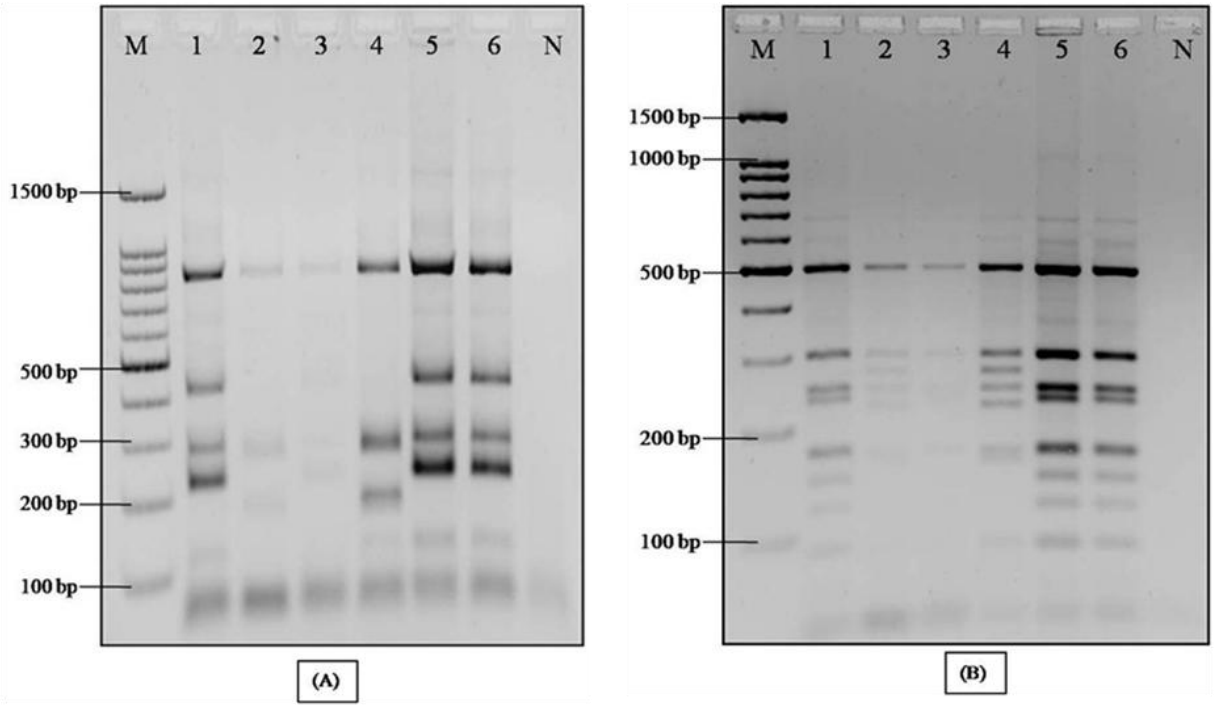

Figure 5. Agarose gels showing restriction patterns of PCR-amplified rDNA digested with (A) *Msp*I and (B) *Hae*III. Lane M, 100 bp DNA ladder; Lane 1-6: *F. oxysporum* f. sp. *psidii* isolates.

Table 2. Restriction fragment size (in base pairs) of *F. oxysporum* f. sp. *psidii* ITS region digested with *Msp*I and *Hae*III.

Culture number	*Msp*I	*Hae*III
Fop-84	120, 230, 300, 420, 900	90, 120, 150, 180, 230, 250, 300, 500
Fop-51	200, 300, 900	90, 180, 230, 250, 280, 300, 500
Fop-30	220, 300, 450	90, 180, 230, 250, 300, 500
Fop-164	150, 200, 300, 450, 900	90, 180, 230, 250, 280, 300, 500
Fop-44	120, 230, 300, 420, 900	90, 120, 150, 180, 230, 250, 300, 500
Fop-48	120, 230, 300, 420, 900	90, 120, 150, 180, 230, 250, 300, 500

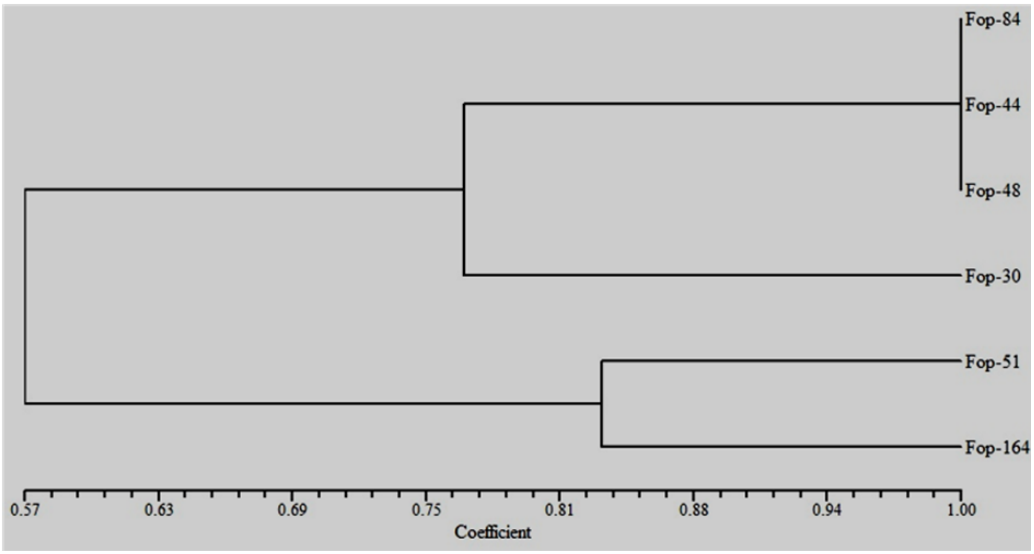

Figure 6. UPGMA dendrogram showing relationships among the 6 isolates of *F. oxysporum* f. sp. *psidii* based on PCR-RFLP data.

phological and cultural characterization studies in early diagnosis of guava wilt disease. Similarly, PCR assays have been successfully implemented for identification and detection of economically important *Fusarium* species such as *Fusarium avenaceum* (Turner et al., 1998), *Fusarium culmorum* (Klemsdal and Elen, 2006), *Fusarium graminearum* (Yoder and Christianson, 1998), *Fusarium langsethiae* (Wilson et al., 2004), *Fusarium moniliforme* (Moller et al., 1999), *Fusarium subglutinans* (Moller et al., 1999), *Fusarium poae* (Parry and Nicholson, 1996), *Fusarium sambucinum* (Yoder and Christianson, 1998), *Fusarium sporotrichioides* (Wilson et al., 2004) and *Fusarium venenatum* (Yoder and Christianson, 1998). Most of these molecular assays are based on the development of species-specific primers. In the experiment analysis, Lee et al. (2000) suggested that the ITS with 5.8S is more useful in the differentiation of *Fusarium* species.

In conclusion, we have developed molecular diagnostic marker for the identification of *F. oxysporum* f. sp. *psidii* isolated from the different agro-ecological regions of northern India. Additionally, we have demonstrated the genetic biodiversity and relationships among these isolates. The PCR assay can provide a definitive diagnosis of the guava wilt pathogen in soils within hours. It can be further used to more accurately survey the occurrence and distribution of the pathogens in soil. It is a very easy, rapid, sensitive and specific for diagnostic of *F. oxysporum* for better wilt management in guava.

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