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ORIGINAL ARTICLE

Identification and characterization of *Fusarium* sp. using ITS and RAPD causing fusarium wilt of tomato isolated from Assam, North East India

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Abstract *Fusarium oxysporum* which causes wilt is a serious pathogen. *Fusarium* isolates were isolated from Assam located in North East region of India. Morphological identification of *Fusarium* isolates was done using conidial and hyphal structures. Molecular identification of *Fusarium* isolates was done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4. All the ITS sequences were compared for gaps and similarity. Further, characterization of random amplified polymorphic DNA (RAPD) was carried out using 40 primers. 15 primers that gave reproducible results were selected. RAPD was used to observe the relatedness among these isolates. Thus, it was concluded that molecular profiling using ITS is an indispensable method for identification studies.

1. Introduction

Fusarium species are known to cause a huge range of diseases on an extraordinary range of host plants [1,2]. This group of cosmopolitan, soilborne filamentous fungi is economically important because many members are the causal agents of vascular wilt or root rot diseases in agricultural and ornamental crops throughout the world [3]. In fact, the near ubiquity of *Fusarium oxysporum* in soils worldwide has led to its inclusion in what has been termed the global mycoflora [4]. The identification of *Fusarium* species is commonly done based on their micro and macroscopic features. However, these features are mostly reported to be unstable [2,5,6]. In disease diagnosis, the most preferred method is microscopic examination of diseased tissues and identification of pathogen based on morphological characters, biochemical and allozyme characteristics etc. which require expert knowledge and estimates are still

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prone to error [7]. These methods are time consuming and have proved to be limited and insufficient. At present identification of eukaryotic organisms is basically done based on the nucleotide sequence information from conserved regions using PCR amplification. Sequences which have been valuable in distinguishing species and origins of *Fusarium* include internal transcribed spacer (ITS) region from the conserved ribosomal RNA genes, intergenic spacer (IGS), translation elongation factor (EF-1α), β-tubulin region and the mitochondrial small subunit (mtSSU) [8–10]. This sequence information has been widely used in the taxonomy and phylogeny of *Fusarium* species. It provides enough resolution at the sub-species level as this variability is harbored mainly in the introns. Besides these conserved regions, some of the DNA sequences that have also been used successfully to distinguish *Fusarium* species include nitrate reductase region (*NIR*), putative reductase, *UTP*-ammonia ligase, trichothecene 3-O-acetyltransferase, and phosphate permease [9,10].

PCR based techniques are regularly used for identification, characterization and early diagnosis of microbes and pathogens. Random amplified polymorphic DNA (RAPD) analysis [11] has been used for identification of fungi. It has been observed to have a high level of variability among many isolates [12–16]. RAPD is simple and relatively faster as compared with other molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and inter small sequence repeats (ISSRs) etc., [14,17]. RAPD is relatively easy to analyze and economical [16,18]. As a result, this technique has been used extensively in molecular characterization of fungi [19–24].

In this paper, we characterize *Fusarium* isolates morphologically and identify it using internal transcribed spacer (ITS). Further, RAPD study has also been carried out to observe the relatedness among the *Fusarium* isolates.

2. Materials and methods

2.1. Isolation of fungal cultures

Fusarium isolates were isolated from wilt infested tomato plants (*Lycopersicon esculentum*, local *var.*) collected from parts of Assam which is located in the North East region of India. Infested stem samples were sterilized by dipping in 10% (w/v) sodium hypochlorite solution for 3–5 min and washed thrice with sterile water. The stem was cut with a sterile blade and four pieces of diseased vascular tissue (*ca.* 5 × 5 mm) were placed on the surface of potato dextrose agar (PDA, Himedia, Mumbai) media. PDA was amended with streptomycin sulfate and chlor-tetracycline HCl to minimize chances of any bacterial growth. Plates were incubated at 28 ± 2 °C and observed periodically. The fungi were identified following sporulation and pure cultures were stored at 4 °C on PDA slants.

2.2. Morphological characterization

For morphological identification, single spore isolates were grown for 10–15 days on PDA medium [6]. Culture characteristics of each isolate were determined from 10 to 15 day old PDA cultures. Microscopic features of conidia, conidiophores and chlamydospores were also determined based on Summeral et al. (2003). Identification studies were further authenticated by Institute of Microbial Technology (IMTECH), Chandigarh.

2.3. Isolation of DNA

Genomic DNA was extracted using DNeasy plant minikit (Qiagen, Germany). DNA concentration was estimated using an UV–vis spectrophotometer (Thermo Spectronic UV1). It was then stored at −20 °C until further use.

2.4. Molecular characterization using ITS

Molecular identification of *Fusarium* cultures were carried out based on conserved ribosomal internal transcribed spacer (ITS) region. We amplified the ITS regions between the small nuclear 18S rDNA and large nuclear 28S rDNA, including 5.8S rDNA using universal primer pairs ITS1 (5′-TCCGTA GGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) [25]. Amplification was performed on a Thermal Cycler (Applied Biosystems 9700) with 25 μl reaction mixtures containing 2.5 μl of 10X buffer (10 mM Tris–HCl, pH 8.8); 2.5 mM $MgCl_2$; 2 mM each of dNTP; 25 pmol ml^{-1} primer (each of ITS-1 and ITS-4); 1U of Taq DNA Polymerase; 60–100 ng genomic DNA. The amplification cycle consists of an initial denaturation at 95 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 8 min. Amplified PCR products were separated on an agarose gel (1.5% w/v) in 1X TAE buffer at 65 V for 150 min. They were then eluted and further sequencing was carried out at Bangalore Genei, Bangalore. All reagents were procured from Fermentas, MBI, USA.

2.5. ITS data analysis

The ITS nucleotide sequences for each isolate were then compared to those in the public domain databases NCBI (National Center for Biotechnology information; www.ncbi.nih.gov) using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). Alignment of ITS DNA sequences was done using Clustal_W program [30]. Phylogenetic tree was created using CLC Sequence Viewer Version 6.3 based on UPGMA (unweighted pair group method for arithmetic analysis). The confidence of the branching was estimated by bootstrap analysis.

2.6. Molecular characterization using RAPD

PCR conditions were optimized by varying concentrations of template DNA, Taq DNA Polymerase, dNTPs and $MgCl_2$. An initial screening was done with 40 ten *mer* random primers (OPA and OPN series, synthesized by Sigma–Aldrich, Bangalore). Only 15 primers that gave reproducible and scorable amplifications were further used in the analysis. Amplification was performed in a thermal cycler (Applied Biosystems 9700) with 25 μl reaction mix containing 2.5 μl of 10X buffer (10 mM Tris HCl, pH 8.8); 5 mM $MgCl_2$; 2 mM each of dNTPs; 25 pmol mL^{-1} primer; 1U of Taq DNA polymerase; 60–100 ng genomic DNA. Amplification cycle consisted of an initial denaturation at 95 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 36 °C for 45 s, and 72 °C for 45 s and a final extension at 72 °C

for 8 min. Amplified products were separated on an agarose gel (1.5% w/v) in 1X TAE buffer at 65 V for 150 min. The gel stained with ethidium-bromide was viewed under UV light and documented with a Gel Doc system (Syngene, UK). All reagents were procured from Fermentas, MBI, USA. Using the software program Numeric Taxonomy Ntsys-pc (Numerical Taxonomy and Multivariate Analysis System) version 2.0 (Exeter Software, Setauket, NY, USA), a similarity triangular matrix was created from each rectangular matrix using the band-based Dice similarity coefficient (SD) [27]. Once the similarity matrix was constructed, the unweighted pair group method with average linkages (UPGMA) [28] was used to cluster the patterns and phylogenetic tree was constructed to group individuals into discrete clusters.

3. Results and discussion

3.1. Isolation and morphological identification of *Fusarium* isolates

8 *Fusarium* isolates were isolated. Based on structures of microconidia (Fig. 1), macroconidia (Fig. 2) and other morphological characters F1 was identified as *F. oxysporum* (MTCC8608), F2 as *F. oxysporum* (MTCC9913), F3 as *F. oxysporum* (MTCC8610), F4 as *Fusarium equisetum*, F5 *Fusarium subglutinans* (MTCC9914), F6 as *Fusarium proliferatum*, F7 as *F. subglutinans* (MTCC9915) and F8 as *F. subglutinans* (MTCC9916) (Table 1).

3.2. Molecular identification based on ITS

The total size of the ITS1 and ITS4 regions, including the 5.8S rDNA gene of the isolates studied varied from 380 to 620 bp (Fig. 3). *Fusarium* sequences obtained from amplification of conserved ribosomal ITS region were compared with sequences from National Center for Biotechnology Information (NCBI) database using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/BLAST). These sequences were identified and deposited in NCBI GenBank (Table 1). F1, F2 and F3 were identified as *F. oxysporum* (HM802271, HM802272 and HM802273 respectively), F4 as *Fusarium equiseti* (HQ332532), F6 as *F. proliferatum* (HQ332533), F5, F7 and F8 as *Fusarium* sp. (HQ332534, HQ332535 and HQ332536 respectively).

3.3. Sequence analysis of ITS

ITS sequences of *Fusarium* isolates F1–F8 were aligned with the consensus region using CLUSTAL W program (Fig. 4). 1000 bootstrap replicates were performed and high bootstrap replication percentages were given on the tree's internal nodes. The topologies of the neighbor-joining trees were constructed using CLC Sequence Viewer Version 6.3. Phylogenetic analysis grouped the *Fusarium* isolates into three clusters (Fig. 5). Cluster I includes *Fusarium* isolates F5 and F8 with a bootstrap support of 82%, cluster II includes *Fusarium* isolates F1 and F3 with a bootstrap support of 73%. Also, *Fusarium* isolate F2 was grouped with F1 and F3 with a bootstrap support of 59%, cluster III includes *Fusarium* isolates F6 and F4 with a bootstrap support of 100% while, *Fusarium* isolate F7 was also grouped in cluster III with a bootstrap support of 53%.

3.4. RAPD analysis

The phylogenetic tree was constructed from the RAPD images consisting of 3 clusters. Cluster I comprised F1, F3 and F6, cluster II consists of F2, F5, F7 and F8. Cluster III comprised F4 only (Fig. 6). Jaccard's similarity coefficient was calculated using the RAPD data and it showed the interrelatedness among *Fusarium* isolates (Table 2).

In the present study, *Fusarium* isolates F4 and F6 were identified as *F. oxysporum* and based on morphological characters. But, it differed considerably with that of the ITS identification. Based on ITS region, F4 was identified as *F. equiseti* and F6 as *F. proliferatum*. Plant pathogenic fungi are usually identified by their growth on selective media or through biochemical, chemical and immunological tests. Furthermore, morphological identification of these fungi on nonselective media is time consuming and requires expert taxonomists. Selective media can help in identification up to the genus level, while it cannot differentiate between different species.

We also observed differences between the results when *Fusarium* isolates were identified morphologically and molecularly based on ITS. F5, F7 and F8 were identified to be *F. subglutinans* based on morphological characters while they were identified as *Fusarium* sp. based on ITS region. Molecular biology techniques particularly PCR have provided an alternative approach for detection and identification of many soilborne pathogenic fungi and plant pathogens [29,30]. ITS rDNA is most frequently studied because of species specificity of this region and they are known to provide better resolution at the sub-species level and thus sequence analysis is a superior choice for phylogenetic studies in the *F. oxysporum* species complex [31–34]. But, O'Donnell & Cigelnik (1997) [35] reported that DNA sequences of the ITS regions are uninformative for *Fusarium* although they are useful in distinguishing species in many eukaryotic organisms. According to O'Donnell and Cigelnik (1997) [35] certain regions of the DNA are

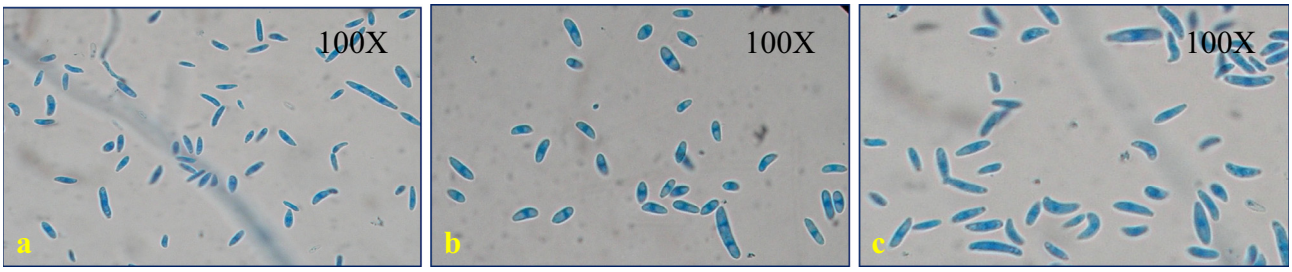

Figure 1 Microconidia structures of (a) *Fusarium oxysporum* F1; (b) *Fusarium oxysporum* F2; (c) *Fusarium oxysporum* F3.

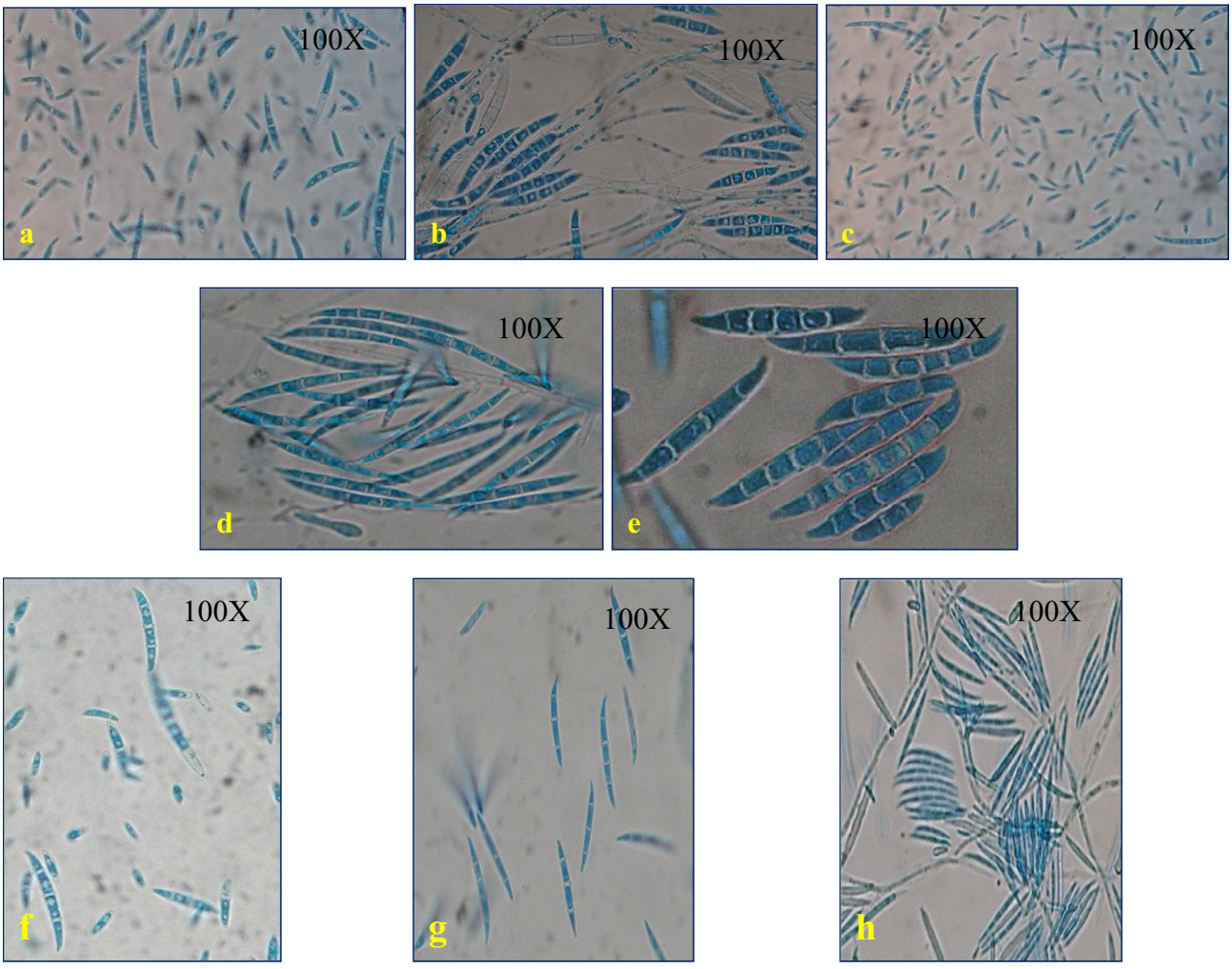

Figure 2 Macroconidia structures of (a) *Fusarium oxysporum* F1; (b) *Fusarium oxysporum* F2; (c) *Fusarium oxysporum* F3; (d) *Fusarium equiseti* F4; (e) *Fusarium* sp. F5; (f) *Fusarium proliferatum* F6; (g) *Fusarium* sp. F7; (h) *Fusarium* sp. F8.

Table 1 List of *Fusarium* isolates with their MTCC and GenBank accession number.

Isolate No.	Host	Morphological identification	MTCC No.	ITS identification	GenBank Accession No.
F1	Tomato	*Fusarium oxysporum*	MTCC8608	*Fusarium oxysporum*	HM802271
F2	Tomato	*Fusarium oxysporum*	MTCC9913	*Fusarium oxysporum*	HM802272
F3	Tomato	*Fusarium oxysporum*	MTCC8610	*Fusarium oxysporum*	HM802273
F4	Tomato	*Fusarium oxysporum*	ND	*Fusarium equiseti*	HQ332532
F5	Tomato	*Fusarium subglutinans*	MTCC9914	*Fusarium* sp.	HQ332534
F6	Tomato	*Fusarium oxysporum*	ND	*Fusarium proliferatum*	HQ332533
F7	Tomato	*Fusarium subglutinans*	MTCC9915	*Fusarium* sp.	HQ332535
F8	Tomato	*Fusarium subglutinans*	MTCC9916	*Fusarium* sp.	HQ332536

'ND' – not deposited. 'MTCC' – Microbial Type Culture Collection, IMTECH Chandigarh.

cladistically uninformative and even misleading. They observed that all the isolates studied harbored two non-orthologous rDNA ITS2 types. Half of the species of the *Gibberella fujikuroi* and *F. oxysporum* lineages studied possessed either type I or type II sequences as the major ITS2 type. The divergence between the two ITS2 sequences was greater than that observed within each type. ITS2 gene trees were therefore discordant with trees inferred from the partial tubulin gene, mt SSU rDNA, nuclear 28S rDNA and nuclear rDNA ITS regions. When both phylogenetic trees generated using ITS and RAPD were analyzed we observed a similarity in the interpretation of inter-relatedness among the *Fusarium* isolates. This suggests the effectiveness and usefulness of molecular techniques for further characterization of fungal and other organisms. Previously, we demonstrated that tomato plants infested with *Fusarium* pathogen have a tendency to

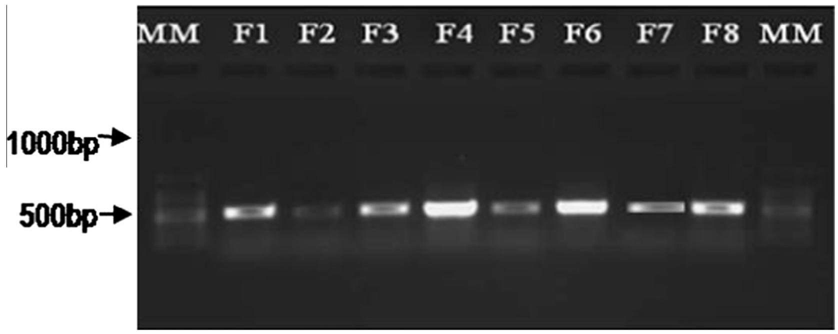

Figure 3 Amplification of conserved ribosomal regions of *Fusarium* sp. using the primers ITS-1 and ITS-4. 'MM' – 100 bp DNA ladder; 'F1–F8' – *Fusarium* isolates.

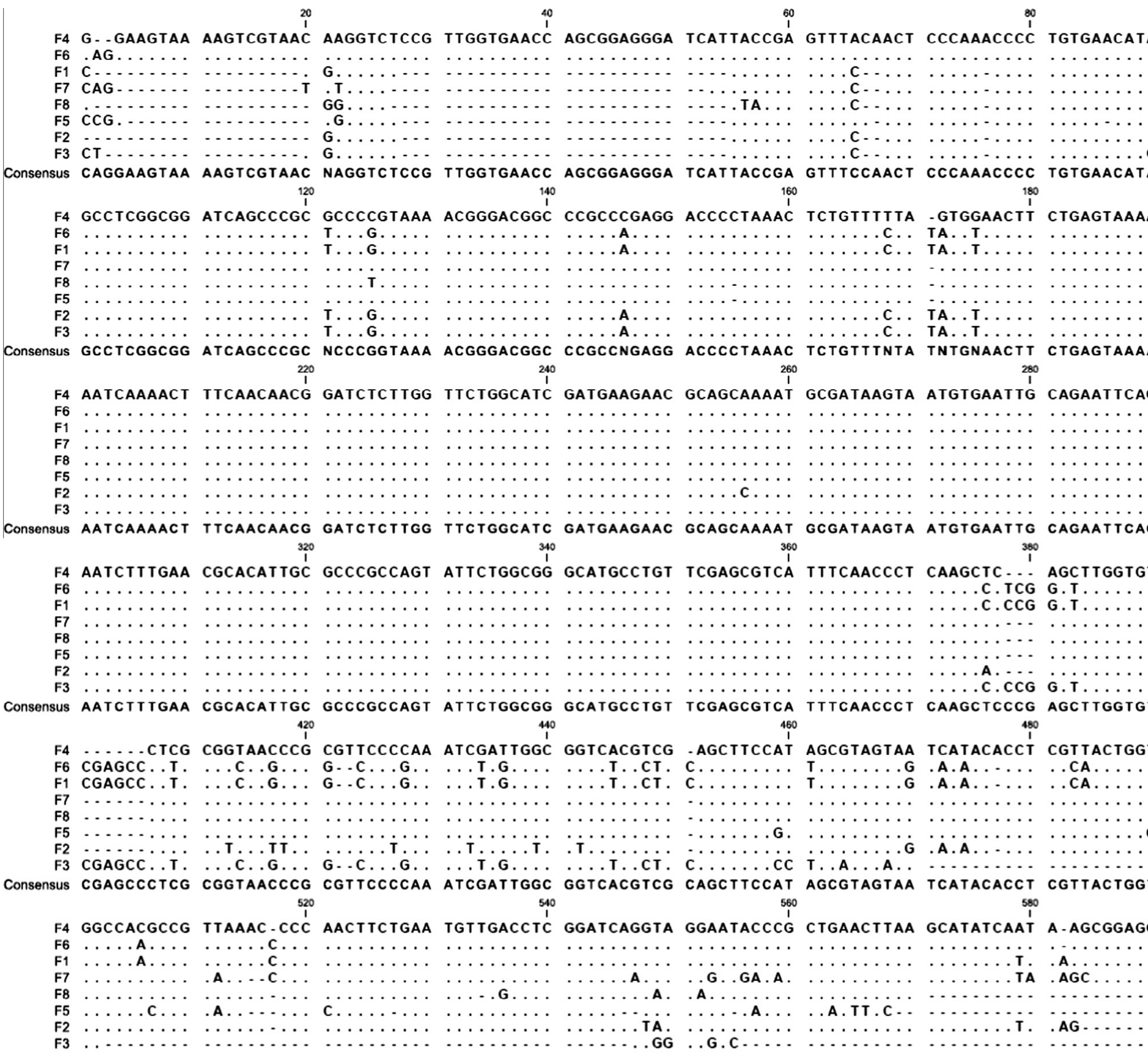

Figure 4 Sequence alignment of *Fusarium* sp. isolates using conserved ribosomal ITS region. Gaps are indicated by dashes (-) and similar ones by dots (.).

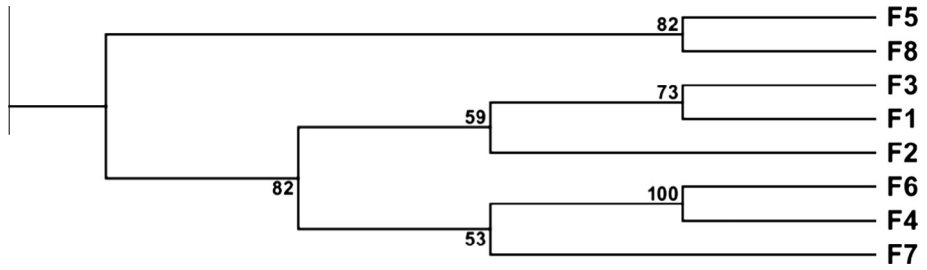

Figure 5 Phyllogenetic tree generated using nucleotide sequence information of the ITS region of the conserved ribosomal DNA of *Fusarium* isolates.

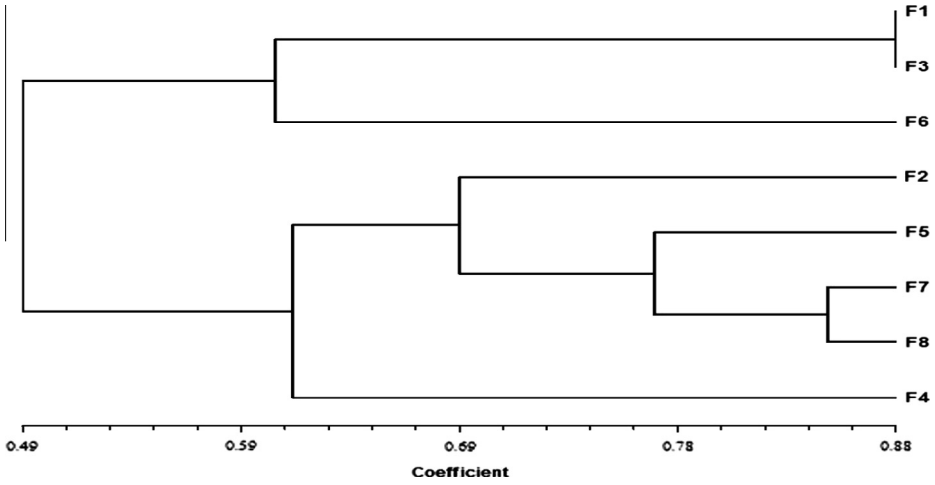

Figure 6 Phyllogenetic tree of *Fusarium* isolates constructed using RAPD data based on UPGMA.

Table 2 Similarity matrix using Jaccard's coefficient based on RAPD data for *Fusarium* isolates.

	F1	F2	F3	F4	F5	F6	F7	F8
F1	1.000							
F2	0.717	1.000						
F3	0.916	0.709	1.000					
F4	0.595	0.587	0.557	1.000				
F5	0.702	0.603	0.725	0.694	1.000			
F6	0.595	0.572	0.618	0.511	0.587	1.000		
F7	0.595	0.541	0.557	0.755	0.664	0.587	1.000	
F8	0.671	0.572	0.694	0.679	0.786	0.603	0.725	1.000

secrete higher levels of phenolic compounds. It was also observed that excess accumulation of phenolic compounds was cytotoxic [36], whereas, in healthy plants this phenolic compounds were secreted normally and got sequestered in the cell wall. This phenolic compound was observed to get accumulated in the vacuoles and thereby gets deposited as lignifications in the cell wall region. This lignification acts as a natural barrier resisting the entry of *Fusarium*.

Thus, there are shortcomings of classical taxonomic and morphological characters for discrimination of species within the genus *Fusarium*. Molecular tools like ITS and RAPD provide necessary information required for a taxonomic purpose for species identification, as well as to elucidate the evolutionary relationships among species.

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