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

Irom Manoj Singha\(^a\)*, Yelena Kakoty \(^b\), Bala Gopalan Unni \(^c\), Jayshree Das \(^d\), Mohan Chandra Kalita \(^e\)

\(^a\) Department of Biotechnology, Asian Institute of Management and Technology, Guwahati 781 023, Assam, India
\(^b\) Biotechnology Division, North East Institute of Science and Technology, Jorhat 785 006, Assam, India
\(^c\) Research Cell, Assam Downtown University, Panikhaiti, Guwahati 781 026, Assam, India
\(^d\) Biotechnology Division, Defence Research Laboratory, Tezpur 784 001, Assam, India
\(^e\) Department of Biotechnology, Gauhati University, Guwahati 781 014, Assam, India

Received 27 February 2016; revised 1 May 2016; accepted 8 July 2016
Available online 25 July 2016

**KEYWORDS**
*Fusarium*; Characterization; ITS; RAPD

**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.

© 2016 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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
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 DNA 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 GGTTGAACCTGCGG-3′) and ITS4 (5′-TCCCTCGCTATT 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 MgCl₂; 2 mM each of dNTPs; 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 MgCl₂. 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 MgCl₂; 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 created, 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.
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 2](image-url)  
**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.

| 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.

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

**Acknowledgement**

We are thankful to Dr. RB Srivastava, former Director DRL, Tezpur and Dr. PG. Rao, former Director NEIST, Jorhat for support and encouragement. This study was part of the PhD thesis that was submitted under Gauhati University, Guwahati. Irom Manoj Singha is thankful to DRDO Delhi for the JRF and SRF fellowship provided during 2007–2011.

**References**

[1] B.A. Summeral, B. Salleh, J.F. Leslie, Plant Dis. 87 (2003) 117–128.

[2] C. Booth, The Genus Fusarium, Commonwealth Mycological Institute, Kew, 1971.

[3] S.L. Woo, C. Noviello, M. Lorito, CAB Int. (1998) 319.

[4] D. Parkinson, Biology of Conidial Fungi, 1, Academic, New York, 1981, pp. 277–294.

[5] A. Szecsi, A. Dobrovolsky, Mycopathologia 89 (1985) 95–100.

[6] P.E. Nelson, T.A. Tousson, W.F.O. Marasas, Fusarium Species, The Pennsylvania State University Press, 1983.

[7] R.K. Kheterpal, Ind. J. Phytopathol. 59 (2006) 397–398.

[8] R.P. Baayen, K. O’Donnell, P.J.M. Bonants, E. Cigelnik, L.P. N.M. Kroon, E.J.A. Roebrock, C. Walwijk, Phytopathology 90 (2000) 991–997.

[9] K. O’Donnell, H.C. Kistler, B.K. Tachke, H.H. Casper, Proc. Natl. Acad. Sci. 97 (2000) 7905–7910.

[10] K. Skovgaard, H.I. Nirenberg, K. O’Donnell, S. Rosendahl, Phytopathology 91 (2001) 1231–1237.
Identification and characterization of Fusarium sp.

[11] J.G.K. Williams, A.R. Kubelik, J.K. Livak, J.A. Rafalski, S.V. Tingey, Nucleic Acids Res. 18 (1990) 6531–6535.
[12] A. Chiocchetti, S. Ghignone, A. Minuto, M.L. Gullino, A. Garibaldi, Q. Migheli, Plant Dis. 88 (1999) 576–581.
[13] M. Pasquali, A. Acquafredda, B. Balmas, Q. Migheli, A. Garibaldi, M. Gullino, J. Phytopathol. 104 (2003) 49–57.
[14] S.D. Wilson, E. Chandler, P. Jenning, P. Nicholson, F.E.M.S. Microbiol. Letters 233 (2004) 69–76.
[15] V. Balmas, B. Scherm, P.D. Primo, D. Ran, A. Marcello, Q. Migheli, Eur. J. Plant Pathol. 111 (2005) 1–8.
[16] H. Bayraktar, F.S. Dolar, S. Maden, J. Phytopathol. 156 (2008) 146–154.
[17] S. Guleria, R. Aggarwal, T.S. Thind, T.R. Sharma, J. Phytopathol. 155 (2007) 654–661.
[18] B. Lievens, L. Claes, D.J. Vakalounakis, A.C. Vanachter, B.P. Thomma, Environ. Microbiol. 9 (2007) 2145–2161.
[19] K.B. Assigbetse, D. Fernandez, M.P. Dubois, J.P. Geiger, Phytopathology 84 (1994) 622–626.
[20] S. Manulis, N. Kogan, M. Reuvan, Y. Ben-Yephet, Phytopathology 84 (1994) 98–101.
[21] A. Tantaoui, M. Quinten, J.P. Geiger, D. Fernandez, Phytopathology 86 (1996) 787–792.
[22] Q. Migheli, E. Briatore, A. Garibaldi, Eur. J. Plant Pathol. 104 (1998) 30–35.
[23] A.M. de Haan, A. Numansen, E.J.A. Roebroeck, J. van Doorn, Plant Pathol. 49 (2000) 89–99.
[24] F.M. Alves-Santos, M. Farnando, B. Ramos, S. Garcia, M. Asuncion, A.P. Eslava, Phytopathology 92 (2002) 237–244.
[25] T.J. White, T. Bruns, S. Lee, J. Taylor, PCR Protocols, San Diego Academic Press, 1990, pp. 315–322.
[26] J.D. Thompson, D.G. Higgins, T.N. Gibson, Nucleic Acids Res. 22 (1994) 4673–4680.

Further reading

[27] F.J. Rohlf, NTSYS-pc numerical taxonomy and multivariate analysis system, version 2.02, Exeter Publication, New York, 1998.
[28] P.H.A. Sneath, R.R. Sokal, Numerical Taxonomy: The Principles and Practice of Numerical Classification, Freeman, San Francisco, 1973.
[29] T. Kagaya, M. Kitao, M. Shinohara, K. Uchida, S. Fukushi, F.B. Hoshino, N. Takeda, K. Katayama, J. Clin. Microbiol. 41 (4) (2003 Apr) 1548–1557.
[30] P. Vincelli, N. Tisserat, Plant Dis. 92 (2008) 660–669.
[31] D.H. Kim, R.D. Martyn, C. Magill, Phytopathology 83 (1993) 91–97.
[32] P.A. Okubara, K.L. Schroeder, T.C. Paultiz, Can. J. Plant Pathol. 27 (2005) 300–313.
[33] H. Terashima, S. Fukuchi, K. Nakai, M. Arisawa, K. Hamada, N. Yabuki, K. Kitada, Curr. Genet. 40 (2002) 311–316.
[34] B. Zhao, X. Wei, W. Li, R.S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z.C. Lai, K.L. Guan, Genes Dev. 21 (2007) 2747–2761.
[35] K. O'Donnell, E. Cigelnik, Mol. Phylogenet. Evol. 7 (1997) 103–116.
[36] I.M. Singh, Y. Kakoty, B.G. Unni, M.C. Kalita, J. Das, A. Naglot, S.B. Wann, L. Singh, World J. Microbiol. Biotechnol. 27 (2011) 2583–2589.