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Diversity and identity of *Fusarium* species occurring on fruits, vegetables and food grains

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Abstract. *Ingle AP. 2017. Diversity and identity of Fusarium species occurring on fruits, vegetables and food grains. Nusantara Bioscience 9: 44-51.* The members of the genus *Fusarium* are the most important pathogens associated with different plants including crop plants, fruits, vegetables, etc. *Fusarium* species are mainly responsible for wilts, blights, root-rots and cankers in these plants. *Fusarium* spp. produces many types of mycotoxins, which can unfavorably affect human and animal health. In the present study, 48 isolates of *Fusarium* were recovered from various fruits, vegetables and food grains. Out of these, 23 isolates were recovered from vegetables, 14 from food grains and 11 from fruits. Further, different isolates recovered from infected plant materials were identified on the basis of morphological, cultural and microscopic characteristics. All the isolates were categorized into 11 groups. viz., *F. semitectum* have maximum number of isolates (10), followed by *F. solani* (7), *F. oxysporum* (5), *F. equiseti* (5), *F. acuminatum* (4), *F. proliferatum* (4), *F. scirpi* (4), *F. moniliforme* (3), *F. tricinctum* (3), *F. graminearum* (2) and *F. culmorum* (1). For the rapid identification and confirmation of identity, ITS (internal transcribed spacer) r-DNA sequencing and their comparison with sequences available in GenBank was performed using online BLAST program. ITS-rDNA sequence comparison can be used as potential and unique molecular marker for the rapid and accurate identification of different *Fusarium* species. Moreover, the rapid identification can help in the management of *Fusarium* infections in plants.

Keywords: *Fusarium*, crop plants, fruits, vegetables, food grains, molecular marker, mycotoxins

INTRODUCTION

The total population of entire world is about 7 billion and projected to over 8 billion by 2025. Out of these about 4.43 billion i.e., more than 60% of population is living only in Asia (Samantarai and Achakzai 2014; UN 2015). The Indian economy is mainly based on agriculture and more than 70% of total population depends on agriculture. Therefore, production of sufficient quantity of food, which can feed the whole population of country has always been a great challenge. Various environmental challenges such as storms, droughts, flood, etc. contribute a major portion of crop yield loss in agriculture (Iizumi and Ramankutty 2015). Apart from these, loss in agricultural production continues to be constrained by a number of biotic and abiotic factors (Rai and Ingle 2012). The important biotic factors affecting agricultural production mostly include soil fungi, insects-pests, parasites and predators. It was reported that the fungal attack has been a major problem associated with these crops and more predominant is the attack of *Fusarium*, which causes tremendous loss in Indian economy (Ingle et al. 2008).

In addition to agricultural loss, consumption of myoctoxins contaminated food (vegetables, fruits and food grains) can cause illness and various disorders in human beings and animals. The most important *Fusarium* mycotoxin, which can frequently occur at biologically significant concentrations in various food products are fumonisins, zearalenone and trichothecenes (deoxynivalenol, nivalenol and T-2 toxin) (Ferrigo et al. 2016; Tima et al. 2016; Tola and Kebede 2016). It was claimed that our knowledge of *Fusarium* began with the discovery of "diseases of cereals" by Bennett in 1928, but Link was the first scientist who described the *Fusarium roseum* as the first species in 1809 (Moss and Smith, 1984). Most *Fusarium* spp. are pathogenic to plants. It is responsible for causing several plant diseases such as crown-rots, head blights, scabs, vascular wilts, root-rots and cankers (Leslie and Summerell 2006).

Banana (*Musa sapientum*) is susceptible to the infection by several fungal diseases of which *Fusarium*- wilt caused by *F. oxysporum* is considered to be the most important and responsible for severe economic losses in banana-producing countries (Butler 2013; Moretti et al. 2004). Leong et al. (2009) also reported the infection caused by *F. oxysporum* in banana and successfully isolated 13 isolates of *F. oxysporum* from Malaysia and two isolates from Indonesia. Recently, Triest et al. (2016) reported that *F. musae* was responsible for the infection of banana fruits and the consumption of such infected fruits may cause fusariosis in human being.

Hawa et al. (2010) reported that Dragon fruit (*Hylocereus polyrhizus*) plants were severely infected with *F. semitectum* leading to the economic loss of many cultivars. They isolated 79 isolates of *F. semitectum* from 3 different plant parts namely from the stem, fruit and root of diseased *H. polyrhizus* from 9 states in Malaysia. Saseetharan and Zakaria (2014) studied the diversity of

Fusarium species in various vegetables crops. They recovered total 83 isolates of different *Fusarium* species including *F. oxysporum* (22 isolates), *F. semitectum* (19 isolates), *F. solani* (19 isolates), *F. proliferatum* (14 isolates), *F. pseudocircinatum* (4), *F. sacchari* (2), *F. equiseti* (2) and *F. verticillioides* (1). Moreover, only 21 isolates were found to be pathogenic when subjected to pathogenicity test. There are many reports on infection of economically important crop plants by different *Fusarium* species. Gupta et al. (2009) reported the wilting in Guava caused by *F. solani* in different places of Northern India. They isolated total 6 isolates of *F. solani*, and found that it was most prevalent fungus than other pathogen responsible for wilting. The wilt pathogen (*F. udum*) was isolated from the pigeon pea, showing vascular- wilt symptoms and collected from different places of the India (Mahesh et al. 2010).

Apart from these, Elhassan (2016) demonstrated the association of *F. oxysporum* in *Fusarium*-wilt disease of date palm (*Phoeniex dactylifera* L.) in Northern State, Sudan and recovered different isolates of *F. oxysporum* from infected plants. In another study, Nirmaladevi et al. (2016) studied the molecular phylogeny, pathogenicity and toxigenicity of various strains of *F. oxysporum* f. sp. *lycopersici* isolated from tomato. Further, they used different molecular markers such as ISSR fingerprinting and ITS sequence analysis for the diversity and identification. . Similarly, *F. proliferatum* was also found to cause wilt disease in greenhouse-grown safflower (*Carthamus tinctorius*) in Jeonju, Korea, which was identified by using morphological and molecular markers including ITS-r DNA and translation elongation factor 1α (Kim et al. 2016). It is evident from all the above studies that the *Fusarium* spp. generally associated with various crop plants causing plant disease.

Considering the above facts, rapid and accurate identification of *Fusarium* species is necessary for the management of *Fusarium*-wilt, etc. For this, detection and identification based on morphological, cultural and microscopic characteristics is not enough. The use of molecular markers is necessary for the confirmation of identity. Therefore, the main aim of the present study was to examine the diversity of *Fusarium* species associated with vegetables, food grains and fruits and also to develop strategies for the rapid and accurate identification of *Fusarium* species using molecular markers.

MATERIALS AND METHODS

Plant materials

Different infected plant materials like food grains (wheat, sorghum, mung bean, black mungo, soybean, ground nut, etc.), fruits (banana, papaya, sapota, grapes, etc.), and vegetables (ladies finger, brinjal, potato, onion, ginger, gherkins etc.) were aseptically collected from the different locations like grain, vegetable and fruit markets of Amravati and Akola districts (M.S.) India. The details of infected materials are given in Table 1.

Isolation of different *Fusarium* species

The infected plant materials were surface sterilized, inoculated on to the surface of sterilized Potato Dextrose Agar (PDA) and incubated in BOD incubator at 28 ± 2^0C for 48-72 hrs. All the petri plates were regularly observed for the growth of *Fusarium*.

Morphological identification of different *Fusarium* spp.

After 7 days, different morphological and cultural characteristics such as dorsal and ventral colour and growth rate of colony, texture of mycelium, etc. were noted. Moreover, microscopic characteristics like septation and size of macro- and microconidia, the presence, absence and types of chlamydospores, etc. were also recorded. The identification of these isolates was made using the criteria provided in *Fusarium* identification key given by Seifert (1996) and *Fusarium* Laboratory Manual (Leslie and Summerell 2006).

DNA extraction

All the isolates of *Fusarium* species were grown on PDA at 28 ± 2^0C for 3 days. The mycelia were harvested and total DNA was extracted using fungal genomic DNA isolation kit purchased from Chromous Biotech Pvt. Ltd, Bangalore, India according to manufacturer's instructions.

ITS amplification and sequencing

ITS regions of different *Fusarium* species were amplified by PCR using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') designed and synthesized by Chromous Biotech Pvt. Ltd, Bangalore, India. Each PCR reaction mixture contained, 2X PCR master mix (12.5µL) (Fermentas Life Sciences, Canada), genomic DNA (5µL), 1µM each of the primers ITS1 and ITS4 (1µL), 25 mM $MgCl_2$ (1.5µL) (provided with PCR master mix) additional Taq DNA polymerase (0.3µL) (Genaxy, 5U/µL) and nuclease free distilled water (3.7µL) (supplied with Fermentas PCR master mix) in a total volume of 25 µL.

PCR was performed on gradient PCR machine (Palm-Cycler from Corbett Research, Australia) and thermal cycler (Eppendorf, Germany). The PCR conditions include initial denaturation at 94^0C for 2 min, 35 cycles with denaturation at 94^0C for 30 sec, annealing 38^0C for 1 min, extension at 72^0C for 2 min and final extension at 72^0C for 5 min with holding temperature at 4^0C for 10 min. Negative control (without template DNA) was maintained for each set of experiment to test for the presence of non-specific banding. All experiments were repeated for three times. PCR products were electrophoresed on 1.5% agarose by using 1X TAE buffer (Fermentas Life Sciences, Canada), stained with ethidium bromide, visualized in a UV-trans illuminator and the gel were photographed using Gel Doc system (AlphaImager, Gel documentation system, USA). After proper amplification, PCR products were sent for sequencing to Chromous Biotech Pvt. Ltd, Bangalore, India.

BLAST analysis

For the confirmation of preliminary identified *Fusarium* species, online BLAST analysis was carried out. After sequencing, the ITS nucleotide sequences for each species were then compared to those in the public domain databases NCBI (National Center for Biotechnology information; www.ncbi.nih.gov).

UPGMA analysis

The sequences obtained were subjected to the Unweighted Pair Group Method with Arithmetic Mean analysis (UPGMA) analysis. The Numerical Taxonomy System of Multivariate Statistical Program (NTSYS) software package was used to obtain the phenotypic cluster and phylogenetic tree stating the genetic relationship among the different isolates of *Fusarium*.

RESULTS AND DISCUSSION

Isolation of different *Fusarium* spp. from infected plant materials

Different infected plant materials (Table 1) were collected and used as a source for isolation of *Fusarium*. Total 48 isolate of *Fusarium* species were recovered from these infected materials. Out of these, 23 isolates from vegetables, 14 from food grains and 11 isolates were recovered from fruits. All these isolates were stored in a refrigerator at 4^{o}C for further use. The above data suggested that *Fusarium* species are most prevalently found in vegetables followed by food grains and fruits in the geographic regions selected for the collection of materials. Saseetharan and Zakaria (2014) reported the prevalence of *Fusarium* species on the vegetable crops. In addition, many other researchers have reported infection of different *Fusarium* species with various plants; for example, *F. udum* with pigeon pea (Joshi 2001), *F. oxysporum* f. sp. *cepae*, *F. solani* and *F. proliferatum* with onion (Klokocar-Smit et al. 2008), *F. musae* with banana (Triest et al. 2016), *F. oxysporum* with date palm (Elhassan 2016), *F. oxysporum* f. sp. *lycopersici* with tomato (Nirmaladevi et al. 2016), etc. Figure 1 shows representative infected material from each vegetable, food grain and fruit.

Morphological and cultural identification of *Fusarium* spp.

The different isolates of *Fusarium* species recovered from infected materials were identified on the basis of morphological, cultural and microscopic characteristics as discussed in methodology. Depending on similarities in these characteristics, all the isolates of *Fusarium* species were categorized into 11 different groups using *Fusarium* identification keys (Seifert 1996) and *Fusarium* Laboratory Manual (Leslie and Summerell 2006). Among these groups, *F. semitectum* have maximum number of isolates (10), followed by *F. solani* (7), *F. oxysporum* (5), *F. equiseti* (5), *F. acuminatum* (4), *F. proliferatum* (4), *F. scirpi* (4), *F. moniliforme* (3), *F. tricinctum* (3), *F. graminearum* (2) and *F. culmorum* (1) (Figure 2). Detailed morphological, cultural and microscopic features reported for identification of all 11 groups have been provided in Table 2.

Sequencing and comparison of ITS regions

For rapid identification and phylogenetic analysis, the ITS regions 1 and 4 have been used as targets because they generally display sequence variation among species and minor variation within isolates of the same species. Moreover, it offers distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. Here, the initial identification of the *Fusarium* isolates based on morphological, cultural and microscopic characteristics was compared with the result of ITS sequencing and all these isolates were also confirmed by ITS sequence analysis.

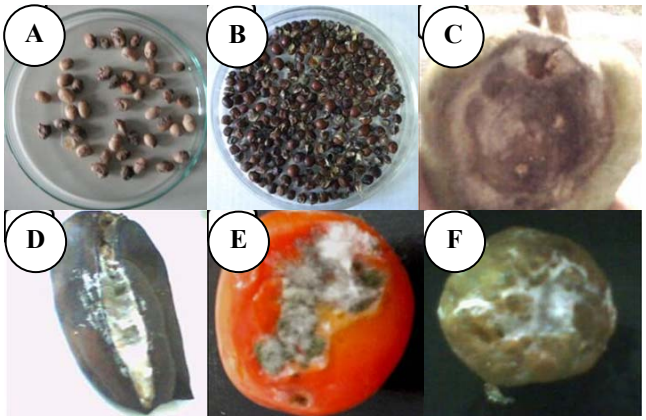

Figure 1. Infected plant materials (A) Soybean (*Glycine max*) seeds (B) Arhar (*Cajanusc ajan*) seed (C) Papaya (*Carica papaya*) fruit (D) Banana (*Musa paradisiaca)* fruit (E) Tomato (*Lycospersicum esculentu*) (F) Round Guard (*Citrullus vulgaris* var. *fistulosus*)

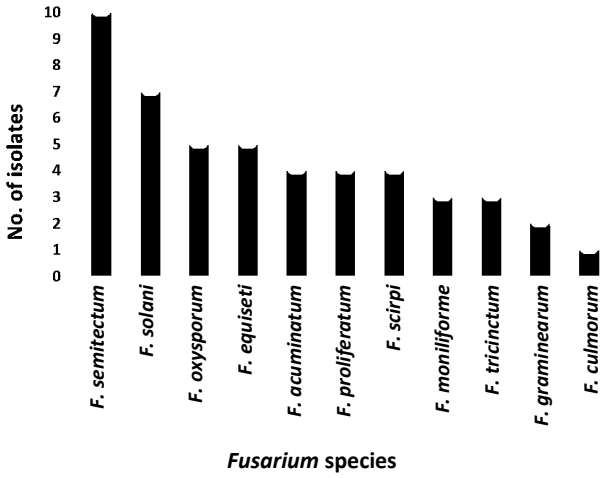

Figure 2. Different *Fusarium* species and their number of isolates

Table 1. List of different infected materials collected from different locations

Host	Botanical name	Type of host
Soybean	*Glycine max*	Grains
Ground nut	*Arachis hypogaea*	Grains
Mung bean	*Vigna radiata*	Grains
Pigeon pea	*Cajanus cajan*	Grains
Cotton	*Gossypium hirsutum*	Grains
Red Chilly	*Capsicum annum*	Vegetable
Cauliflower	*Brassica oleracea*	Vegetable
Ladies finger	*Abelmoschus esculentus*	Vegetable
Papaya	*Carica papaya*	Fruit
Sponge gourd/ turai	*Luffa cylindrica*	Vegetable
Beet	*Beta vulgaris*	Vegetable
Potato	*Solanum tuberosum*	Vegetable
Cucumber	*Cucumis sativus*	Fruit
Gherkins/ little gourd	*Coccinia grandis*	Vegetable
Onion	*Allium cepa*	Vegetable
Brinjal	*Solanum melongena*	Vegetable
Cow pea	*Vigna unguiculata*	Vegetable
Chickpea	*Cicer arietinum*	Grains
Potato-1	*Solanum tuberosum*	Vegetable
Tomato	*Lycospersicum esculentum*	Vegetable
Apple	*Malus domestica*	Fruit
Onion-1	*Allium cepa*	Vegetable
Brinjal-1	*Solanum melongena*	Vegetable
Bitter gourd	*Momordica charantia*	Vegetable
Carrot	*Daucus carota*	Vegetable
Moth bean	*Vigna aconitifolia*	Grains
Green peas	*Pisum sativum*	Vegetable
Ginger	*Zinziber officinale*	Vegetable
Wheat	*Triticum aestivum*	Grains
Round gourd	*Citrullus vulgaris* var. *fistulosus*	Vegetable
Bell Pepper	*Capsicum annuum*	Vegetable
Maize	*Zea mays*	Grains
Banana	*Musa paradisiaca*	Fruit
Sorghum	*Sorghum vulgare*	Grains
Lemon	*Citrus limon*	Fruit
Black gram	*Vigna mungo*	Grain
Bottle gourd	*Lagenaria siceraria*	Vegetable
Ridge gourd	*Luffa acutangula*	Vegetable
Orange	*Citrus sinensis*	Fruit
Maize kernel	*Zea mays*	Fruit
Garlic	*Allium sativum*	Vegetable
Grapes	*Vitis vinifera*	Fruit
Sapota/Chikoo	*Manilkara zapota*	Fruit
Cow pea	*Vigna unguiculata*	Grains
Papaya-1	*Carica papaya*	Fruit
Banana-1	*Musa paradisiaca*	Fruit
Kidney beans	*Phaseolus vulgaris*	Grains
Rice	*Oryza sativa*	Grains

In the present study, all the isolates were preliminary identified and categorized into 11 different groups depending on similarities in morphological and cultural characters. Therefore, for the ITS sequencing study, only one representative isolate from each of 11 groups (total 11 species sequenced) was selected. DNA of 11 *Fusarium* species namely, *F. semitectum*, *F. solani*, *F. oxysporum*, *F. equiseti*, *F. acuminatum*, *F. scirpi*, *F. proliferatum*, *F. tricinctum*, *F. moniliforme*, *F. graminearum* and *F. culmorum* were isolated by using commercial fungal DNA isolation kit and ITS regions were amplified using species specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplifications registered a sole fragment of approximately 550 bp identical for all *Fusarium* species on 1.5 % agarose gel after proper electrophoresis (Figure 3).

The expected 550 bp size of PCR product of ITS region was found to be slightly different after sequencing. The maximum size of sequence was found in *F. proliferatum* (511 bp) followed by *F. graminearum* (510 bp), *F. tricinctum* (502 bp), *F. semitectum* (498 bp), *F. scirpi* (496 bp) and *F. culmorum* (493 bp), while *F. solani* (492 bp) and *F. moniliforme* (492 bp) showed the similar size sequences followed by *F. acuminatum* (491 bp) and *F. oxysporum* (486 bp). However, the minimum size of sequence was reported in case of *F. equiseti* (485 bp). All the above 11 sequences after complete validation were submitted to the European Molecular Biology Laboratory (EMBL) through their online submission procedure. EMBL accepted these sequences and assigned a specific accession number to all these sequences. The details of sequences submitted to EMBL database and their accession numbers are given in Table 3.

BLAST Analysis

ITS-4 sequences thus obtained for all the *Fusarium* species were subjected to Basic Local Alignment Search Tool (BLAST) network service, so as to determine the homology. As the BLAST service is free and available online, the nucleotide sequences (ITS-4) were compared (aligned) with all the sequences available with nucleotide databases (e.g. NCBI). Homology search was performed within the non-redundant databases of GenBank using the BLAST algorithm at http: //www.ncbi.nlm.nih.gov/BLAST/ of NCBI.

For each sequenced PCR product, sequences were put in window given for test sequence in a FASTA format and aligned using online BLAST search tool in NCBI databases. The resulting output showed the best similarity matches with the sequences available in databases. The example has been shown in Figure 4 and similar results have been obtained for all species, except *F. semitectum*. It showed the best matches with different *Fusarium* spp. (identified up to species level only). The possible reason is unavailability of ITS sequences of *F. semitectum* in databases. The BLAST results reported for all sequences of the present study revealed that each sequence of 11 *Fusarium* species have homology with nucleotide sequences of same species present in the databases at different similarity level ranges between 98-100 %. Each of the above tested *Fusarium* species showed the maximum hits with the different strains of the same species. BLAST analysis confirmed that all the 11 groups of *Fusarium* species classified on the basis of morphological, cultural and microscopic characters were correct.

Table 2. Morphological, cultural and microscopic characteristics of 11 different *Fusarium* species

Identified *Fusarium* species	Colony colour		Type of Mycelium	Colony diameter (cm)*	Common macro-conidial septation	Length and width of macroconidia in (µm)**		Septation in microconidia	Chlamydo-spores
	Dorsal	Ventral				Length	Width		
F. semitectum	White	Brown	Aerial	7.5	3-5	40.80 ± 0.52	2.40± 0.14	0-1	Chains
F. solani	Cream	Violet	Aerial	8.0	3-5	48.54 ± 2.12	4.20 ± 0.16	0-1	Chains
F. oxysporum	White	Violet	Aerial	6.5	3-5	28.00 ± 0.24	3.20 ± 1.26	0	Chains
F. equiseti	White	Cream	Aerial	8.0	5-7	78.60 ± 0.16	2.40 ± 0.40	Absent	Clumps
F. acuminatum	White	Reddish	Aerial	8.0	3-5	52.60 ± 1.92	2.60 ± 0.20	0-1	Chains
F. proliferatum	White	Faint violet	Aerial	8.0	3-5	48.60 ± 1.26	3.20 ± 1.90	0	Absent
F. scirpi	White	Cream	Aerial	8.0	6-7	76.40 ± 1.24	2.40 ± 1.20	0-2	Clumps
F. moniliforme	Faint pink	Yellowish	Aerial	5.0	3-5	36.40 ± 2.20	3.50 ± 0.20	0	Absent
F. tricinctum	White	Pale orange	Aerial	6.0	3-4	42.80 ± 2.20	3.20 ± 1.28	0	Chains
F. graminearum	Off white	Dark red	Aerial	7.5	5-6	56.00 ± 0.52	4.80 ± 0.86	Absent	Chains
F. culmorum	White	Pink	Aerial	8.0	4-5	28.50 ± 1.52	4.80 ± 0.20	Absent	Single/chain

Note: *Diameter of colony after 7 days of growth at 28°C; ** Mean values of 10 random conidia ± standard deviation

Table 3. List of ITS-4 sequences of *Fusarium* species and their GenBank accession numbers

Fusarium species	Anamorphs/ synonyms	Host		Place of collection	EMBL GenBank accession No.
		Common name	Botanical name		
F. semitectum	*F. incarnatum*	Soybean	*Glycine max*	Akola (M.S.) India	FR851230
F. oxysporum	*F. oxysporum*	Tomato	*Lycospersicum esculentum*	Akola (M.S.) India	FR851229
F. equiseti	*F. equiseti*	Brinjal	*Solanum melongena*	Amravati (M.S.) India	FR851233
F. acuminatum	*F. acuminatum*	Ginger	*Zinziber officinale*	Amravati (M.S.) India	FR851231
F. proliferatum	*Giberella intermedia*	Sorgum	*Sorghum vulgare*	Akola (M.S.) India	FR851236
F. moniliforme	*Giberella moniliformis*	Maize kernel	*Zea mays*	Amravati (M.S.) India	FR851235
F. tricinctum	*F. tricinctum*	Sapota	*Manilkara zapota*	Amravati (M.S.) India	FR851237
F. graminearum	*Giberella zeae*	Kidney beans	*Phaseolus vulgaris*	Akola (M.S.) India	FR851234
F. culmorum	*F. culmorum*	Rice	*Oryza sativa*	Amravati (M.S.) India	FR851232
F. solani	*F. solani*	Potato	*Solanum tuberosum*	Amravati (M.S.) India	FR878062
F. scirpi	*F. scripi*	Black gram	*Vigna mungo*	Akola (M.S.) India	FR878061

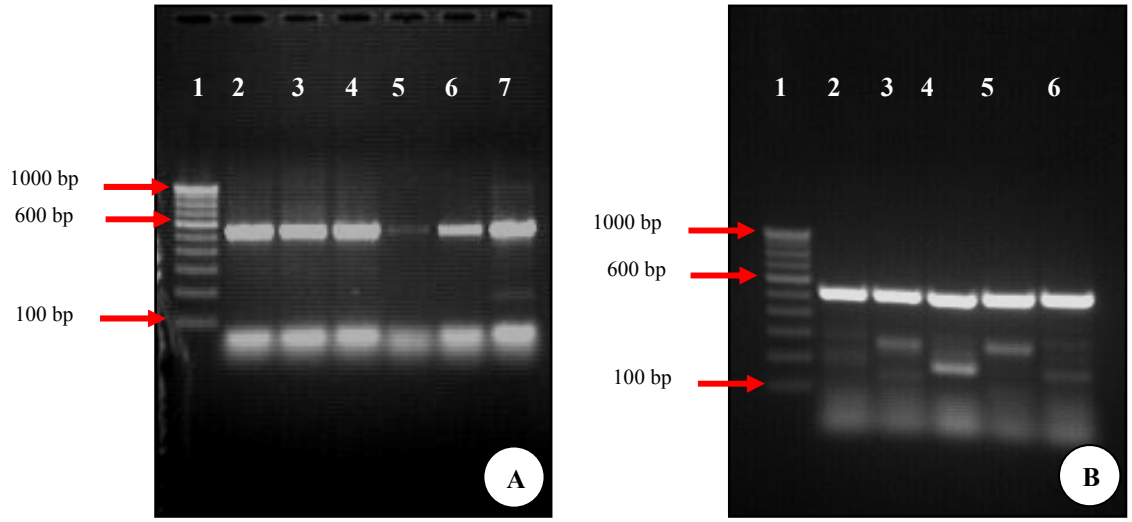

Figure 3. Agarose gel showing PCR amplification product for 11 different *Fusarium* species. A. Lane 1: Marker (100 bp); Lane 2: *F. tricinctum*; Lane 3: *F. culmorum*; Lane 4: *F. proliferatum*; Lane 5: *F. moniliforme*; Lane 6: *F. graminearum*; Lane 7: *F. semitectum*. B. Lane 1: Markers (100 bp); Lane 2: *F. oxysporum*; Lane 3: *F. scirpi*; Lane 4: *F. equiseti*; Lane 5: *F. solani*; Lane 6: *F. acuminatum*

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ824032.1	Fusarium oxysporum strain YY-1 18S ribosomal RNA gene, partial sec	896	896	99%	0.0	100%
FJ605247.1	Fusarium oxysporum isolate UFMGCB 1316 18S ribosomal RNA gene,	896	896	99%	0.0	100%
HQ451891.1	Fusarium oxysporum strain FOCCB-4 internal transcribed spacer 1, p	894	894	99%	0.0	100%
FJ157216.1	Fusarium oxysporum strain LZ070103 18S ribosomal RNA gene, partia	893	893	99%	0.0	100%
FJ156282.1	Fusarium oxysporum f. sp. niveum 18S ribosomal RNA gene, partial s	893	893	100%	0.0	99%
HQ649825.1	Fusarium oxysporum isolate s049 18S ribosomal RNA gene, partial se	891	891	99%	0.0	99%
HQ649820.1	Fusarium oxysporum isolate r407 18S ribosomal RNA gene, partial sec	891	891	99%	0.0	99%
HQ649817.1	Fusarium oxysporum isolate r171 18S ribosomal RNA gene, partial sec	891	891	99%	0.0	99%
HQ649816.1	Fusarium oxysporum isolate r160 18S ribosomal RNA gene, partial sec	891	891	99%	0.0	99%
HQ649814.1	Fusarium oxysporum isolate r057 18S ribosomal RNA gene, partial sec	891	891	99%	0.0	99%
HM043738.1	Fusarium oxysporum f. sp. lycopersici isolate ED-3 18S ribosomal RN	891	891	99%	0.0	99%
HM042319.1	Fusarium oxysporum strain R6 internal transcribed spacer 1, partial s	891	891	99%	0.0	100%
GU565571.1	Fusarium oxysporum strain CPRI15 18S ribosomal RNA gene, partial s	891	891	99%	0.0	99%
GU724514.1	Fusarium oxysporum isolate Fo15 18S ribosomal RNA gene, partial se	891	891	99%	0.0	99%
GU724513.1	Fusarium oxysporum isolate Fo13 18S ribosomal RNA gene, partial se	891	891	99%	0.0	99%
FJ919630.1	Fusarium oxysporum isolate For2 18S ribosomal RNA gene, partial sec	891	891	99%	0.0	99%
AB470894.1	Fusarium oxysporum genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28	891	891	99%	0.0	99%
FJ196602.1	Fusarium sp. FPGLXJ01 18S ribosomal RNA gene, partial sequence; ir	891	891	99%	0.0	99%
FJ233193.1	Fusarium oxysporum 18S ribosomal RNA gene, partial sequence; inte	891	891	99%	0.0	99%
FJ157215.1	Fusarium oxysporum strain YH070403 18S ribosomal RNA gene, partia	891	891	99%	0.0	100%
EU727455.1	Fusarium oxysporum isolate 10 18S ribosomal RNA gene, partial sequ	891	891	99%	0.0	99%

Figure 4: BLAST analysis of ITS-4 sequence of *F. oxysporum* showing the best matches with the other *F. oxysporum* strains with maximum identification percentage of 100%

UPGMA analysis

Sequence based UPGMA analysis was performed for the determination of genetic diversity among the 11 *Fusarium* species isolated in the present study. For the phylogenetic analysis of these species, initially multiple sequence alignment was carried out using a program Clustal-W available online. Thus obtained alignment file was used for the construction of dendrogram using UPGMA tool of phylogenetic analysis program NTSYS pc. 2.02.

The UPGMA cluster analysis clearly grouped these isolates into 7 major clusters (Figure 5) and established their relationship of similarity. Genetic relationship calculated in the form of similarity coefficient from the dendrogram showed high level of genetic similarity among all different species of *Fusarium*, which ranges from 0.00 to 0.04. Similarity coefficients (Jaccard's coefficients) for genetic similarities among different isolates were determined and the genetic relationship between each isolate was shown in Table 4. Cluster analysis obtained from the ITS-4 sequences clearly distinguished all the 11 *Fusarium* species in 7 different main clades.

Out of 7, first clade includes 2 species, *F. solani* and *F. equiseti* having the similarity coefficient of 0.00. Second clade contains *F. tricinctum* and *F. semitectum* which also have similarity coefficient of 0.00 showing that these species are closely related to each other. Third clade includes only *F. scirpi*. Fourth clade was represented by *F. moniliforme* and *F. oxysporum* having similarity coefficient 0.02. Each of fifth and sixth clusters contains single *F. culmorum* and *F. acuminatum* species respectively. The last and seventh cluster was found to have two *Fusarium* species (*F. graminearum* and *F. proliferatum*) having similarity coefficient of 0.01. Sequence- based BLAST and phylogenetic analysis is found to be very significant for the rapid identification of *Fusarium* species.

Table 4. Similarity coefficients (Jaccard's coefficients) of 11 species of *Fusarium* isolated from different hosts

	***Fusarium* species**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	*F. solani*											
2	*F. scirpi*	0.01										
3	*F. equiseti*	0.00	0.02									
4	*F. tricinctum*	0.00	0.01	0.00								
5	*F. semitectum*	0.01	0.02	0.00	0.00							
6	*F. culmorum*	0.05	0.06	0.05	0.05	0.04						
7	*F. moniliforme*	0.05	0.06	0.05	0.05	0.04	0.07					
8	*F. oxysporum*	0.04	0.05	0.04	0.04	0.04	0.07	0.02				
9	*F. graminearum*	0.11	0.11	0.11	0.10	0.10	0.11	0.08	0.09			
10	*F. proliferatum*	0.10	0.11	0.10	0.09	0.09	0.10	0.07	0.07	0.01		
11	*F. acuminatum*	0.10	0.11	0.10	0.10	0.10	0.09	0.12	0.11	0.07	0.06	

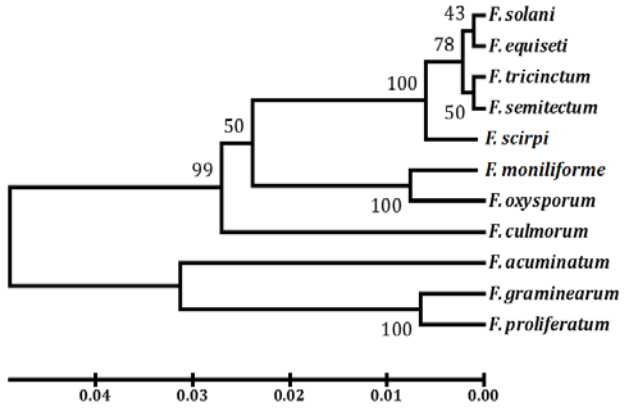

Figure 5: ITS-4 sequence based tree (rectangular) of 11 different species of *Fusarium* constructed using UPGMA. The numbers at branch node indicate the confidence value of bootstrap replications.

Many studies in the past have proved the importance of various molecular makers including ITS sequence comparison. Therefore, the findings reported in the present study are of utmost importance. Gurjar et al. (2009) reported identification of Indian pathogenic *F. oxysporum* f. sp. *ciceris* species recovered from wilted chickpea plants using ITS-1 and ITS-4 species specific primers and sequenced. Similarly, other studies carried out by Leong et al. (2009), Dissanayake et al. (2009), Palmore et al. (2010), Chehri et al. (2011) on identification of different *Fusarium* species using ITS-rDNA as a molecular marker provide evidences that this marker was helpful for the identification of *Fusarium* species. Our findings corroborate with these studies, which confirmed that ITS-rDNA markers can be used for the rapid and accurate identification of *Fusarium* species. Lin et al. (2014) used ITS as a molecular marker for identification of the *F. verticillioides* and *F. proliferatum* associated with sugarcane infection in Pokkah Boeng, China. Recently, Singha et al. (2016) demonstrated that molecular profiling using ITS is an indispensable method for rapid identification of *Fusarium* sp. isolated from *Fusarium*-wilt of tomato from Assam, North East India. Further, they also reported that BLAST was useful tool, which can be used to compare test sequences with sequences available in the GenBank. In addition, UPGMA analysis was necessary to find out the similarity among the different species. Similarly, in another study Kumar et al. (2016) also emphasized on the importance of ITS, BLAST and phylogenetic analysis in the identification and characterization of *F. mangiferae* as pathogen of mango malformation in India.

From the present study, it can be concluded that vegetables, food grains and fruits are susceptible towards the infection of various *Fusarium* species. Moreover, its associations are more prevalent in vegetables followed by food grains and fruits. Identification of *Fusarium* species based on morphological, cultural and microscopic features is found to be significant for the preliminary identification. However, it is difficult in case of *F. equiseti* and *F. scirpi* to identify on the basis of these markers due to similarities in their morphological and microscopic characters. In such cases, use of molecular markers for rapid and authentic identification would be promising. ITS-rDNA sequence comparison followed by BLAST analysis are the potential and unique molecular markers for the rapid identification of *Fusarium* species. However, the knowledge on occurrence of *Fusarium* spp. on vegetables, fruits, and food grain crops, can provide a basis for proper harvesting and storage practices as unsuitable harvesting practices and poor storage conditions may cause growth and proliferation of the mycotoxin-producing *Fusarium* species.

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