

Molecular identification of pathogenic *Fusarium* species, the causal agents of tomato wilt in western Iran

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Abstract: *Fusarium* species are causal agents of fungal diseases occurring frequently in numerous agriculturally important plants, including potato, garlic and are one of the common pathogens of tomato, causing root rot in the west part of Iran. Therefore, the objectives of this study were to isolate and identify disease-causing *Fusarium* species from infected tomatoes based on the morphological and molecular characteristics. Twenty-five isolates of *Fusarium* were obtained from infected root of tomato plants collected from the fields in different regions of western Iran. Based on morphological features, the strains were classified into four following *Fusarium* species: *F. oxysporum*, *F. redolens*, *F. proliferatum* and *F. verticillioides*. The phylogenetic trees based on *tef1* and *tub2* dataset clearly distinguished closely related species. All of the isolates were evaluated for their pathogenicity on healthy tomato seedlings in the greenhouse. This is the first report on molecular identification of *Fusarium* species isolated from tomato plants cultivated in Iran.

Key words: distribution and pathogenicity, *Fusarium* spp., morphology, phylogenetic analysis

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the economically important vegetables cultivated throughout the world (Madhavi and Salunkhe 1998). Many diseases and disorders can affect tomatoes during the growing season and pathogens such as *Fusarium* spp., *Rhizoctonia* spp., *Phytophthora* spp., *Sclerotium* spp., and *Macrophomina* spp. are the most common. These pathogens lead to reductions in quantity and quality of yield all over the world (Thapa and Sharma 1978; Grattidge and O'Brien 1982; Jones *et al.* 1991; Ketelaar and Kumar 2002; Steinkellner *et al.* 2005; Rozlianah and Sariah 2010). *Fusarium* species are frequently isolated from soil and organic substrates, and are responsible for many economically important plant diseases such as root rot, fruit rot, and crown rot. They can also cause major storage rots on food and feeds contaminating the substrates with harmful substances known as mycotoxins (Etcheverry *et al.* 2002; Fandohan *et al.* 2003; Mohd Zainudin *et al.* 2008). Moreover, some of the species are also, increasingly associated with opportunistic infections of humans and animals (Guarro and Gene 1995; Leslie and Summerell 2006).

Fusarium species are highly destructive pathogen of both greenhouse and field crops in tomato plantation areas of the world (Jones *et al.* 1991). *Fusarium verticillioides*, *F. oxysporum* and *F. equiseti* are the most common pathogens of tomato plants in worldwide vegetable production. They can infect tomato at all growth stages and enter plants through the root system and crown. Plants showing necrosis are frequently found among tomato plants affected by the crown rot (Rozlianah and Sariah

2010). Some of the species such as *F. oxysporum* can grow in the vascular bundles and infected plants show an early wilting syndrome a few weeks after inoculation (Kaiser *et al.* 1993; Alves-Santos *et al.* 1999; Steinkellner *et al.* 2005; Gupta *et al.* 2011). Therefore, the objectives of this study were to isolate and identify disease-causing *Fusarium* species from infected tomato roots based on the morphological data with those derived from the molecular techniques and to find the phylogenetic relationships among the strains.

Materials and Methods

Isolation and identification of *Fusarium* spp.

Twenty-five sick tomato plants were collected from different regions of western Iran during 2013–2014 growing seasons (Table 1). Each sample of tomato plant with disease symptoms was collected in a paper envelope and brought to the laboratory. The pathogens were isolated by direct culturing of infected tomato roots according to Chopada *et al.* (2015) with minor modifications. Briefly, the infected roots were washed with running tap water to remove all adhering soil particles, and then cut into small pieces prior to surface sterilization using 96% ethanol for 30 s. All the sterilized pieces were placed onto Peptone-Pentachloronitrobenzene Agar (PPA) plates (Nash and Snyder 1962). All the plates were incubated under the standard incubation conditions (Chehri *et al.* 2010) for 48 h and the resulting single-spore of *Fusarium* colonies were transferred to fresh Potato Dextrose Agar

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Table 1. Location of sample collection, GenBank accession numbers and rank of the tomato root rot condition eight weeks after incubation from each sample

No.	Isolate number	Species identified	Location in western Iran	Pathogenicity/virulence ^a	^b <i>tef1</i>	^b <i>tub2</i>
1	FOSC 143	<i>F. oxysporum</i>	Kermanshah	e	KT276320	KT276312
2	FOSC 239	<i>F. oxysporum</i>	Kermanshah	e	KT276321	KT276313
3	FOSC 249	<i>F. oxysporum</i>	Kermanshah	e	–	–
4	FOSC 206	<i>F. oxysporum</i>	Biseton	d	–	–
5	FOSC 207	<i>F. oxysporum</i>	Biseton	d	–	–
6	FOSC 214	<i>F. oxysporum</i>	Sahneh	d	–	–
7	FOSC 216	<i>F. oxysporum</i>	Asad Abad	b	–	–
8	FOSC 217	<i>F. oxysporum</i>	Asad Abad	b	–	–
9	FOSC 215	<i>F. oxysporum</i>	Kamyaran	b	–	–
10	FOSC 203	<i>F. oxysporum</i>	Kamyaran	c	–	–
11	FOSC 202	<i>F. oxysporum</i>	Kangavar	c	–	–
12	FOSC 213	<i>F. oxysporum</i>	Kangavar	a	–	–
13	FOSC 224	<i>F. oxysporum</i>	Asad Abad	a	–	–
14	FOSC 229	<i>F. oxysporum</i>	Asad Abad	a	–	–
15	FOSC 218	<i>F. oxysporum</i>	Harsin	a	–	–
16	FOSC 273	<i>F. redolens</i>	Harsin	b	KT276317	KT276308
17	FOSC 293	<i>F. redolens</i>	Harsin	b	KT276316	KT276309
18	FOSC 287	<i>F. redolens</i>	Harsin	b	–	–
19	GFSC 201	<i>F. proliferatum</i>	Asad Abad	b	KT276314	KT276306
20	GFSC 204	<i>F. proliferatum</i>	Biseton	b	KT276315	KT276307
21	GFSC 202	<i>F. proliferatum</i>	Harsin	a	–	–
22	GFSC 203	<i>F. proliferatum</i>	Harsin	a	–	–
23	GFSC 205	<i>F. proliferatum</i>	Kermanshah	a	–	–
24	GFSC 126	<i>F. verticillioides</i>	Kermanshah	a	KT276318	KT276310
25	GFSC 221	<i>F. verticillioides</i>	Kermanshah	a	KT276319	KT276311

^ameans with different letters are significantly different from each other ($p < 0.05$): a – healthy, no visible symptoms nonvirulent; b – hypovirulent; c – moderately virulent; d – virulent; and e – high virulent

^bGenBank numbers for *tef1* and *tub2* genes sequences

(PDA) plates for further studies. The species were identified on the basis of macroscopic and microscopic characteristics such as growth rates, pigmentations of colony, types of conidiogenous cells, shape and size of conidia, and presence or absence of sporodochia and chlamydo-spore. Identification to the species level was based on the descriptions of Leslie and Summerell (2006).

Pathogenicity test

All selected *Fusarium* isolates were used for pathogenicity assays on tomato. Inocula were produced on autoclaved cornmeal-sand (CMS) in flasks incubated in the dark at 25°C for two weeks. Soil mixture (clay loam/sand/peat at 1 : 1 : 1, vol/vol/vol) was autoclaved and mixed with the infested CMS substrates. The inoculum density was approximately 10^6 cfu · g⁻¹ of soil for each of the tested *Fusarium* species. The cultivar Beliy naliiv-241, susceptible to *F. oxysporum* was used in this experiment. Five week-old tomato seedlings were grown in small pots filled with the CMS-soil mixture of an examined isolate. Equal amounts of CMS-soil mixture were used in all treatments. Control tomato seedlings were grown in the noninfested CMS-soil mixture. The seedlings were regularly watered during the experiment. The experiments were carried out in the greenhouse conditions maintained at 22 to 28°C, 60–70% relative humidity (RH). The experiments were

arranged in a completely randomized design with three replications. Development of symptoms on the plants inoculated by fungi and the controls were monitored continuously at weekly intervals for eight weeks. The disease development index was assessed by the method described in Grattidge and O'Brien (1982) using a scale from 0 to 5: 0 – (0) of leaves yellowed and wilted; 1 – (1–24%) of leaves yellowed and wilted; 2 – (25–49%) of leaves yellowed and wilted; 3 – (50–74%) of leaves yellowed and wilted; 4 – (75–99%) of leaves yellowed and wilted; 5 – (100%) dead plant. Re-isolations from all isolate were performed on PPA medium (Nash and Snyder 1962). Statistical analysis was performed using SPSS 16 software according to Chehri (2015).

Molecular methods

DNA extraction process, polymerase chain reaction (PCR) and sequencing of the partial *tef1* and *tub2* genes were performed as described previously (Chehri *et al.* 2014; Chehri 2015). The partial *tef1* and *tub2* genes were amplified with primer pairs ef1 and ef2 (O'Donnell *et al.* 1998), and T1 and T2 (O'Donnell and Cigelnik 1997), respectively. Amplification reactions were performed in a total volume of 50 µl, by mixing 0.4 µl of template DNA with 16.35 µl ddH₂O, 8 µl of each primer; 1 µl of deoxy-nucleotide triphosphate (dNTP) (Promega); 0.25 µl of *Taq*

DNA polymerase (Promega); 8 µl of PCR 5X reaction buffer (Promega, Madison, WI, USA) and 8 µl of MgCl₂ (Promega). DNA sequences from a portion of the partial *tef1* and *tub2* genes were generated and analyzed according to the procedures described in previous studies (O'Donnell and Cigelnik 1997; O'Donnell *et al.* 1998). Maximum-parsimony analyses were performed on the aligned DNA sequences of the individual and combined datasets using MEGA4.0 version 4.0 (Tamura *et al.* 2007). DNA sequences have been deposited in GenBank (Table 1).

Results

Totally 25 strains of *F. oxysporum* were isolated from 25 infected roots of tomato plants collected from fields in different regions of western Iran. All strains were characterized through morphological approach (Table 2). For species determination, the descriptions by Leslie and Summerell (2006) were adopted. Based on morphological features, 18 isolates were identified to two known *Fusarium* species among *F. oxysporum* species complex (FOSC), namely *F. oxysporum* (15) and *F. redolens* (3), and seven isolates were classified into two known *Gibberella fujikuroi* species complex (GFSC), namely *F. proliferatum* (5) and *F. verticillioides* (2). All of the isolates were evaluated for their pathogenicity on healthy tomato plants in the greenhouse conditions. Tomato seeds (*L. esculentum*) cv. Bely naliv-241) susceptible to *F. oxysporum* were used in this experiment. Three out of the 15 isolates (FOSC 143, FOSC 239, FOSC 249) that belonged to *F. oxysporum* with putative wilting symptom, were highly pathogenic to tomato plants and caused 100% of leaves yellowed. The isolates FOSC 206, FOSC 207, and FOSC 214 were also pathogenic (75–99%) and FOSC 202 and FOSC 203 were moderately pathogenic (50–74%) to tomato roots. The isolates FOSC 215, FOSC 216, and FOSC 217 were considered as hypovirulent group and caused 1–24% of leaves yellowed. *Fusarium oxysporum* isolates: FOSC 213, FOSC 224, FOSC 229 and FOSC 218, showed no external symptoms eight weeks after soil inoculation test and were considered as nonvirulent (Table 1). All three isolates that based on morphological features were identified as *F. redolens* (FOSC 273, FOSC 287 and FOSC 293), eight weeks after soil inoculations were considered as hypovirulent group and caused 1–24% of leaves yellowed (Table 1).

The results of the pathogenicity test revealed that two isolates (GFSC 201 and GFSC 204) out of the five isolates that belonged to *F. proliferatum* with wilting symptom were weak pathogenic and caused 1–24% of leaves yellowed and three other isolates (GFSC 202, GFSC 203 and GFSC 205) were non-pathogenic (Table 1). Also, two isolates that based on morphological features were identified as *F. verticillioides* (GFSC 126 and GFSC 221) showed no external symptoms and were considered as nonvirulent group (Table 1).

Eight strains were selected for DNA sequence analysis using the *tef1* and *tub2* genes (Table 1). A single band of DNA fragments 500-bp and 700-bp was amplified for the *tub2* and *tef1* genes, respectively, from all tested *Fusarium* spp. isolates. The obtained sequences were compared with those available on the FUSARIUM-ID da-

tabases (Geiser *et al.* 2004) and NCBI, *Fusarium* MLST (O'Donnell *et al.* 2012). Based on similarities searched at FUSARIUM-ID and NCBI database, identification of all *Fusarium* spp. was confirmed with statistical significance. This also was confirmed by a phylogenetic analysis of the combined dataset of *tef1* and *tub2* genes data (Fig. 1). The phylogenetic tree generated from the combined dataset of *tef1* and *tub2* genes revealed a monophyly among two isolates (FOSC 201 and FOSC 204) included in Table 2, and *F. oxysporum* (FCC 3460 and NRRL 22902) obtained from GenBank. All these strains showed a well moderately supported (77% MP) relationship. The tree also showed two isolates (FOSC 273 and FOSC 287) with 99% bootstrap support are placed in distinct lineage of *F. redolens*. The tree showed a well supported relationship (97% MP bootstrap) between *F. proliferatum* (NRRL 22944 and NRRL 31071) obtained from GenBank and two isolates included in Table 2 that based on morphological features were identified as *F. proliferatum*. The tree also showed a monophyly between *F. verticillioides* (NRRL 25600) and isolates GFSC 126 and GFSC 221 (99% MP), and based on morphological features, which all strains were identified as *F. verticillioides*.

Discussion

The aim of this study was to identify pathogenic *Fusarium* species associated with root areas of tomato plants. This study is the comprehensive research for identification and genetic diversity of *Fusarium* spp., affecting the important tomato plantation areas in western Iran. *Fusarium oxysporum* was the most prevalent with a frequency of 60%, followed by *F. proliferatum* (20%), *F. redolens* (12%), and *F. verticillioides* (8%). These results support findings in other studies that characterized *F. oxysporum* as a predominant and most important fungal species in tomato plantation areas in different countries of the world (Grattidge and O'Brien 1982; Jones *et al.* 1991; Steinkellner *et al.* 2005; Amini 2009; Rozlianah and Sariah 2010). Based on morphological features, occurrence of *F. oxysporum* was reported in tomato plantation areas in different provinces in Iran (Fassihiani 1985; Amini 2009). To the best of our knowledge, this is the first report on molecular identification of *Fusarium* species isolated from tomato plants cultivated in Iran and *F. proliferatum*, *F. verticillioides* and *F. redolens* were also identified for the first time in tomato growing areas of Iran.

Pathogenicity test showed that three isolates of *Fusarium oxysporum* were highly pathogenic, whereas *F. proliferatum* and *F. redolens* were found to be weakly virulent. These results corresponded to those of previous studies regarded *F. oxysporum* as the important virulent species in tomato fields in different countries of the world (Steinkellner *et al.* 2005; Amini 2009; Rozlianah and Sariah 2010). The result of pathogenicity test also demonstrated that four of 15 isolates of *F. oxysporum* were found to be nonpathogenic to tomato plants. We believe that this study will develop proper management strategies to control tomato root rot by nonpathogenic strains (Bao and Lazarovits 2001; Forsyth *et al.* 2006; Jian *et al.* 2009; Iakovos *et al.* 2009). The ability of nonpathogenic

Table 2. Morphological characteristics of eight strains of *Fusarium* spp. isolated from the infected root rot of tomato plants collected from western Iran

Culture no.	Species identified	Chlamydospores	Types of conidiogenous cells	Sporodochia colour	Shape of microconidia	Shape of basal cell and apical cell	Length × width of macroconidia [μm] ^a	
							3- and 4-septate	5-septate
FOSC 143	<i>F. oxysporum</i>	+	monophialidic	pale orange	oval, elliptical	foot shaped to pointed and tapered and curved	43.5 ± 1.5 × 4.8 ± 0.2	47.5 ± 2.5 × 5.8 ± 0.2
FOSC 239	<i>F. oxysporum</i>	+	monophialidic	pale orange	oval, elliptical	foot shaped to pointed and tapered and curved	43.5 ± 1.5 × 5.0 ± 0.2	46.5 ± 2.5 × 5.9 ± 0.2
FOSC 273	<i>F. redolens</i>	+	monophialidic	pale brown	oval, elongated oval and often pointed on one end	foot shaped and hooked	47.5 ± 2.5 × 4.0 ± 0.5	51.5 ± 2.5 × 5.9 ± 0.2
FOSC 293	<i>F. redolens</i>	+	monophialidic	pale brown	oval, elongated oval and often pointed on one end	foot shaped and hooked	47.5 ± 1.5 × 4.9 ± 0.5	51.5 ± 2.5 × 5.8 ± 0.2
GFSC 201	<i>F. proliferatum</i>	-	polyphialidic	pale orange	pyriform, clavate	poorly developed foot shape and curved	45.5 ± 1.5 × 4.9 ± 0.5	52.5 ± 2.5 × 5.8 ± 0.2
GFSC 204	<i>F. proliferatum</i>	-	polyphialidic	pale orange	pyriform, clavate	poorly developed foot shape and curved	44.5 ± 1.5 × 4.9 ± 0.5	48.5 ± 2.5 × 5.8 ± 0.2
GFSC 126	<i>F. verticillioides</i>	-	monophialidic	orange	clavate	notch or foot shape and curved	32.5 ± 1.5 × 4.8 ± 0.2	49.5 ± 2.5 × 5.9 ± 0.2
GFSC 221	<i>F. verticillioides</i>	-	monophialidic	orange	clavate	notch or foot shape and curved	34.5 ± 1.5 × 4.8 ± 0.2	50.5 ± 2.5 × 5.8 ± 0.2

^amean values of 30 random conidia ± standard deviation

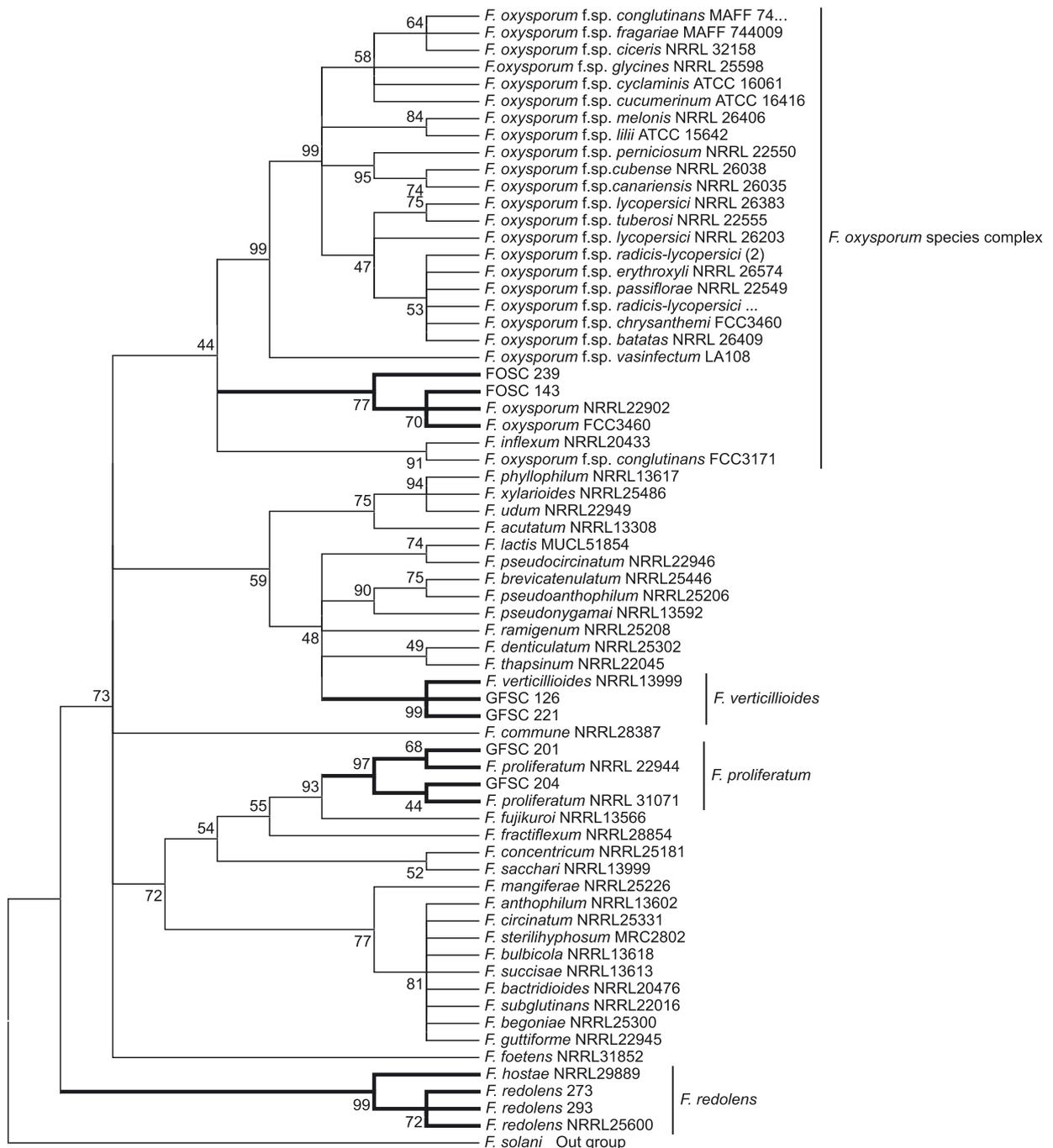


Fig. 1. A maximum parsimony phylogeny for 67 taxa of the *Fusarium oxysporum* species complex (FOSC) and *Gibberella fujikuroi* species complex (GFSC) inferred from combined *tef1* and *tub2* gene sequences. Bootstrap tests were performed with 1,000 replications. *Fusarium solani* (NRRL 22389) obtained from GenBank was treated as the outgroup

F. oxysporum and pathogenic *F. oxysporum* belonging to a different formae speciales than the pathogen, to induce plant resistance to fusarioses has been demonstrated in several studies (Bao and Lazarovits 2000; Forsyth *et al.* 2006; Jian *et al.* 2009; Iakovos *et al.* 2009).

In this study, phylogenetic analysis based on *tef1* and *tub2* dataset distinctly separated all morphological taxa and therefore proved to constitute a rapid and suitable way to group closely related *Fusarium* spp. such as *F. oxysporum* and *F. redolens* and to estimate the genetic relationships between the groups, and it is a complement to the morphological studies for description of *Fusarium* species.

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