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Genetic Relationship among *Fusarium oxysporum* f. sp. *melonis* Vegetative Compatibility Groups and Their Relatedness to Other *F. oxysporum formae speciales*

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ABSTRACT

Fusarium wilt of melon is a destructive fungal disease throughout the world. In this study, the evolutionary relationships among isolates of different *formae speciales* of *Fusarium oxysporum* was examined, with a special emphasis on the *forma specialis melonis*. Bootstrapped maximum likelihood analysis of the elongation factor-1α (EF-1α) sequence was conducted on 16 Iranian and 11 foreign isolates of *F. o. melonis* that included representatives of different vegetative compatibility groups (VCGs 0130-0136). The tree inferred from the dataset resolved five evolutionary lineages that were correlated with the *F. o. melonis* VCGs, with the exception of VCGs 0130 and 0131, which could not be differentiated with EF-1α sequences. Furthermore, based on EF-1α sequences, specific associations were found between *F. o. melonis* VCGs and the other *formae speciales* whose sequences were obtained from the GenBank. Taken together, these results support a polyphyletic origin for *F. o. melonis*, meaning that the ability of this *forma specialis* to cause disease on melon has emerged multiple times.

Keywords: *Cucumis melo*, Iran, Polyphyletic, VCG.

INTRODUCTION

Fusarium oxysporum Schlechtend. Fr. is a major vascular wilt pathogen of many economically important crops (Snyder and Hansen, 1940). Within the species, however, there is a high level of host specificity with over 120 described *formae speciales* and races (Gordon and Martyn, 1997). Each *forma specialis* consists of isolates with the ability to cause wilt on a unique host or set of plant host species. Because the hosts of a given *forma specialis* usually are closely related, it has been assumed that members of a *forma specialis* are also closely related and may have arisen from a common ancestor (Correll, 1991; Kistler, 1997).

Additional characterization of sub-specific groups in *F. oxysporum* was reported by Puhalla (1985), who developed the method, based on the genetic of the fungus rather than on the host-pathogen interaction, to identify vegetative compatibility groups (VCGs) within this species. He found that isolates in the same VCG belonged to the same *forma specialis* and isolates in different *formae speciales* were in different VCGs, and they may represent genetically isolated populations. Molecular and genetic analyses have shown that isolates within a VCG are genetically more similar than isolates in different VCGs, and it has been suggested that each VCG may represent a clonal population (Gordon and Martyn, 1997).

An understanding of the evolutionary history of the *formae speciales* and races within *F. oxysporum* requires knowledge of the phylogenetic relationships among

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isolates (Apple and Gordon, 1996). Phylogenetic analyses based on DNA sequences of genes such as the elongation factor-1α (EF-1α) have helped to reveal the genetic and evolutionary relationships within and among *formae speciales* of *F. oxysporum* (O'Donnell *et al*., 1998; Baayen *et al*., 2000; Mbofung *et al*., 2007). Such studies showed that a limited number of *F. oxysporum formae speciales* were monophyletic such as *formae speciales lilii* and *tulipae* (Baayen *et al*., 2000), while many others were found to be polyphyletic such as *formae speciales cubense*, *asparagi*, *dianthi*, *lycopersici* and *vasinfectum* (O'Donnell *et al*., 1998; Baayen *et al*., 2000; Skovgaard *et al*., 2001, Cai *et al*., 2003). These findings suggest that pathogenicity towards a specific crop has evolved several times independently.

One of the economically important and destructive *formae speciales* is the causal agent of Fusarium wilt of melon (*Cucumis melo*) (Armstrong and Armstrong, 1978). This *forma specialis* is known to contain four races (0, 1, 2, and 1, 2), which differ in pathogenicity to differential melon cultivars described by Risser *et al*. (1976). In *F. o. melonis* nine VCGs have been identified worldwide (Jacobson and Gordon, 1990a; Katan *et al*., 1994). In Iran, races 1 and 1, 2 and VCG 0134 have been reported (Banihashemi, 1968b, 1982, 1989; Sarpeleh and Banihashemi, 2000).

Understanding genetic heterogeneity within the *F. oxysporum* group is important for understanding the evolution of pathogenic forms, especially for polyphyletic *formae speciales*. Although the previous study based on restriction digest analyses of mtDNA provided knowledge concerning the diversity between different VCGs of *F. o. melonis* (Jacobson and Gordon, 1990b), the genetic relatedness among the lineages identified in this study remains uncertain. It is also not clear how the different VCGs of *F. o. melonis* are related to one another and to other *formae speciales* of *F. oxysporum*. Therefore, the main objective of this study was to elucidate the relationships among the *F. o. melonis* VCGs based on EF-1α sequences and determine their relationships with other *formae speciales* based on sequences of *EF-1α* gene obtained from the GenBank.

MATERIALS AND METHODS

Fungal Isolates

Melon plants with typical Fusarium wilt symptoms were collected from various melon fields in the major melon-producing regions of Iran. Diseased stems were washed under tap water, surface disinfested with 1% sodium hypochlorite for 5 minutes, rinsed in distilled water and air dried. Stems were cut aseptically into 2-3 mm segments and plated in rows on acidified TMN-PDA medium (trimethylnonanol-potato dextrose agar) (Banihashemi, 1968a). Plates were incubated at 25°C for 4-6 days and colonies exhibiting the characteristic morphology of *F. oxysporum* were transferred to Petri plates containing acidified PDA (pH 4.2). Single spore cultures were prepared from most of the isolates and identified according to standard keys (Nelson *et al*., 1983; Leslie and Summerell, 2006). One *F. o. melonis* race 1 isolate, recovered in 1966 (Banihashemi, 1968a), from the fungal collection of Plant Protection Department (Shiraz University, Iran) was included in this study. Eleven isolates of *F. o. melonis* from the USA, France, Japan, and Israel (provided by T. R. Gordon, G. Risser and T. A. Zitter) were also included as reference isolates.

Pathogenicity and Race Determination

Seeds of differential melon cultivars used by Risser *et al*. (1976), including 'CM 17187' (*Fom* 2), 'Doublon' (Fom 1) and 'Charantions-T' (no resistance gene), as well as the local cultivars 'Shahd-e-Shiraz' (resistant to race 1 but not to race 0) and 'Kharboze-Mashhad' (resistant to races 0 and 2) (Banihashemi 2010), were surface-

disinfested with 1% sodium hypochlorite for 5 min, rinsed twice using sterile distilled water and planted in plastic trays filled with autoclaved vermiculite. Plant seedlings were grown in vermiculite at room temperature for ten days until the third true leaf began to emerge.

Single spore cultures of *F. oxysporum* were grown on acidified PDA (pH 4.2). After 5 days, mycelial blocks were taken from the actively growing colony margins of each isolate and transferred into a 250 ml flask containing 50 ml of Potato Dextrose Broth (PDB). Cultures were incubated at room temperature on a rotary shaker at 60 rpm for 3-4 days. The content of each flask was filtered through two layers of sterile cheesecloth to remove the mycelia mats. The conidia were centrifuged down at low speed and washed three times with sterile distilled water and the conidial concentration was adjusted to 10^6 conidia ml^{-1} using a haemocytometer (Banihashemi and deZeeuw, 1975).

The ten days old melon seedlings were removed and their roots were washed with tap water to clean excess vermiculite. Virulence test of all isolates was performed using the root dip method described by Wellman (1939). The roots of melon seedlings were dipped in a conidial suspension for 1 min and the inoculated seedlings were transferred to plastic pots filled with sterilized soil and incubated under greenhouse conditions (25- 28°C, 14-hour photoperiod). For each isolate, ten seedlings of each cultivar were inoculated. Seedlings dipped in water served as control. Both the Iranian and foreign reference isolates were used in the inoculation trials to provide controls for race determination. Yellowed, wilted, and dead plants were recorded daily up to 21 days after inoculation and the pathogen was re-isolated from the stem of inoculated plants to confirm Koch's postulates. Isolates that did not induce wilt were tested twice more in three replicates of five seedlings. If no wilt occurred, such isolates were categorized as nonpathogenic to melon. Based on the reaction of the differential hosts, races were assigned to individual isolates.

Vegetative Compatibility Tests

Vegetative compatibility tests with the Iranian isolates of *F. o. melonis* were conducted following the protocols described by Puhalla (1985) and Correll *et al.* (1987). Nitrate nonutilizing (*nit*) mutants were generated on potato sucrose agar containing 1.5-4% $KClO_3$ and were assigned to different physiological phenotypes (*nit1*, NitM and *nit3*) on the basis of their growth on basal medium amended with different nitrogen sources (nitrate, nitrite, hypoxanthin, uric acid or ammonium tartrate). The formation of heterokaryons was recognized as a line of aerial mycelium where two complementary *nit* mutants grew together on MM (Correll *et al.*, 1987). *Nit* mutants were generated from each isolates until two complementary *nit* mutants were found that formed a vigorous heterokaryon when paired in MM. These *nit* mutants were used as heterokaryon tester *nit* mutants for that isolate in subsequent inter-isolate pairings. Furthermore, tester *nit* mutants were paired with tester mutants of each of the established VCGs obtained from T. R. Gordon (University of California, Davis, USA). When testers from two different isolates successfully formed a heterokaryon, they were placed in the same VCG. Before complementation tests among isolates, vegetative self-incompatibility of each isolate was examined (Jacobson and Gordon, 1988).

DNA Extraction

For DNA extraction, single-spored culture of *F. oxysporum* isolates were grown in PDB medium for 7 days at 25°C and then the mycelia were harvested through filtration. The fungal mycelia were thoroughly and repeatedly washed with sterilized distilled water, frozen, lyophilized and stored at -20°C. Lyophilized mycelia were ground in liquid nitrogen to a fine powder. Total genomic DNA

was extracted using Genomic DNA Purification kit (Fermentas) according to the manufacturer's instructions.

PCR Amplification and Sequencing

The *EF-1α* gene of *F. o. melonis* isolates was targeted. For this purpose, primer set EF1 and EF2 (O'Donnell *et al*., 1998) was used. Each 20 µl amplification reaction mixture contained 5-10 ng of total DNA, 2 µl of 10 × reaction buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 1 U of Taq polymerase (CinnaGen) and 0.8 µM of each primer. Amplification conditions consisted of 34 cycles of denaturation at 94°C for 40 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 2 minutes. Each PCR reaction included an initial denaturation step at 95°C for 2 minutes and final extension step at 72°C for 5 minutes. The amplification products were purified with GeneJet PCR Purification Kit (Fermentas) to remove excess of primers and nucleotides. Subsequently, purified products were sequenced in both directions using the primers used for PCR amplification and the sequencer.

Phylogenetic Analyses

For all isolates listed in Table 1, phylogenetic analyses were performed using DNA sequences of the *EF-1α* gene (~ 640 bp) that were either retrieved from published EF-1α sequences in the GenBank or determined in this study (Table 1). DNA sequences were edited with DNASTAR (Seq Man II) and aligned with ClustalX 1.8 (Larkin *et al*., 2000). Manual adjustment of sequence alignments was performed to accommodate insertions/deletions. Phylogenetic analyses were conducted in MEGA 5 (Tamura *et al*., 2011) by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-932.3905) is shown (Figures1-2). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ (Gascuel, 1997) method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, Parameter= 0.3994)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sequence of *Fusarium* sp. (NRRL28378) (obtained from the GenBank) was used as outgroup. All EF-1α sequences have been submitted to the GenBank (Table 1).

RESULTS

Pathogenicity and Race Classification

Fifteen of the 17 isolates of *F. oxysporum*, which were collected from different melon fields in Iran, were virulent on all differential hosts including 'CM17187', 'Doublon', 'Charentais-T', 'Shahde-Shiraz' and 'Kharboze-Mashhad,' and were classified as *F. o. melonis* race 1, 2 (Table 1). After symptoms development, the inoculated pathogens were re-isolated from randomly selected plants to confirm Koch's postulates. The remaining two isolates induced no symptoms on any seedling and were classified as nonpathogenic.

Vegetative Compatibility

All isolates were self-compatible and formed a zone of wild-type growth with aerial mycelium where the complementary *nit* mutant colonies met. Of the 15 isolates of *F. o. melonis*, nine were assigned to VCG 0134 (Table 1). The remaining *F. o. melonis* isolates were incompatible with *nit* mutants of any of the isolates from known VCGs of *F. o. melonis* (0130-0136). These isolates were compatible with each other and were therefore

Table 1. *Fusarium oxysporum* isolates used in this study and associated sequences with GenBank accession number noted[a]. Isolate's vegetative compatibility group and race are indicated where known.

Isolate	Species, *Formae speciales*	Geographic origin	Race/VCG[b]	GenBank accession
I-17	*F. oxysporum* f. sp. *melonis*	Iran	1/0134	KF548147
Yazd2	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548158
KT2a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548156
MT13-3a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548145
Seif3a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548144
K109b	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548159
gh30	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548157
P13	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548146
2Ma18-4a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/0134	KF548148
660A/1	*F. oxysporum* f. sp. *melonis*	France	0/0134	KF548166
Sample65	*F. oxysporum* f. sp. *melonis*	USA	1/0134	KF548160
NYFom62	*F. oxysporum* f. sp. *melonis*	USA	1/0134	KF548161
P2/6	*F. oxysporum* f. sp. *melonis*	USA	2/0130	KF548164
18L	*F. oxysporum* f. sp. *melonis*	USA	2/0130	KF548165
PT3/1	*F. oxysporum* f. sp. *melonis*	USA	2/0131	KF548163
Sample37	*F. oxysporum* f. sp. *melonis*	USA	2/0131	KF548162
T61/1	*F. oxysporum* f. sp. *melonis*	Japan	2/0132	KF548168
R12/13	*F. oxysporum* f. sp. *melonis*	France	1,2/0133	KF548167
I1/1	*F. oxysporum* f. sp. *melonis*	Israel	0/135	KF548170
Taik9a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA[c]	KF548151
Taipb1	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA	KF548152
Khaf76a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA	KF548149
MZ16a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA	KF548154
Nasr 8	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA	KF548153
Tai3	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA	KF548150
MaH2-3a	*F. oxysporum* f. sp. *melonis*	Iran	1,2/GNA	KF548155
K419/5	*F. oxysporum* f. sp. *melonis*	Mexico	1/0136	KF548169
2Ma4-5a	*F. oxysporum*	Iran	nonpathogen	KF548171
TO1	*F. oxysporum*	Iran	nonpathogen	KF548172
NRRL22518	*F. oxysporum* f. sp. *melonis*	NA	NA[d]	**FJ985265**
NRRL36472	*F. oxysporum* f. sp. *melonis*	NA	NA	**FJ985357**
NRRL26406	*F. oxysporum* f. sp. *melonis*	NA	NA	**AF008504**
CAV343	*F. oxysporum* f. sp. *melonis*	Israel	NA	**FJ664912**
NRRL26178	*F. oxysporum* f. sp. *melonis*	USA	NA	**AF008503**
NRRL22519	*F. oxysporum* f. sp. *melonis*	NA	NA	**FJ985266**
TX388	*F. oxysporum* f. sp. *melonis*	USA	NA	**DQ837696**
NRRL26871	*F. oxysporum* f. sp. *spinaciae*	Japan	NA	**DQ837687**
NRRL26874	*F. oxysporum* f. sp. *spinaciae*	USA	NA/0330	**AF246849**
NRRL26875	*F. oxysporum* f. sp. *spinaciae*	USA	NA/0331	**AF246850**
NRRL26876	*F. oxysporum* f. sp. *spinaciae*	USA	NA/0332	**AF24685**
FOV14	*F. oxysporum* f. sp. *vasinfectum*	USA	NA	**DQ837695**
BBA69716	*F. oxysporum* f. sp. *vasinfectum*	China	7/NA	**AF362163**
BBA69521	*F. oxysporum* f. sp. *vasinfectum*	India	4/NA	**AF362139**
BBA69711	*F. oxysporum* f. sp. *vasinfectum*	China	8/NA	**AF362161**
BBA65655	*F. oxysporum* f. sp. *vasinfectum*	USA	2/NA	**AF362149**
BBA65634	*F. oxysporum* f. sp. *vasinfectum*	USA	1/NA	**AF362145**
BBA69712	*F. oxysporum* f. sp. *vasinfectum*	China	3/NA	**AF362162**
BBA65650	*F. oxysporum* f. sp. *vasinfectum*	Sudan	5/NA	**AF362154**
BBA62374	*F. oxysporum* f. sp. *vasinfectum*	Egipt	3/NA	**AF362142**
FOL-24L	*F. oxysporum* f. sp. *lycopersici*	Israel	1/0030	**FJ790383**

[a] All elongation factor-1α (EF-1α) sequences were determined in this study, with the exception of those with accession numbers in bold; [b] VCG= Vegetative Compatibility Group; [c] GNA= Group Not Assigned, [d] NA= Not Available.

Table1 continued…

Table 1. continued

Isolate	Species, *Formae speciales*	Geographic origin	Race/VCG[b]	GenBank accession
CA92/95	*F. oxysporum* f. sp. *lycopersici*	USA	3/0030	**FJ790387**
14844	*F. oxysporum* f. sp. *lycopersici*	Australia	3/0030	**FJ790386**
FOL-93H	*F. oxysporum* f. sp. *lycopersici*	Israel	2/0030	**FJ790385**
281	*F. oxysporum* f. sp. *lycopersici*	Spain	2/0030	**FJ790384**
NRRL26037	*F. oxysporum* f. sp. *lycopersici*	NA	NA	**AF008498**
NRRL26200	*F. oxysporum* f. sp. *lycopersici*	NA	NA	**AF008499**
FOL-MM59	*F. oxysporum* f. sp. *lycopersici*	USA	2/0030	**FJ790388**
FOLR2	*F. oxysporum* f. sp. *lycopersici*	USA	NA	**DQ837692**
OSU-451	*F. oxysporum* f. sp. *lycopersici*	USA	2/0031	**FJ790392**
E175	*F. oxysporum* f. sp. *lycopersici*	Netherlands	1/0031	**FJ790391**
NRRL26203	*F. oxysporum* f. sp. *lycopersici*	Italy	NA	**AF008501**
NRRL26437	*F. oxysporum* f. sp. *lycopersici*	USA	3/0033	**AF008502**
NRRL26437	*F. oxysporum* f. sp. *cucumerinum*	USA	NA	**AF362168**
NRRL29870	*F. oxysporum* f. sp. *pisi*	USA	NA	**AF362170**
NRRL36471	*F. oxysporum* f. sp. *niveum*	NA	NA	**FJ985356**
NRRL36331	*F. oxysporum* f. sp. *niveum*	NA	NA	**FJ985346**
NRRL36275	*F. oxysporum* f. sp. *niveum*	NA	NA	**FJ985340**
NRRL38552	*F. oxysporum* f. sp. *niveum*	NA	NA	**FJ985410**
NRRL22546	*F. oxysporum* f. sp. *medicaginis*	SE Asia	nd	**DQ837690**
CAV786	*F. oxysporum* f. sp. *cubense*	Australia	NA/0124	**FJ664959**
CAV009	*F. oxysporum* f. sp. *cubense*	South Africa	NA/0120	**FJ664940**
NRRL26379	*F. oxysporum* f. sp. *radicis-lycopersici*	NA	NA	**AF008508**
FOA50	*F. oxysporum* f. sp. *asparagi*	Australia	NA	**DQ837691**
NRRL26954	*F. oxysporum* f. sp. *tulipae*	Netherlands	NA	**AF246838**
BMP1307	*F. oxysporum* f. sp. *lactucae*	USA	1/NA	**DQ837661**
F9501	*F. oxysporum* f. sp. *lactucae*	Japan	2/NA	**DQ837693**
NRRL26411	*F. oxysporum* f. sp. *fabae*	USA	NA	**DQ837684**
NRRL22538	*F. oxysporum* f. sp. *cepae*	Germany	NA	**DQ837681**
NRRL26622	*F. oxysporum* f. sp. *albedinis*	Morocco	NA	**DQ837688**
NRRL26445	*F. oxysporum* f. sp. *phaseoli*	USA	NA	**DQ837686**
NRRL28378	*F. commune*	Netherlands	NA	**AF246832**

[a] All elongation factor-1α (EF-1α) sequences were determined in this study, with the exception of those with accession numbers in bold; [b] VCG= Vegetative Compatibility Group; [c] GNA= Group Not Assigned, [d] NA= Not Available.

designated in a separate unassigned VCG. The oldest Iranian isolate I-17, which was previously reported to be in VCG 0134, was confirmed to be vegetatively compatible with *nit* testers of VCG 0134. The two nonpathogenic isolates, TO1 and 2Ma4-5a, were compatible with each other but not with any of the *F. o. melonis* testers.

Phylogenetic Analyses

In this study, ~ 640 bp of the region encoding the *EF-1α* gene was sequenced from 27 Iranian and foreign isolates of *F. o. melonis* belonging to different VCGs (0130-0136), as well as two nonpathogenic isolates.

Based on EF-1α sequences, the *F. o. melonis* isolates in this study were divided into six lineages (Figure 1). Lineage 1 included all of the Iranian and foreign isolates belonging to VCG 0134; lineage 2 consisted of one isolate from Japan within VCG 0132; and lineage 3 consisted of four isolates from USA belonging to VCGs 0130 and 0131. One isolate from France within VCG 0133, although not identical to,

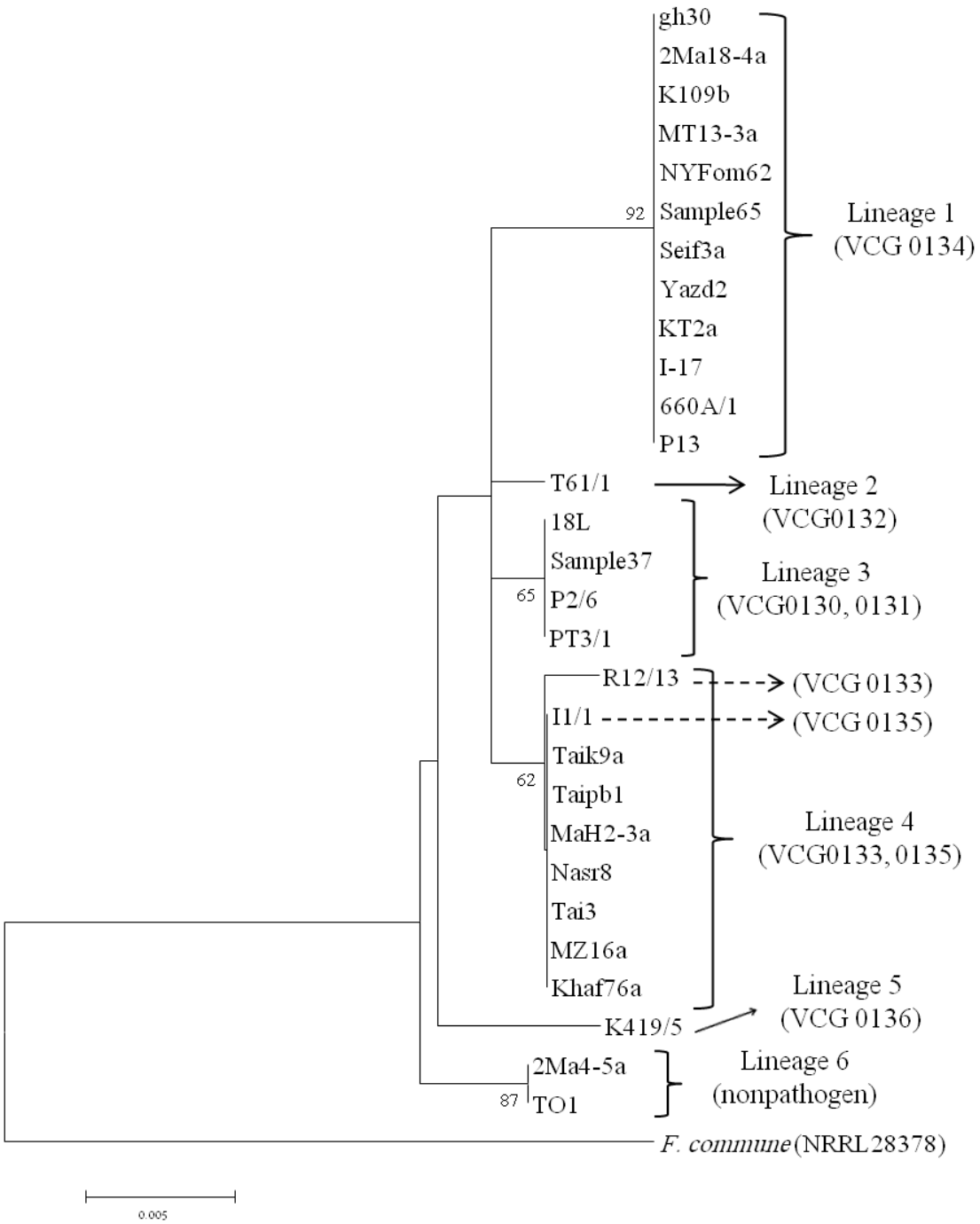

Figure 1. Maximum likelihood phylogenetic tree inferred from EF-1α sequences of different vegetative compatibility groups of *Fusarium oxysporum* f. sp. *melonis*. *F. commune* isolate NRRL28378 is included as outgroup. Bootstrap values (> 60%) are shown as percentages of 1,000 replicates.

clustered together with the seven Iranian isolates belonging to a separated unassigned VCG and with an isolate from Israel (VCG 0135) in lineage 4. Lineage 5 was constituted by a single isolate from Mexico within VCG 0136, and lineage 6 included the two nonpathogenic isolates to melon from Iran.

To assess the relationships of different *F. o. melonis* VCGs with the other *formae speciales*, the *EF-1α* gene sequences from the representative of all seven VCGs of *F. o. melonis* and the two nonpathogenic isolates were compared with 51 *EF-1α* gene sequences obtained from the GenBank (Table1, Figure 2). These sequences were from isolates of 17 different *formae speciales* (*melonis*, *spinaciae*, *vasinfectum*, *cucumerinum*, *pisi*, *niveum*, *medicaginis*, *cubense*, *lycopersici*, *radicis-lycopersici*, *asparagi*, *tulipae*, *fabae*, *cepae*, *albedinis*, *phaseoli*, *lactucae*) and were chosen to represent the main lineages of *F. oxysporum* (O'Donnell *et al*., 1998; Baayen *et al*., 2000; Skovgaard *et al*., 2001; Cai *et al*., 2003; Mbofung *et al*., 2007; Fourie *et al*., 2009; Lievens *et al*., 2009)

As shown in Figure 2, races 4 and 5 of *F. o. vasinfectum* were grouped with *F. o. melonis* VCGs 0130 and 0131. Lineage 1 of *F. o. melonis* corresponding to VCG 0134 had close relationships with *F. o. vasinfectum* race 1 and 2. *F. o. melonis* VCG 0136 and races 1, 2, 3 belonging to *F. o. lycopersici* VCG 0030 were grouped together. Nonpathogenic isolates in lineage 6 were identical to *F. o. lycopersici* (NRRL26203) and closely related to *F. o. lycopercisi* VCGs 0031 and 0033.

DISCUSSION

The primary focus of this study was to elucidate phylogenetic relationships among *F. o. melonis* VCGs. Based on the DNA sequence information of *EF-1α* gene, five evolutionary lineages were found among 27 Iranian and foreign isolates of *F. o. melonis* which were correlated with the *F. o. melonis* VCGs (0130-0136), with the exceptions of VCGs 0130 and 0131 which grouped together in EF-1α lineage 3, as well as one isolate from France within VCG 0133, that clustered together with VCG 0135 and the isolates in the nonassigned VCG in lineage 4. This study did not include isolates of VCG 0137 (of which only one isolate is available) (Jacobson and Gordon, 1990 a) and VCG 0138.

The separation of *F. o. melonis* VCGs into distinct phylogenetic lineages correlates with previous studies using restriction digest analyses of mtDNA (Jacobson and Gordon, 1990b). The identical mtDNA and *EF-1α* gene sequence of VCG 0130 and 0131 may lead to the conclusion that divergence of these VCGs occurred quite recently and that VCG 0130 could have derived from the more widespread 0131 in North America (Jacobson and Gordon, 1990b).

These results support the hypotheses that isolates within a VCG of *F. oxysporum* are genetically more similar than those in different VCGs, regardless of races (Elias and Schneider, 1991; Gordon and Martyn, 1997), and that isolates in each VCG may be clonally derived from a common ancestor (Kistler, 1997).

The present study also provides evidence for multiple evolutionary origins of some *formae speciales* of *F. oxysporum*, including *melonis*, *vasinfectum*, *niveum* and *lycopersici*. Based on phylogenetic analysis, isolates of *F. o. melonis* in the current study were found to be more closely related to some isolates of other *formae speciales* than to isolates of *F. o. melonis* in the other VCGs. O'Donnell *et al.* (1998) examined 2 isolates of *F. o. melonis* and, on the basis of DNA sequences of nuclear and mitochondrial genes, found that these isolates nested in two lineages with independent evolutionary origins.

Evidence for a polyphyletic origin was presented for the first time by O'Donnell *et al*. (1998) for *F. o. cubense*. All of the polyphyletic *formae speciales* identified to date, such as *formae speciales cubense, vasinfectum* and *lycopersici*, are composed

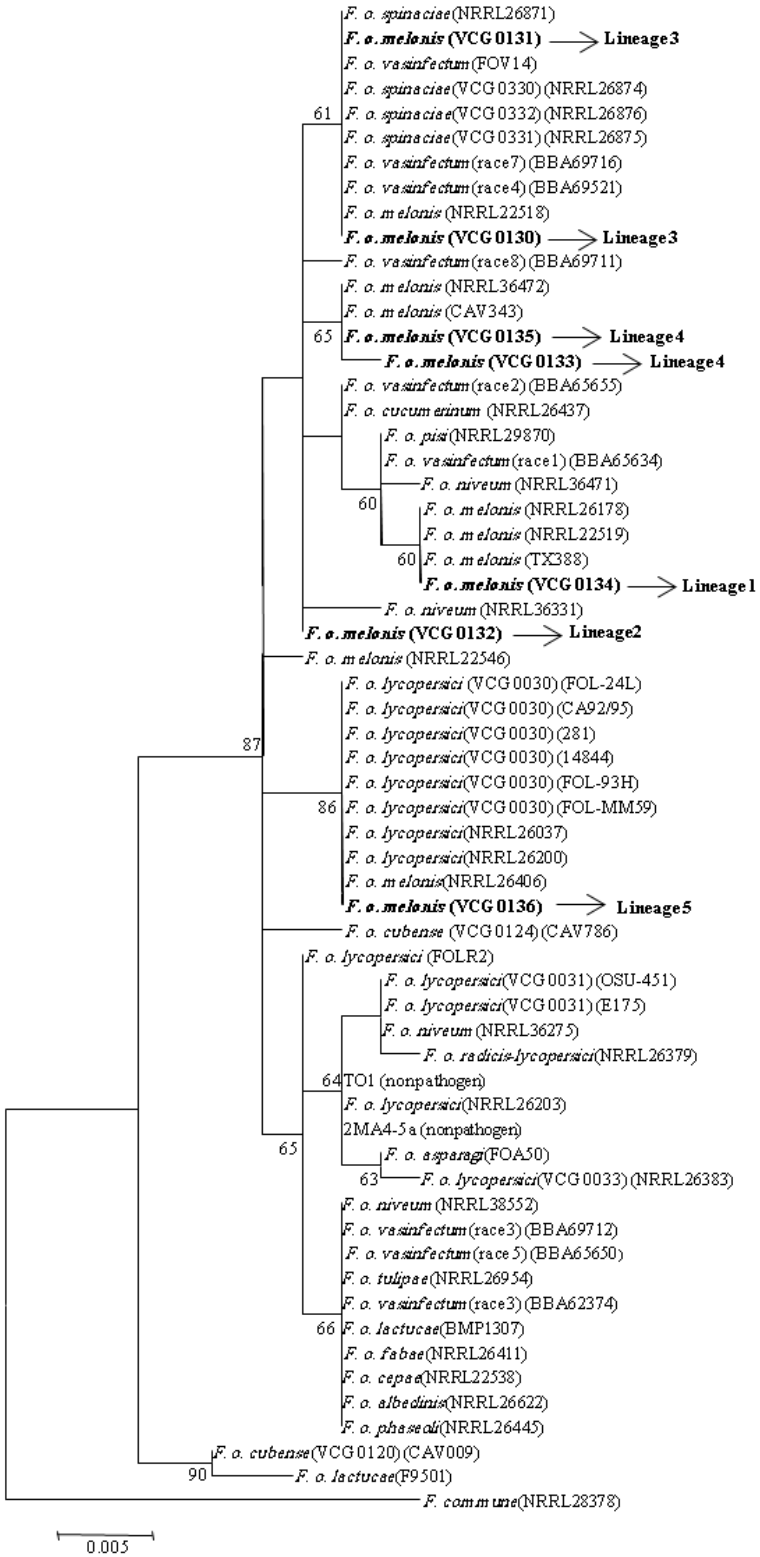

Figure 2. Maximum likelihood phylogenetic tree of *Fusarium oxysporum* f. sp. *melonis* and other isolates from different *Fusarium oxysporum formae speciales* inferred from EF-1α sequences. *F. commune* isolate NRRL28378 is included as outgroup. Bootstrap values (> 60%) are shown as percentages of 1,000 replicates. Different lineages of *Fusarium oxysporum* f. sp. *melonis* are indicated in bold.

of two or more VCGs (O'Donnell *et al*., 1998; Skovgaard *et al*., 2001, Lievens *et al*., 2009). By comparison, *formae speciales* with a single VCG, such as *tulipae* and *lilii*, are genetically assumed to be clonal.

In accordance with previous observations (Cai *et al*., 2003; Kawabe *et al*., 2005; Lievens *et al*., 2009), isolates of *F. o. lycopersici* examined in this study were divided into three groups. The first group composed of VCG 0030, the second included VCG 0031, and the third included VCG 0033 isolates.

In this study, Iranian and foreign isolates within races 0, 1, 1, 2 and 2 from VCG 0134 had identical *EF-1α* gene sequences, that could be explained by the adaptation to host resistance from the same clonal lineage (Jacobson and Gordon, 1991). The race structure within most *formae speciales* of *F. oxysporum* is often complex, which demonstrates that no particular race defines a genetically homogenous group of isolates (Gordon and Martyn, 1997). A notable exception is *F. o. vasinfectum* that has a one-on-one relationship between VCG and race (Assigbetse *et al.,* 1994; Katan and Katan, 1988). Using molecular markers such as sequencing of *EF-1α* gene and mitochondrial small subunit (mtSSU) rDNA (Skovgaard *et al*., 2001), most of the *F. o. vasinfectum* races could differentiate at different levels. Interestingly, based on *EF-1α* gene sequencing, a specific association was found between VCGs belonging to *F. o. melonis* and some VCGs or races from other *formae speciales*. For instance, *F. o. melonis* VCGs 0130 and 0131 were identical to all three VCGs of *F. o. spinaciae* and race 4 and 7 of *F. o. vasinfectum*. *F. o. melonis* VCG 0136 were grouped with *F. o. lycopersici* VCG 0030 and *F. o. melonis* VCG 0134 were closely related to *F. o. vasinfectum* races 1 and 2. For the first time, these specific relationships among *F. o. melonis* VCGs and the other *formae speciales* is reported in this study, which supports a polyphyletic origin for this *forma specialis*, meaning that *F. o. melonis* could emerge independently multiple times in different areas to yield *F. o. melonis* isolates with an independent genetic background. In addition to co-evolution with the host in their centers of origin, the divergence and evolution of *formae speciales* in *F. oxysporum* complex may be influenced by other factors such as genetic exchange or recombination due to parasexuality or heterokaryosis, which reflects the existence of more recently selected pathogenic forms. (Gordon and Martyn, 1997; Fourie *et al*., 2009)

The genetic basis of the *forma specialis* concept in *F. oxysporum* proposed by Snyder and Hansen (1940) is unknown (Baayen *et al*., 2000). The categorization of strains by host range may lead to an artificial subdivision within the species, meaning that unrelated isolates could be clustered together. Thus, individuals in a *forma specialis* may not be closely related or evolved from a common ancestor (O'Donnell *et al*., 1998). It is, therefore, not surprising that many researchers regard *F. oxysporum* as a complex of several different species (Kistler, 1997; O'Donnell and Cigelnik, 1997; Baayen *et al*., 2000). As a result, classification based solely on host range could not commonly reveal genetic diversity and biological differences (Kistler, 1997). Accordingly, detailed knowledge of the genetic variation, evolutionary history and relationships of *formae speciales* within the *F. oxysporum* complex is very important for the development of appropriate disease management strategies and effective breeding programs.

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رابطه ژنتیکی گروه های سازگاری رویشی *Fusarium oxysporum* f. sp. *melonis* و ارتباط آنها با سایر فرم گونه های *F. oxysporum*

م. میرطالبی و ض. بنی هاشمی

چکیده

پژمردگی فوزاریومی از مهمترین بیماریهای خربزه و طالبی در سراسر جهان می باشد. در این مطالعه رابطه تکاملی بین جدایه های مختلف فرم گونه های *F. oxysporum* با تاکید خاص روی *F .o. melonis* مورد مطالعه قرار گرفت. با استفاده از ژن فاکتور کشیدگی یک آلفا(EF-1α)، واکافت فیلوژنتیکی بر طبق رهیافت بیشینه درست نمایی و آزمون بوت استراپ روی ۱۶جدایه ایرانی و ۱۱ جدایه خارجی *F .o. melonis* شامل گروه های سازگاری رویشی مختلف (VCGs 0130-0136) انجام شد. درخت تکاملی به دست آمده از داده ها، پنج شاخه تکاملی را بوجود آورد که با گروه های سازگاری رویشی *F .o. melonis* مربوط بودند. در این مطالعه تنها گروه های سازگاری رویشی ۰۱۳۰ و ۰۱۳۱ با توالی سنجی فاکتور کشیدگی یک آلفا قابل تفکیک نبودند. علاوه بر این رابطه خاص بین گروه های سازگاری رویشی *F .o. melonis* و سایر فرم گونه های *F. oxysporum* که توالی ژن فاکتور کشیدگی یک آلفای آنها از بانک ژن اخذ شده بود به دست آمد. این نتایج منشا چند نیایی *F .o. melonis* را حمایت می کند به این معنا که این فرم گونه در چندین زمان تکامل یافته است.