Original Scientific paper
10.7251/AGRENG1903091A
UDC 632.4

MOLECULAR PHYLOGENY OF *FUSARIUM OXYSPORUM* SPECIES COMPLEX ISOLATED FROM EGGPLANT AND PEPPER IN TURKEY

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ABSTRACT

Members of *Fusarium oxysporum* species complex (FOSC) are economically most important plant pathogenic fungi found in a large number of plants in different families, while individual strains have strong specificities for particular hosts. Fusarium wilt caused by *F. oxysporum* f. sp. *melongenae* (Fomg) and *F. oxysporum* f. sp. *capsici* (Foc) is a worldwide soil-borne disease that causes yield losses in eggplant and pepper (Solanaceae) growing regions of Turkey. The intra-species or inter-species genetic diversity and phylogenetic relationships in Fomg and Foc isolates obtained from Turkey were investigated by utilizing the sequence data obtained through the designed primers belonging to three protein coding gene regions; beta-tubulin (BT), calmodulin (CAL) and chitin synthase (CHS). Phylogenetic analyses were carried out with BT, CAL and CHS gene regions with a selected subset of Fomg and Foc, along with other non-host *F. oxysporum* and outgroup isolates. The clustering trees successfully separated the Fomg and Foc from the outgroup *Fusarium* isolates. On the other hand, sequence data for BT, CAL and CHS gene regions displayed limited variation among Fomg and Foc isolates and found inefficient to distinguish reliably among several *F. oxysporum* forma specialis (f. sp.) groups. The sequence variation on these 'housekeeping gene' regions in the core genome was not considered adequate to differentiate FOSC populations.

Keywords: *Fusarium wilt, phylogenetic analysis, DNA sequence*

INTRODUCTION

The pathogenic *Fusarium oxysporum* species complex (FOSC) causes major yield losses in the family Solanaceae including on tomato, pepper and eggplant productions in Aegean and Mediterranean Regions of Turkey where the vegetable growing is concentrated. *Fusarium oxysporum* is a ubiquitous asexual species of soil-borne plant pathogens and responsible for vascular wilt or root rot disease in a variety of host plants. These pathogenic strains have been grouped into formae speciales (ff. spp.) and races based on plant and cultivar specificity (Armstrong and Armstrong, 1981). Fusarium wilt caused by *F. oxysporum* f. sp. *melongenae*; Fomg) is one of the most destructive and widely distributed pathogen of eggplant (*Solanum melongena* L.) in Turkey (Altinok, 2005). Fusarium wilt is caused by *Fusarium oxysporum* (Schlect.) emend. Synd. and Hans. f. sp. *capsici* Riv. (Foc), which and it has recently emerged in local pepper fields in Turkey. The characteristic symptoms both Fusarium wilt diseases include wilting, stunting, leaf chlorosis, interveinal yellowing of the outer leaflets, then vascular necrosis and finally death of the above ground parts of the plant (Black and Rivelli, 1990; Agrios, 2005; Altinok, 2005). These pathogens may remain viable for a long period of time in soil as chlamydospores, even after rotation with non-host crops (Nelson *et al.,* 1983; Katan, 1999; Altinok *et al.,* 2018). Currently the preferred and efficient method for control of soil-borne fungal diseases is to take advantage of resistant cultivars. Resistant commercial cultivars yet to be developed, however, some grafted plants are presenting a good level of resistance against Fomg (Gisbert *et al.,* 2011; Altinok *et al.,* 2014). Conventional control of FOSC is inadequate in most cases. DNA-based diagnostic tools allow genetic diversity and phylogeny of *Fusarium* species. Many methods have been used to characterize the genetic diversity within and among populations and evolutionary origin of FOSC, including restriction fragment length polymorphisms (RFLPs), random-amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) and comparisons of DNA sequences from conserved genomic regions (Assigbetse *et al.,* 1994; Appel and Gordon, 1995; Jarne and Lagoda, 1996; O'Donnell *et al.,* 1998; Baayen *et al.,* 2000). Sequence-based identification of fungi, namely, DNA barcoding using the ITS region and protein coding regions of nuclear DNA by sequence alignments have also been used extensively in fungi phylogenetic relationships. The most commonly used sequence-based markers in *F. oxysporum* include 'housekeeping gene'. Sequence analysis of the nuclear ribosomal DNA (rDNA), and nuclear rDNA intergenic spacer (IGS), translation elongation factor 1-α (TEF-1α), β-tubulin, histone, actin, chitin synthase, and calmodulin genes, have been used to determine the genus *Fusarium* (Jiménez-Gasco *et al.,* 2002; Schmitt *et al.,* 2009). TEF-1α and actin genes are able to detect polymorphisms of the DNA sequence of a genome and involved in basic cellular functions encode highly conserved proteins identified in fungi (Schmitt *et al.,* 2009; Altinok *et al.,* 2018).

In this study, intra-species or inter-species genetic diversity and phylogenetic relationships in Fomg and Foc isolates obtained from Turkey were investigated by

utilizing the sequence data obtained through the designed primers belonging to three protein coding gene regions; beta-tubulin (BT), calmodulin (CAL) and chitin synthase (CHS) gene sequences analyzed with the hierarchical maximum likelihood clustering method.

MATERIALS AND METHODS

Fungal isolates A subset of 40 Fomg and 40 Foc isolates, along with non-host f. sp. isolates (other FOSC) were selected for phylogenetic analyses based on beta tubulin (BT), calmodulin (CAL) and chitin synthase (CHS) sequences. Designed primers (Operon Technologies GmbH, Cologne, Germany). were given in Table 1. All isolates were obtained from the culture collection of Mycology Laboratory of Plant Protection Department of Erciyes University, Turkey (Project No: TUBITAK-TOVAG-109O524 and TUBITAK-COST-114O866).

DNA extraction, PCR amplification, sequencing and phylogenetic analysis Monoconidial Fomg and Foc isolates representing various geographical locations were selected as a subset. For DNA extraction, the method described by Altinok *et al*. (2018) was used. The PCR reactions were prepared in a total volume of 20 µL containing 10x PCR buffer, 2 mM of dNTPs, 10 mM of each primer, 1 U of Taq DNA Polymerase and genomic DNA (25 ng). A negative control with double-distilled sterile water instead of DNA template was used. PCR conditions; started with 5 min of denaturation at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. Amplification products were electrophoresed on agarose gel in 0.5X Tris-borate EDTA buffer, and stained with the fluorescent dye ethidium bromide and visualized (DNR Bio-Imaging System) under UV light. PCR products were sequenced by Ref-Gen Biotechnology (Ankara, Turkey). BT, CAL and CHS consensus sequences were generated using Geneious software (v10.0.5) (Biomatters, NZ, USA). Multiple alignments of consensus sequences were made with ClustalW (Larkin, 2007). The Megablast algorithm was used to perform similarity (Hall, 1999; Morgulis *et al.,* 2008). Dendrogram was created with MEGA 7 using UPGMA method, with 2000 bootstrap repetitions (Sneath and Sokal, 1973; Huelsenbeck and Ronquist, 2001).

Table 1. Primer codes and sequences used for the analysis of the beta tubulin, calmodulin and chitin synthase genes of the *Fusarium oxysporum* f. sp. *melongenae* and the *Fusarium oxysporum* f. sp. *capsici* in this study.

Primer	**Code**	**Sequence 5´ to 3´**
Beta-tubulin*	Bt-F	5'-CAA GAA CTC AGA GTA TTT CGT C-3'
	Bt-R	5'-TAT GAA CTA GAA GGG GAT GAA G-3'
Calmodulin*	CAL-F	5'-CTC ACT TAC TGA AGA GCA AGT CT-3'
	CAL-R	5'-TTA GTA CTG ACC GTC CTC TAA TC-3'
Chitin synthase*	CHS-F	5'-CTA CAT TCG CTC CTA CCT ACT C-3'
	CHS-R	5'-TGGAAGAACCATCTGTGAGAGTTG-3'

*Primers were designed from the full genome *Fusarium oxysporum* within the scope of our project (TUBITAK-COST 114O866).

RESULT AND DISCUSSION

The Fomg and Foc isolates yielded a single amplicon with a size of 490-500 bp for BT, CAL and CHS in conventional PCR. Amplification products obtained by chitin synthase primer of Fomg (P) and Foc (B) isolates on agarose gel were presented in Figure 1. Geographical origin of Fomg and Foc isolates recovered from greenhouses/fields in Turkey is presented in Table 2. Phylogenetic analyses were carried out using BT, CAL and CHS protein coding gene regions with a selected subset of Fomg and Foc, along with other non-host *F. oxysporum* (*F. oxysporum* f. sp. *lycopersici*-Fol, *F. oxysporum* f. sp. *radicis-lycopersici*-Forl and *F. oxysporum* f. sp. *melongenae*-Fomg) and outgroup *Fusarium* isolates from GenBank (Foc-BT tree *F. solani*-AM419425.1. and *F. incarnatum*-JN614905.1, Fomg-BT tree *F. incarnatum*-JN614905.1 and *F. sublunatum*-KM232076.1, Foc/Fomg-CAL tree *F. proliferatum*-AJ560773.1 and *F.incarnatum*-HQ412341.1, Foc/Fomg CHS tree *F. culmorum*-KP195141.1 and *F. graminearum* AJ314860.1).

The BT, CAL and CHS gene region sequences were yielded and clustering trees successfully separated the FOSC (Fomg, Foc, Fol and Forl) from the outgroup *Fusarium* isolates. One of the cluster contained the whole subset of FOSC, while the other clusters contained the outgroup *Fusarium* isolates. The dendrograms (cluster tree) exhibited mostly similar branching, for that reason Foc-CAL tree is represented as examples in Figure 2. The BT, CAL and CHS gene region was found to be inadequate to provide an intraspecific differentiation for the FOSC. All of the FOSC were grouped together and there was no significant and meaningful discrimination among non-host *F. oxysporum* isolates (Fol and Forl). On the other hand, sequence data for these gene regions displayed limited variation among Fomg and Foc isolates. The sequence similarity for these gene regions was not related to the virulence or geographical origin of the Fomg and Foc isolates. The sequence variation on these 'housekeeping gene' regions in the core genome were not considered adequate to differentiate FOSC populations. Host-specificity is associated with the presence of accessory or lineage-specific (LS) chromosomes and pathogenicity chromosomes are composed of 'transposable elements'. These chromosomes contain effector genes associated with pathogenicity. Pathogenic effector proteins are important proteins for virulence, and these proteins interfere with host proteins (De Wit *et al.,* 2009). Horizontal transfer of accessory chromosomes seems to act as a powerful force on pathogen populations resulting in evolution of new virulent pathotypes (Ma *et al.,* 2010). For that reason the FOSC shares highly similar core chromosomes. The uniform genetic structure of the Fomg and Foc population may be considered as an advantage in plant breeding studies to be carried out to generate resistant pepper cultivars. Basic factors of detection and genetic differentiation of plant pathogens are reported to be virulence ability, host-pathogen relationship, presence of alternative hosts, saprophytic capability and/or long-term resistance to unsuitable environmental (Parker and Gilbert, 2004). In general, plant varieties have two types of resistance; monogenic (vertical), controlled by a single resistance (R) gene and oligogenic (horizontal), controlled by multiple genes. Monogenic resistance has been more effective than

polygenic resistance in other crops and practical to use in modern plant breeding (Beckman and Roberts, 1995; Agrios, 2005).

Table 2. Geographical origin of *Fusarium oxysporum* f. sp. *melongenae* (Fomg) and *Fusarium oxysporum* f. sp. capsici (Foc) isolates collected from greenhouses and fields.

Number	Isolates	Sampling location	Number	Isolates	Sampling location
P1*	HSM-17	Hatay-Samandag	B*1	MSGM-94/1	Manisa-Gölmarmara
P2	Fomg109	Izmir-Menemen	B2	IZTB-80/D-1	Izmir-Torbalı
P3	Fomg102	Izmir-Odemis	B3	ANMK-43/3	Antalya-Kumluca
P4	UBR-9	S.Urfa-Birecik	B4	ANKS-49/8	Antalya-Kas-Demre
P5	BRK-42	Bursa-M.Kemalpasa	B5	MSGM-95/10	Manisa-Gölmarmara
P6	BMK-38	Bursa-M.Kemalpasa	B6	MGML-56/5	Mugla-Milas
P7	DBS-22	Diyarbakir-Bismil	B7	ANSR-42/2	Antalya-Serik
P8	Fomg220	Izmir-Odemis	B8	ANKS-49/2	Antalya-Kas-Demre
P9	ANC-9	Aydin-Incirliova	B9	FOC-2	Mersin-Tarsus
P10	Fomg97	Manisa-Merkez	B10	ANSR-40/2	Antalya-Serik
P11	Fomg122	Izmir-Bayindir	B11	ANGP-21/2	Antalya-Gazipaşa
P12	MS-6	Manisa-Salihli	B12	MRBZ-1/6	Mersin-Bozyazı
P13	ANZ-36	Aydin-Nazilli	B13	MSGM-94/6	Manisa-Gölmarmara
P14	Fomg154	Mersin-Tarsus	B14	ANSR-39/3	Antalya-Serik
P15	Fomg94	Antalya-Serik	B15	ANSR-39/5	Antalya-Serik
P16	Fomg160	Mersin-Tarsus	B16	ANLY-29/4	Antalya-Alanya
P17	UMR-12	S.Urfa-Merkez	B20	ANKS-49-11	Antalya-Kas-Demre
P18	MF-26	Mugla-Fethiye	B21	AYBZ-65-12	Aydın-Bozdoğan
P19	ML-5	Mugla-Milas	B22	IZTB-81/6	Izmir-Torbali
P20	MS-1	Manisa-Salihli	B23	ANSR-42/1	Antalya-Serik
P21	MRM-21	Mersin-Merkez	B24	ANLY-26/1	Antalya-Alanya
P22	ANC-11	Aydin-Incirliova	B25	AYNZ-60/4	Aydin-Nazilli
P24	ML-17	Mugla-Milas	B26	ANMK-43/2	Antalya-Kumluca
P25	MS-9	Manisa-Salihli	B27	IZMN-76/1	Izmir-Menderes
P26	ANZ-40	Aydin-Nazilli	B28	AYBZ-70/5	Aydin-Bozdogan
P27	UMR-28	S.Urfa-Birecik	B29	AYNZ-60/5	Aydin-Nazilli
P28	HSM-21	Hatay-Samandag	B30	AYNZ-60/7	Aydin-Nazilli
P29	ANC-6	Aydin-Incirliova	B31	AYNZ-60/3	Aydin-Nazilli
P30	MY-19	Mugla-Yatagan	B32	AYBZ-65/7	Aydın-Bozdogan
P31	BMK-50	Bursa-M.Kemalpasa	B33	MSSR-85/12	Manisa-Saruhanli
P35	BMK-32	Bursa-M.Kemalpasa	B34	ANGP-18/2	Antalya-Gazipasa
P36	HD-7	Hatay-Dortyol	B35	ANGP-18/A-1	Antalya-Gazipasa
P37	HSK-14	Hatay-Iskenderun	B36	MSSR-86/7	Manisa-Saruhanlı
P38	SCR-45	Samsun-Carsamba	B37	MRBZ-1/9	Mersin-Bozyazi
P39	DBS-21	Diyarbakir-Bismil	B38	IZMN-76/5	Izmir-Menderes
P40	HSK-14	Hatay-Iskenderun	B39	ANLY-29/6-B	Antalya-Alanya
P42	MRM-25	Mersin-Merkez	B40	ANLY-29/6-A	Antalya-Alanya
P43	BRO-55	Bursa-Orhangazi	B41	ANGP-15/1	Antalya-Gazipasa
P44	Fomg172	Izmir-Menemen	B42	ANKS-50/3	Antalya-Kas-Demre
P45	HSM-29	Hatay-Samandag	B43	MGML-65/5	Mugla-Milas

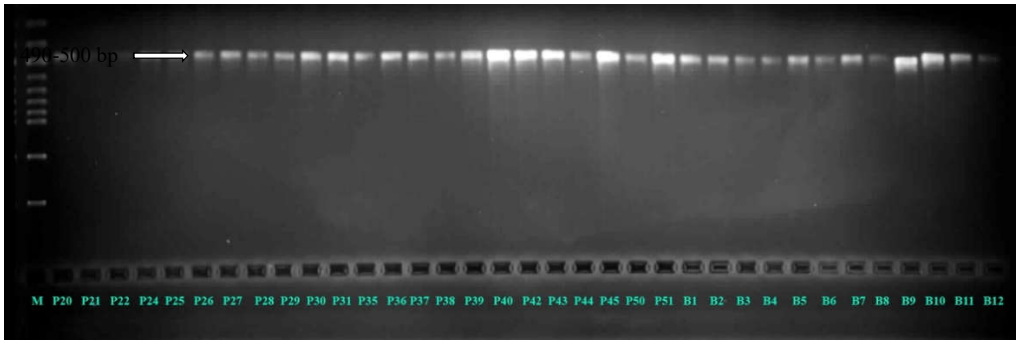

Figure 1. PCR products obtained by chitin synthase primer of Fomg (P) and Foc (B) isolates on agarose gel. Marker (M); 100 bp.

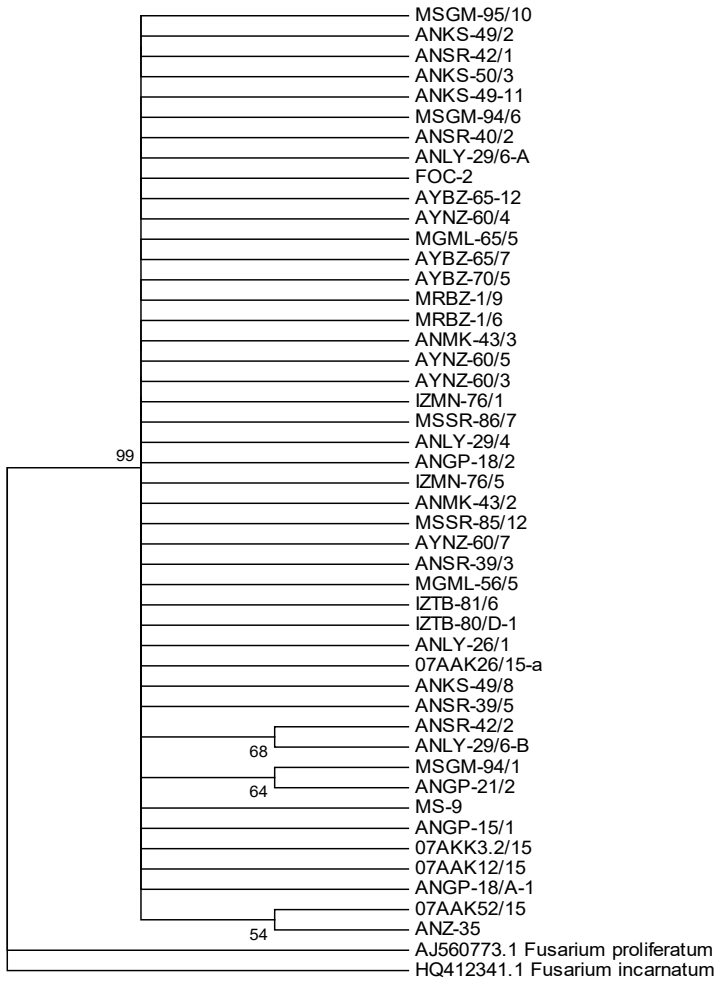

Figure 2. Phylogenetic tree constructed using the UPGMA clustering method with 2000 bootstrap replicates and drawn as condensed, based on sequences of the calmodulin (CAL) sequences gene regions of *Fusarium oxysporum* f. sp. *capsici* isolates (Foc). Non-host *F. oxysporum* Fol-07AAK52/15 and 07AKK3.2/15, Forl-07AAK26/15-a and 07AAK12/15, Fomg-MS-9 and ANZ-35. Out group Fusarium from GenBank *F. proliferatum*-AJ560773.1 and *F.incarnatum*-HQ412341.1.

CONCLUSIONS

The results obtained from the BT, CAL and CHS gene regions were not found to be informative for genetic diversity among Fomg and Foc sampled from pepper greenhouses/and fields in Turkey. On the other hand, sequence data for BT, CAL and CHS gene regions displayed limited variation among Fomg and Foc isolates and found inefficient to distinguish reliably among several FOSC. The sequence variation on these 'housekeeping gene' regions in the core genome was not considered adequate to differentiate FOSC populations. This study showed that the Fomg and Foc isolates exhibited possible monophyletic ontogeny, and there was no-significant variation in phylogenetic analyses of the BT, CAL and CHS regions to form genetically different groups. The uniform genetic structure of the Fomg and Foc populations in Turkey may be considered as an advantage in plant breeding studies to be carried out to generate resistant cultivars.

ACKNOWLEDGMENTS

This study is a part of the project fully supported by the Scientific and Technical Research Council of Turkey (TUBITAK-COST project number: 114O866).

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