Turkish Journal of Agriculture - Food Science and Technology, 7(7): 1040-1045, 2019
DOI: https://doi.org/10.24925/turjaf.v7i7.1040-1045.2541

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology

First Report on Identification of *Fusarium graminearum* Species Complex Members from Turkey and Iran

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ARTICLE INFO

Research Article

Received : 18/03/2019
Accepted : 25/06/2019

Keywords:
DNA Sequencing
Fusarium graminearum
Fusarium Head Blight
Multilocus Genotyping
Species Complex

ABSTRACT

Fusarium graminearum species complex is the major *Fusarium* head blight disease pathogen in worldwide. *Fusarium* head blight disease lead to damages on small grain cereals. The identification of the *F. graminearum* species complex is important for improving disease control and management. Traditional identification methods such as macroscopy and microscopy analysis need to be supported by biochemical and genetics assays. Thus, DNA sequencing-based methods is one of the most preferred, reliable, low priced in the identification methods. In the study, 54 *F. graminearum* isolates obtained from diseased wheat, barley and corn fields in Turkey and Iran were identified by morphological characteristics and then characterized by species-specific SCAR marker. β-tubulin, Tef1-α, 28s rDNA and Histone H3 genes amplified, purified and then sequenced. The merged multiloci length was obtained as last of all 2215 bp. These sequencing results was used to multiloci genotyping assays. Last of all, 20 isolates were determined as *F. graminearum sensu stricto* by multiloci genotyping analysis. Remaining isolates were identified as *F. asiaticum* or *Fusarium* sp. The findings are important in terms of revealing the first-time identification in Turkish and Iranian isolates as *F. graminearum* species complex members by amplification four (β-tubulin, Tef1-α, 28s rDNA and Histone H3) highly conserved DNA regions.

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Introduction

Fusarium head blight (FHB) is a major destructive disease of some small grain cereals and is closely related to primary food safety concern. Many *Fusarium* species can lead to FHB and epidemics of these species and their associated mycotoxins have been occurred in many different regions (Parry et al., 1995; Logrieco et al., 2002; Goswami and Kistler, 2004). *Fusarium graminearum* is known to be the important causal agent of FHB from many regions worldwide (Pasquali and Migheli, 2014). Several cereals, such as wheat, barley and maize, are infected by *F. graminearum* and substantial yield losses reaching up to billions of dollars, reduction in crop quality and health problems with severe levels are observed. *F. graminearum*, as called *F. graminearum* species complex (FGSC), has been reported to show high level of genetic diversity in the worldwide and this variable population genetics potential led to a continuous increase in genetic characterization studies. (Miedaner et al., 2008, 2013; Matny, 2015; Taheri, 2018).

Since identification of phytopathogens at species level precisely is important in disease management, researchers have been focused on genetic characterization of *F. graminearum* more than 30 years. The first striking data for this species was about to distinguish *F. graminearum* and *F. pseudograminearum* as two different species (Aoki and O'Donnell, 1999). After that, second important advance in *F. graminearum* characterization was the lineage differentiation up to seven members by nuclear gene sequencing and SCAR markers (O'Donnell et al., 2000; Carter et al., 2002). Recently, the number of phylogenetically distinct species for *F. graminearum* species complex is 15. Nuclear gene sequence variation has been clearly related to chemotype and geographic distribution up to now and the cosmopolitan member has been reported as *F. graminearum sensu stricto* (O'Donnell et al., 2000; Wang et al., 2008; Yli-Mattila et al., 2009; Przemieniecki et al., 2014).

The close association between *F. graminearum* and FHB have been reported many European and Asian countries including Turkey in last two decades (Miedaner et al., 2008; Pasquali and Migheli, 2014; Matny, 2015; Taheri, 2018). The identification of FGSC is a critical step to control of mycotoxin contamination and improving disease management. Conventional methods based on morphological characters, are commonly used in for discrimination of species in *Fusaria.* However, these methods could require labour and insufficient alone. Thus, DNA sequencing-based methods have been preferred for credible, fast, reliable and cost-effective strategies for species identification currently. In this study, for the first time, *F. graminearum* isolates causing FHB and crown rot diseases from Turkey and Iran have been subjected to FGSC member identification by multiple sequencing assays with amplification of β-tubulin, Histone H3, TEF1-α (Translation elongation factor) and ITS-28S rDNA regions.

Materials and Methods

Fungal Materials

Isolates were obtained from wheat, barley and maize fields from plant crown and ear parts near plant maturity between the years 2006-2017. Surface sterilization, *in vitro* growth and microscopic assays were carried out as described by Tunali et al. (2006). Single spore isolations were carried out by using a common protocol described by Burgess et al. (1994). Totally, 54 single spore isolates of *F. graminearum* were used in further analysis. Characteristics of *F. graminearum* isolates are given in Table 1.

Genomic DNA Extraction

Genomic DNA (gDNA) was isolated from 7-day-old cultures grown on Czapek-Dox Agar (CDA) at 26±2ºC using the sodium dodecyl sulphate-based protocol. 50-100mg fresh mycelium was collected from petri dishes and washed with 96% ethanol for 3 mins. Dried mycelium was homogenized in liquid nitrogen with mortar and pestle. The homogenate was transferred to a sterile microtube and the binding 500 µL lysis buffer (100 mM Tris-Cl, 100 mM EDTA, 1 M NaCl, 1% SDS and 1/500 β-mercaptaetanol) was added to tubes, and homogenization was carried out by throughout vertexing. 10 µL of RNase A (10 mg/mL) was added to tubes and incubated at 65°C for 15 mins. 700 µL chloroform:isoamylalchol (24:1) was added to tubes and gently mixed. After centrifugation at 13.000 rpm for 1 min, supernatant was transferred to new tube and 1:1 volume of chloroform:isoamylalchol (24:1) was added. After centrifugation 1:10 volume NaOAc and 2 volumes of cold 96% ethanol was added to tubes. Samples were incubated at -80°. Samples were then centrifuged at 13.000 rpm for 5 min. Tubes were washed 70% ethanol with two times and after centrifugation at 13.000 rpm for 2 min, ethanol was removed and gDNA was eluted in 50 µL ddH_2O. The purity and the quantity of extracted gDNA molecules were checked with spectrophotometer (Thermo, USA) and 1% agarose gel electrophoresis. gDNA was photographed underusing a gel documentation system (Maestrogen, Taiwan) by staining with 0.2-0.5 µg/mL ethidium bromide under UV light. Electrophoresis was carried out under 70V for 45 min. Intact gDNA molecules were diluted to 10 ng/µL dilution series in order to use them in further assays.

Table 1 *F. graminearum* isolates used in this study. ‘’*’’ means positive *EcoRV* digestion.

Code	Host	Location	Year	Code	Host	Location	Year
F5	Wheat	Sakarya	2006	FgM6	Wheat	Mazandaran	2007
F6 *	Wheat	Sakarya	2006	FgM7	Wheat	Mazandaran	2007
F7 *	Wheat	Sakarya	2006	FgM9	Wheat	Mazandaran	2007
F8	Wheat	Sakarya	2006	FgM10	Wheat	Mazandaran	2007
F9 *	Wheat	Balikesir	2006	Fg4	Wheat	Mazandaran	2007
1F	Wheat	Bolu	2010	FgM5	Wheat	Mazandaran	2007
2F	Wheat	Cankiri	2009	Fg5	Wheat	Sari	2007
3F	Maize	Samsun	2010	Fg18 *	Wheat	Moghon	2007
4F	Barley	Bolu	2010	Fg49	Wheat	Moghon	2007
5F *	Maize	Samsun	2010	Fg56	Wheat	Gorgan	2007
6F	Maize	Samsun	2010	Fg165 *	Wheat	Kordkooy	2007
7F	Maize	Samsun	2010	Fg170	Wheat	Gorgan	2007
14F*	Wheat	Kastamonu	2009	Fg174 *	Wheat	Gorgan	2007
15F *	Wheat	Sakarya	2010	Fgsh1	Wheat	Mazandaran	2007
14-4TR	Wheat	Tokat	2014	Fgsh4 *	Wheat	Mazandaran	2007
14-5TR*	Wheat	Amasya	2014	Fgsh5	Wheat	Mazandaran	2007
14-6TR *	Wheat	Tokat	2014	Fgsh7	Wheat	Mazandaran	2007
14-7TR *	Wheat	Amasya	2014	Fgsh10	Wheat	Mazandaran	2007
10-2TR *	Maize	Samsun	2010	FgSh13	Wheat	unknown	2007
17-9TR	Wheat	Samsun	2017	Fgsh15	Wheat	Unknown	2007
17-11TR *	Wheat	Samsun	2017	FgT2	Wheat	Mazandaran	2007
17-12TR *	Wheat	Samsun	2017	FgT3 *	Wheat	Mazandaran	2007
17-13TR *	Wheat	Samsun	2017	FgT7	Wheat	Mazandaran	2007
17-14TR *	Wheat	Samsun	2017	FgT9 *	Wheat	Mazandaran	2007
17-15TR *	Wheat	Samsun	2017	FgT10	Wheat	Mazandaran	2007
17-16TR *	Wheat	Samsun	2017	FgT11 *	Wheat	Mazandaran	2007
FgM1 *	Wheat	Neka	2007	FgT16	Wheat	Mazandaran	2007

Species Specific Identification by Polymerase Chain Reaction (PCR) Assays

Species-specific identification was carried out by two different species-specific SCAR primers.

UBC85F/UBC85R primer set developed by Schilling et al. (1996) was used in order to amplify monomorphic bands of 332 bp DNA. PCRs were carried out in a volume of 25 µL containing 50 ng genomic DNA, 1X PCR master mix (Takara, Japan) and 5 pmol of each primer. PCR conditions were performed at 98ºC for 2 min for pre-denaturation, 35 cycles at 94ºC for 30 s, 61ºC for 30 s, 72ºC for 30 s and at 72ºC for 2 min for final extension. The amplicons were electrophoresed in 1.5 % agarose gels and stained with EtBr. The gel images were obtained as described before.

Multiloci Genotyping by PCR and Bioinformatics Assays

To discriminate the members of *F. graminearum* species complex specific and highly conserved DNA regions were targeted in multiple alignment assays. For this purpose, β-tubulin, Histone H3, TEF1-α (Translation elongation factor) and ITS-28S rDNA regions were amplified. PCR primers (Starkey et al. 2007) are given in Table 2. 50 µL volume of PCRs mixtures included 1X PCR mix (Takara, Japan), 10 pmol of each primer, and 50ng of gDNA. PCR cycling conditions were as follows: pre-denaturation at 98°C for 3 min; 35 cycles of 98°C for 30s, 50-55°C for 30s, 72°C for 1-2 min; final extension at 72°C for 3 min. PCR bands were analyzed as described before on 1.5% agarose gels. After checking the correct bands on agarose gels, bands were purified by using a commercial PCR product clean up kit (BioBasic, Canada). Agarose gel pieces of 100 mg were cut form the gels and the protocol provided by the manufacturer was followed in amplicon purification.

Sanger dideoxy termination method-based sequencing process was carried out using "DYEnamic ET Terminator Cycle Sequencing" kit (Amersham, USA) on ABI PRISM 310 device. The nucleotide sequence related signal pics were displayed on chromatograms using the "Chromas Lite Edition" software. The sequences translated to FASTA format were subjected to BLASTN analysis. After BLASTN check, the nucleotide sequences generated via forward and reverse primers were aligned and assembled into a single sequence via DNA Dragon software. These sequences were subjected to multiple alignment and neighbor joining (NJ) topology assays by Mega 6.0 software (Tamura et al., 2013). Accession numbers reported from different investigations of (O'Donnell et al., 2000; 2008; Yli-Mattila et al., 2009; Starkey et al., 2007; Tóth et al 2008; Desjardins and Proctor 2011) for each gene of every species complex members were used as positive control in alignment assays. Each gene was both aligned single and multiple as a single FASTA file by using multiple alignment assays. Histone H3 sequences obtained from Turkish and Iranian isolates were also subjected to PCR-RFLP based sequencing strategy developed by O'Donnell et al. (2004) was also used in order to confirm the presence of *F. graminearum sensu stricto* or *F. asiaticum* members in isolates used in this study.

Table 2 Primers used in this study.

Primer set	Forward sequence (5'-3')	Reverse sequence (5'-3')	Gene	Band size (bp)
Ef1/2	atgggtaaggargacaagac	ggargtaccagtsatcatgtt	TEF1-α	700
btubcodonF/R	gtcattacaccgagggtgct	gaggcagccatcatgttctt	β-tubulin	603
HistonH3.2F	aggtccactggtggcaag	atgtccttggactggatggt	Histone H3	482
ITS5/NL4	ggaagtaaaagtcgtaacaagg	ggtccgtgtttcaagacgg	ITS-28S rDNA	1200

Results and Discussion

F. graminearum isolates obtained from diseased wheat, barley and maize samples of Turkey and Iran were confirmed by morphological characteristics as reported by Tunali et al. (2006). After morphologic characterization by microscopy, isolates were confirmed at species level by SCAR markers. Each isolate yielded 332 bp amplicon by UBC85F/R primer set (data not shown). These results showed that each isolate, used in this study, could be accepted as *F. graminearum* species complex.

After species-specific identification of *F. graminearum* isolates by common methods, multiloci genotyping was used to further identify isolates as members for FGSC. Totally four genes were amplified from 54 isolates. Predicted amplicon sizes, given in Table 2, were obtained by PCR assays (Figure 1). Each amplicon was cleaned up and then sequenced. After obtaining two directional sequences for each isolate; the data was assembled. Before final version of trimmed sequences, each nucleotide sequence was independently subjected to BLASTN analysis. BLASTN analysis resulted in highly conserved DNA regions with reference *F. graminearum* nucleotide sequences on GenBank (bit scores > 50 and E value <0.05). After BLASTN assays, the sequences were trimmed and the trimmed final nucleotide sequence length for β-tubulin, Histone H3, TEF1-α and ITS-28S rDNA regions were extracted as approximately 285, 375, 605 and 950 bp length, respectively. The merged total sequence length was approximately 2215 bp. The deletions/insertions and substitutions were detected by multiple alignment for some isolates leading to genetic polymorphisms (Figure 2). The isolates containing same deletions/insertion were positioned in the same sub-division with reference sequence. In maximum likelihood topology analysis, two distinct divisions (Div-1 and Div-2) were obtained via 1000 hierarchical replicates (Figure 3). Div-1 included 5 isolates while Div-2 contained remaining 49 isolates from Turkey and Iran. Two reference strains, *F. graminearum sensu stricto* and *F. asiaticum*, were clustered in Div-2. 14 Turkish (17-14TR, 17-13TR, 17-12TR, 17-11TR, 17-15TR, F6, 15F, 14F, 14-7TR, 14-6 TR, 14-5 TR, 5F, 10-

2TR and F7) and 6 Iranian (Fg18, Fg165, Fg174, FgT3, FgT11 and Fgsh4) isolates were closely related to reference *F. graminearum sensu stricto* in Subdiv-1 of Div-2. Twenty-four Turkish and Iranian isolates were co-clustered with *F. asiaticum* reference strain in sub-div-2 of Div-2. *EcoRV* digestion of Histon H3 nucleotide sequence dependent assay resulted so similar results from multiloci genotyping assay. *EcoRV* digestion positive isolates were given in Table 1 with asterisk. *F graminearum sensu stricto* positive isolates were co-clustered in sub-div-1 of Div-2. These results clearly showed that only 20 isolates used in this study (17-14TR, 17-13TR, 17-12TR, 17-11TR, 17-15TR, F6, 15F, 14F, 14-7TR, 14-6TR, 14-5 TR, 10-2TR, 5F, F7, Fg18, Fg165, Fg174, FgT3, FgT11 and Fgsh4**)** belonged to *F. graminearum sensu stricto* while 25 isolates (4F, F8, F5, 2F, 6F,7F, Fg170, FgSh5, FgM9, Fg5, FgSh15, Fg56, Fg49, FgT7, FgT10, 1F, FgSh7, FgT2, FgM6, FgM10, FgT16, FgM5, FgSh13, FgM7 and FgSh10) were closely related to *F. asiaticum*. Remaining 9 isolates (17-9TR, FgM1, FgT9, Fg4, F9, 17-16TR, 3F, FgSh1 and 14-4TR) clustered in Div-1 and Div-2 could be accepted as *Fusarium* sp. as reported by Przemieniecki et al. (2014).

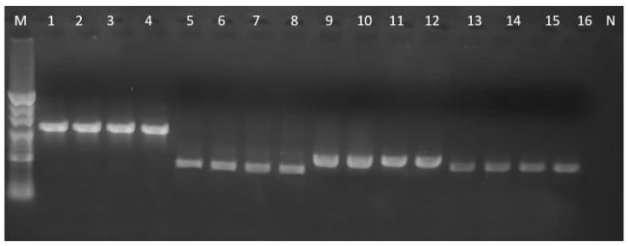

Figure 1 β-tubulin, Histone H3, TEF1-α, 28S rDNA genes amplified from *F.graminearum* isolates.
N: negative control, M: 100bp size marker (Genemarkbio, Taiwan), 1-4: 28S rDNA, 5-8: Histone H3, 9-12: TEF1-α, 13-16: β-tubulin PCR product.

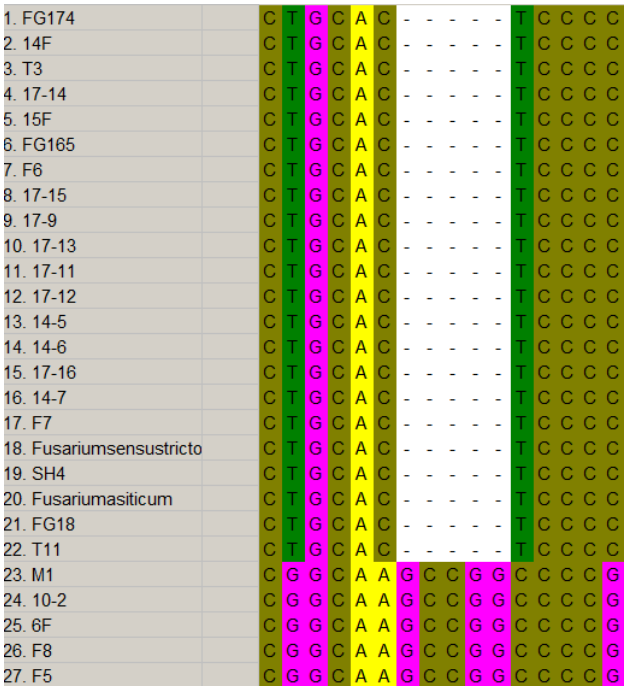

Figure 2 Deletion/insertion and substitutions on multiple alignment profile obtained by Mega 6.0 software

In phylogenetics analysis obtained from multiple alignment of multiloci sequencing, isolates belonged to the collection years 2006, 2014 and 2017 showed strong correlation in clustering analysis. Isolates from these years were co-clustered in the same sub divisions together in phylogram. This result was compatible with previous reports including *Fusarium* isolates from different locations by different molecular marker analysis including RAPD, PCR-RFLP and allele-specific PCR assays (Carter et al., 2000; Saharan et al., 2007; Çepni et al., 2013; Yörük et al., 2018). Similarly, isolates from the same or near agro-ecological location (s) were clustered so closely in phylogram. Even if the precise correlation between geographic origin and clustering of *Fusarium* isolates can't be stated whenever, locations can be accepted as a major characteristic behind the variation of *Fusarium* isolates (Carter et al., 2000, 2002; Mishra et al., 2004; Miedaner et al., 2001, 2008; Yörük and Albayrak, 2013). Most of the isolates used in this study were obtained from wheat as the host. As seen in Figure 3, isolates obtained from maize were phylogenetically closely located at dendrogram. It seems that, characteristics for phylogenetical differentiation of FGSC isolates used in this study should be considered together. In other words, location, collection year and belonging to different species complex member could play collectively. By this way, location is predominating factor to differentiate among the isolates. However, collection year was another strong factor to identify FGSC isolates comprehensively. Chemotype and diseases could be variable within FGSC members. The head blight disease is the most frequently observed disease for both *F. asiaticum* and *F. graminearum sensu stricto* While these two species were detected in Asia, but *F. graminearum sensu stricto* has been reported as global pathogen worldwide. Both species could produce 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and nivalenol while some other members can't produce these three myxotoxins (Przemieniecki et al., 2014).

Fifteen members for *F. graminearum* species complex have been present, recently (Przemieniecki et al., 2014). In this study, twenty isolates were clearly identified as *F. graminearum sensu stricto* by *Eco*RV digestion and multiloci genotyping analysis. Remaining isolates were identified as *F. asiaticum* or *Fusarium* sp. This study is important in terms of presenting the detailed differentiation of Turkish and Iranian *F. graminearum* isolates as FGSC members. FGSC identification studies included confusing, hesitant and inexact information up to now (Yörük and Yli-Mattila, 2015). Thus, to support the data obtained from SCAR markers, multiloci genotyping and genealogical concordance analysis by next generation sequencing assays (including genome wide haplotyping) would be useful to obtain more detailed knowledge in further studies. This study presents knowledge about the presence of FGSC members in Turkey and Iran. The studies including the increased number of Turkish and Iranian *F. graminearum* isolates would provide detailed and comprehensive data for FGSC characterization in Turkey and Iran.

Acknowledgements

This work was supported by the Board Regents of Istanbul Yeni Yuzyil University under Grant number 6047.

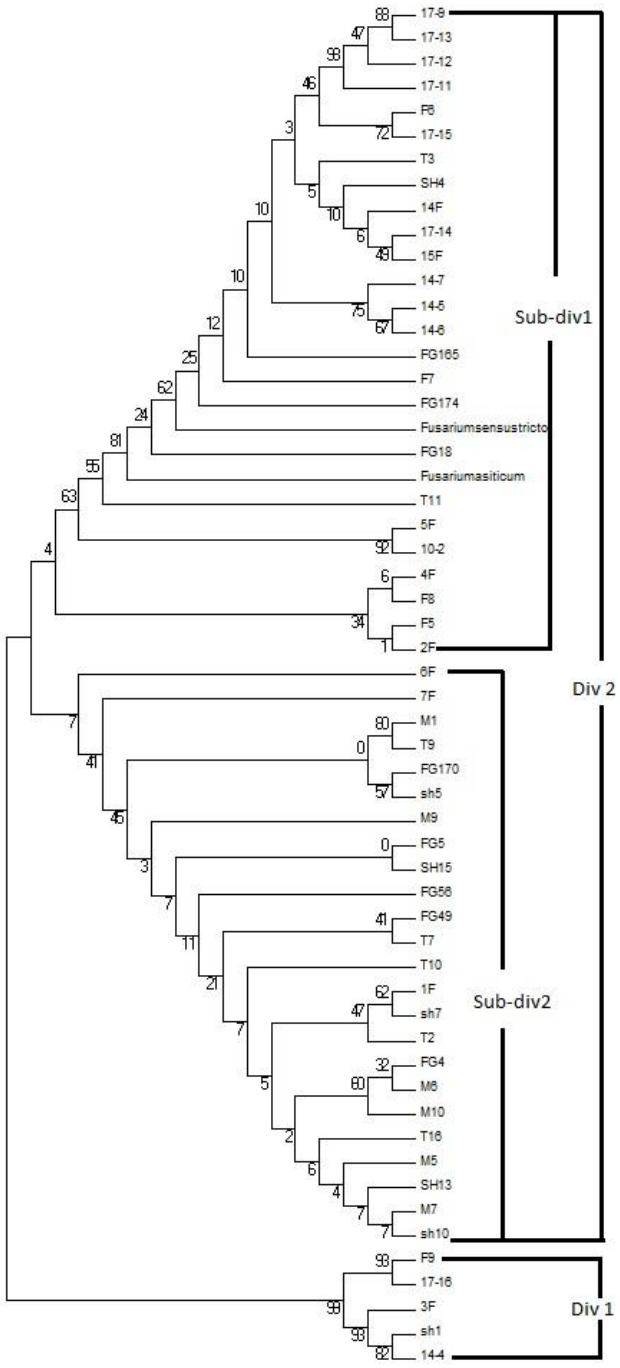

Figure 3 Filogenetic tree of *F. graminearum* isolates was obtained by maximum likelihood analysis

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