

**Molecular Identification, Virulens and Genetic Diversity  
of *Fusarium* species on Wheat**

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**Abstract**

Surveys were conducted in Konya, Ankara, Eskisehir, Kayseri, Nevsehir, Aksaray, Yozgat, Kirsehir and Kirikkale province in 2011 and 2012 growing seasons and 1256 diseased wheat samples were collected from 2770 wheat fields. As a result of isolation from this samples 668 *Fusarium* isolates were obtained belonging to 18 different species. In consequence of DNA sequence analysis, obtained isolates were determined *Fusarium acuminatum*, *F. avenaceum*, *F. brachygibbosum*, *F. cerealis*, *F. chlamyosporum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. incarnatum*, *F. lacertarum*, *F. longipes*, *F. nivale* (*Microdochium nivale*), *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. solani*, *F. sporotrichioides*, *F. tricinctum*. Pathogenicity tests performed and it was determined that the most virulent group were respectively, 86,72-96%, 84,67-94% ve 80,67-94,67% with diseases severity *F. culmorum*, *F. tricinctum* and *F. nivale*. The most commonly isolated species was *F. nivale*. Using phylogenetic analyses based on the internal transcribed spacer sequence data, it was examined by genetic variations between species of *Fusarium*.

**Keywords:** *Fusarium* spp., *Triticum aestivum*, molecular, pathogenicity

**INTRODUCTION**

Wheat is a major agricultural crop and the main cereal consumed by humans in Turkey. Total wheat production is forecast at 18 million tonnes in 2016 in Turkey. Central Anatolia Region is the most wheat producing region with 2.9 million hectares of wheat cultivation area. *Fusarium* is one of economically important and destructive fungal diseases of wheat in Central Anatolia ( Tunali *et. al*, 2008). *Fusarium* spp. cause root and crown rot and head blight on wheat.

The most important reported *Fusarium* species are *F. graminearum* and *F. culmorum* while several others such as *F. acuminatum*, *F. avenaceum*, *F. nivale* and *F. crookwellense* have also been reported (Burgess *et al.* 2001, Fernandez & Chen 2005, Smiley & Patterson, 1996). *F. graminearum* predominates in North America and in Southern Europe, while *F. culmorum* and *M. nivale* predominate in cooler climates in Europe (Parry *et al.* 1995). The causal agents of *Fusarium* head blight are primarily *F. graminearum*, *F. culmorum* and *F. avenaceum* (Lemmens *et al.*, 2004; Stepien *et al.*, 2008) The *F. graminearum* species complex, which consists of at least 11 phylogenetically distinct species, is the predominant species causing *Fusarium* head blight worldwide (O'Donnell *et al.*, 2000). Apart from reducing the yield, head blight damages grain quality by contamination from toxic

secondary metabolites (mycotoxins), which cause a health risk to both humans and animals. The toxins produced by *Fusarium* spp. are Deoxynivalenol (DON); Diacetoxyscirpenol (DAS), Monoacetyl-deoxynivalenol; Nivalenol (NIV); Zearalenone (ZEN) and Fusarenone-X (Bottalico and Perrone, 2002). More frequently occurring species are *F. culmorum*, *F. pseudograminearum*, *F. acuminatum*, *F. solani*, *F. avenaceum* and *F. verticilloides* (Yıldırım *et al.*, 1999 ;Tunali *et al.*, 2008) in Central Anatolia Region. In these studies conducted especially Konya, Karaman, Niğde, Aksaray Ankara Provinces in Central Anatolia Region. In this study some province e.g. Kayseri, Kırşehir, Nevşehir and Yozgat were firstly investigated in terms of *Fusarium* species. Traditional diagnostic methods for detection and identification of *Fusarium* spp. in culture are based on micro and macro morphological features. This process is time consuming, requires training, and it can often be difficult to distinguish between similar species. Molecular methods, more sensitive and faster are also employed to the specific identification of *Fusarium* species. The majority of the diagnostic assays are random amplified polymorphic DNA (RAPD) analysis (Voight *et al.*, 1995), specific diagnostic PCR primers (Nicholson *et al.* 1998), or DNA sequencing (Appel & Gordon 1996, O'Donnell *et al.* 1998). Several studies have demonstrated that ITS primers are useful targets for identification of some species complexes of *Fusarium* (Tan & Niessen, 2003, Mishra *et al.* 2003, O'Donnell *et al.* 2008).

In the present investigation morphological and molecular techniques were used to identify some *Fusarium* species isolated from wheat fields in Central Anatolia Region, as well as evaluating the genetic diversity of the identified species using the CLC Main Workbench 7.7.3 program.

## **MATERIALS and METHOD**

### **Sample Collection**

Surveys were conducted in Konya, Ankara, Eskisehir, Kayseri, Nevsehir, Aksaray, Yozgat, Kirsehir and Kirikkale province in 2011 and 2012 growing seasons in Central Anatolia Region, Turkey (Figure 1). 1256 diseased wheat samples were collected from 2770 wheat fields.



**Figure 1.** Location of survey area in Central Anatolia Region

## **Isolation of *Fusarium* species**

Segments of root and crown rot were surface sterilized for 1 min. in 1% sodium hypochlorite (NaOCl) solution, then washed thoroughly with sterile water and air dried in a laminar flow hood prior to place on potato dextrose agar (PDA, Merck, Germany) containing 50 mg/l streptomycin sulfate. Ten segments were placed on each plate and five petri dishes were used for each sample. Then dishes were incubated under a combination of long-wave ultraviolet and fluorescent light (12 h light: 12 h dark) for 7 days. Temperature was kept  $23 \pm 2$  °C under the light and dark conditions, respectively. After incubation, the cultures were transferred onto Synthetic Nutrient Agar (SNA). for species identification.

## **Pathogenicity Tests**

Pathogenicity tests were performed with agar plate assay. Isolates were incubated on PDA at  $23 \pm 2$  °C for 2 days, mycelial discs (4mm) from an actively growing edge of the fungal culture were transferred to 2% WA and incubated at the same conditions for 2 days. Seeds of the susceptible wheat cultivar (cv. Sultan 95) were disinfected by dipping in 1% NaOCl for 5 min, rinsed with sterile distilled water and aseptically blotted then six seeds were placed onto adjacent to the growing edge of the isolates in each Petri plate. Five plates were used for each isolate. After incubation for 7–8 days at  $23 \pm 2$  °C under photoperiod of 12 h, disease assessment was rated on a scale of 0-5, based on the relative size of a necrotic area on the hypocotyl as follows :0=no disease, 1= 1-10%, 2= 11-30%, 3=31-50%, 4=51-80 % and 5=the entire hypocotyl infected (Ichielevich-Auster *et al.*, 1985). These scale values were converted to disease severity values using the following formula (Karman 1971).

**Disease Severity**=  $[\sum (\text{no. of plant in category} \times \text{category value})] \times 100 / \text{Total no. of plants} \times \text{max. category value}$ .

## **Morphological Identification**

For morphological identification, single spore isolates were grown for 10–15 days on PDA medium. Culture characteristics of each isolate were determined from 10 to 15 day old SNA cultures. Microscopic features of conidia, conidiophores and chlamydospores were also determined based on Leslie & Summerell (2006).

## **Molecular Identification**

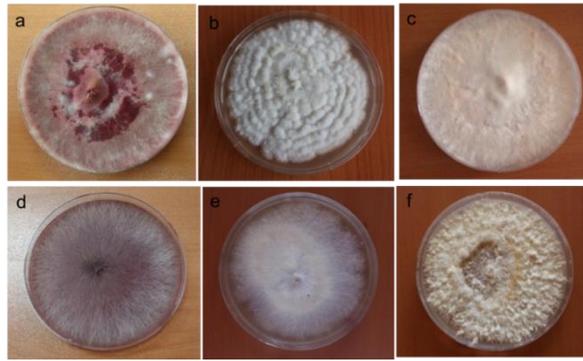
Approximately, 300 mg mycelium were harvested and ground with liquid nitrogen in a sterile mortar for DNA extraction from culture medium. Genomic DNA was extracted using a Qiagen DNeasy ® Plant Mini Kit, as specified by the manufacturer, and stored at -20 °C prior to use. PCR reaction mixtures and condition were modified from previous studies (Aroca & Raposo 2007; Cobos & Martin, 2008). The ITS regions of the isolates were amplified using the universal primers ITS-1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS-4 (5' TCC TCC GCT TAT TGA TATGC 3') as described by White *et al.* (1990). The reaction mixtures of PCR, a final volume of 50 µl, contained 5µl of 10X buffer [75 mM Tris HCl, pH 9.0, 50 mM KCl, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], 2 µl of 5 µM each primers, 5 µl of 1.5mM MgCl<sub>2</sub>, 2 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 U Taq polymerase (Fermentas), 5 µl of DNA template for each reaction and 5 µl of bovine serum albumin (BSA: 10 mg/ml). DNA amplifications were carried out in a Techne TC-5000 thermal cycler by the following program: 94 C for 2 min, followed by 34 cycles of (1) denaturation (94°C for 30 s), (2) annealing (60°C for 30 s) and (3) extension (72 °C for 30 s), and a final extension step 10 min at 72°C. The PCR products were separated in 1.5 % agarose gels stained with ethidium bromide, and visualized

under UV light. They were sequenced by GENOKS (Gene Research and Biotechnology Company, Ankara, Turkey). The nucleotide sequences were subjected to Basic Alignment Search Tool (BLAST) analysis (<http://www.ncbi.nlm.nih.gov>) and compared to other sequences in GenBank. The phylogenetic neighbour joining trees were created using the CLC Main Workbench 7.7.3 Programme.

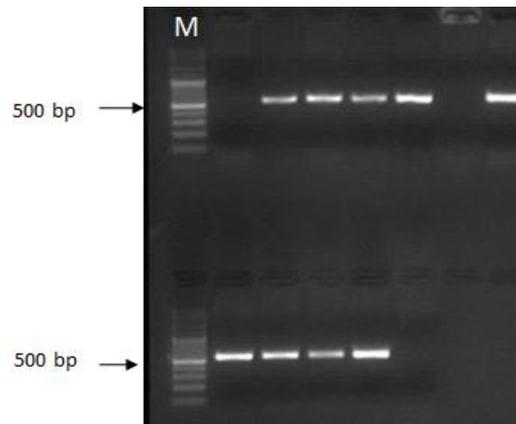
### **RESULTS and DISCUSSION**

It was detected that 668 *Fusarium* isolates recovered from 1256 diseased wheat samples collected from Konya, Ankara, Eskisehir, Kayseri, Nevsehir, Aksaray, Yozgat, Kirsehir and Kirikkale province (Table 1). In consequence of morphologic identification and DNA sequence analysis, isolates obtained from infected plants were determined as *F. avenaceum*, *F. brachygibbosum*, *F. cerealis*, *F. chlamydosporum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. incarnatum*, *F. lacertarum*, *F. longipes*, *F. nivale* (*Microdochium nivale*), *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. solani*, *F. sporotrichioides*, *F. tricinctum* (Figure 2). The total size of the ITS1 and ITS4 regions, including the 5.8S rDNA gene of the isolates studied varied from 550 to 600 bp (Figure 3).

*Fusarium* sequences obtained from amplification of conserved ribosomal ITS region were compared with sequences from National Center for Biotechnology Information (NCBI) database using BLAST 2.0 (<http://www.ncbi.nlm.nih.gov/> BLAST). Identified species showed 98-100% similarity with the isolates belong same species in NCBI. The most commonly isolated species was *F. nivale*. As with this report, Demirci & Dane detected *F. acuminatum*, *F. oxysporum*, *F. equiseti* and *F. nivale* in *Anatolia Region* in their study. In contrast, Tunali *et al.* (2008) reported that *F. culmorum* and *F. pseudograminearum* were the most common species in *Central Anatolia Region*. At the same time they were detected that more than 20 species of *Fusarium* e.g. *F. acuminatum*, *F. avenaceum*, *F. chlamydosporum*, *F. compactum*, *F. equiseti*, *F. lateritium*, *F. heterosporum*, *F. oxysporum*, *F. poae*, *F. proliferatum*, *F. semitectum*, *F. solani*, *F. sporotrichioides*, *F. tricinctum* and *F. verticillioides* were isolated from roots and crowns of wheat in their study. Bentley *et al.* (2006) isolated 16 *Fusarium* species from wheat stem bases across 25 sites in parts of the Aegean, Marmara, and Black Sea regions, with *F. oxysporum*, *F. equiseti*, *F. acuminatum*, *F. sambucinum*, *F. culmorum*, and *F. armeniacum* being the most frequent. *F. solani*, *F. pseudograminearum* and other species were found at low frequencies.



**Figure 2.** *Fusarium* spp. on PDA, (a) *F. graminearum*, (b) *F. chlamydosporum*, (c) *F. nivale*,  
(d) *F. culmorum*, (e) *F. tricinctum*, (f) *F. equiseti*

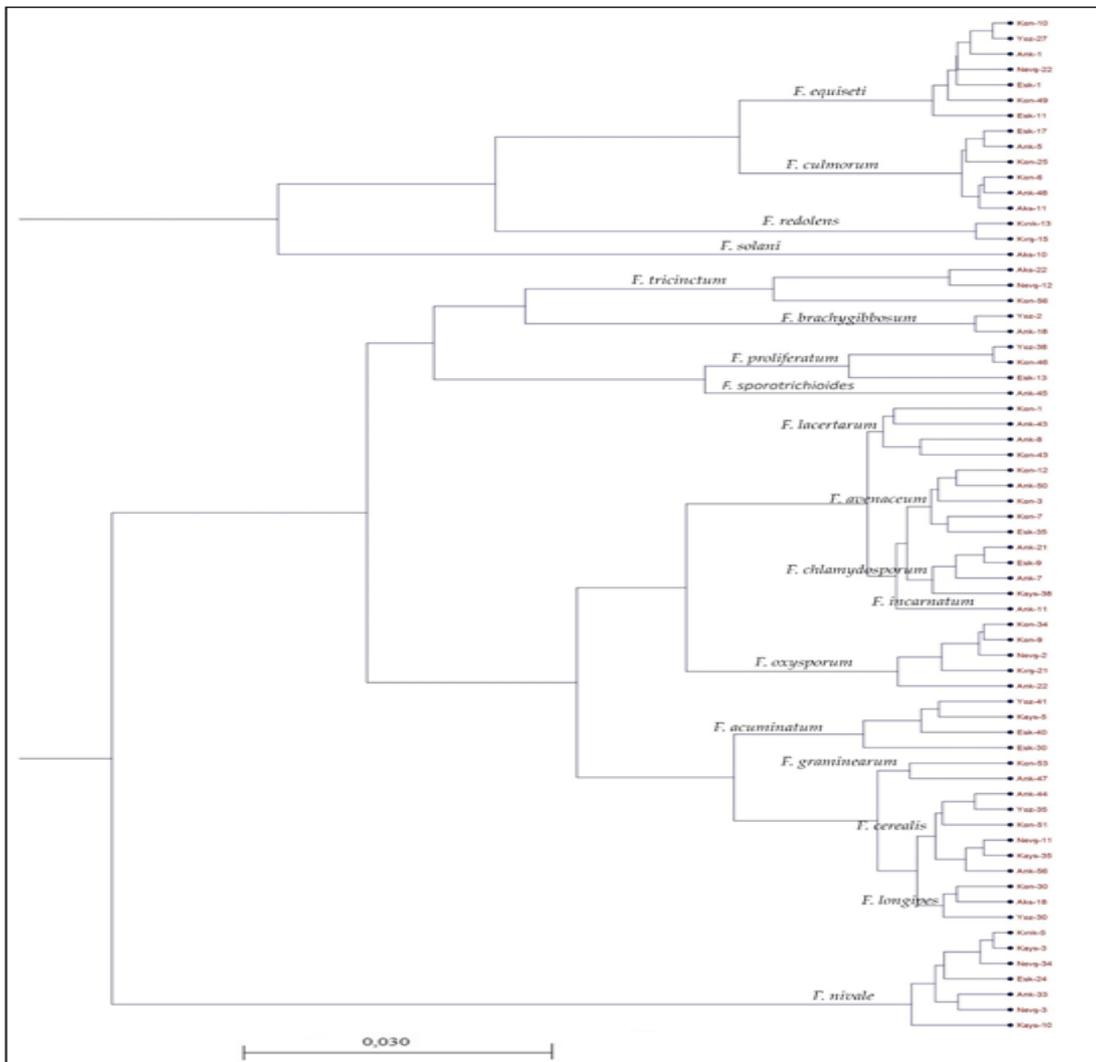


**Figure 3.** Amplification of conserved ribosomal regions of *Fusarium* spp. using the primers ITS-1 and ITS-4 (M: Markör Gene Ruler 100 bp DNA ladder MBI Fermentase)

**Table 1.** Diseases severity, origin, and number of *Fusarium* species isolated from wheat fields in Central Anatolia Region

<i>Fusarium</i> Species	Origin	Number of Isolate	Disease severity (%)
<i>F. acuminatum</i>	Konya, Ankara, Eskişehir, Yozgat, Kayseri, Kırıkkale, Kırşehir,	26	23-80
<i>F. avenaceum</i>	Konya, Ankara, Eskişehir, Yozgat, Kayseri, Aksaray, Nevşehir	27	61-78
<i>F. brachygibbosum</i>	Ankara, Eskişehir, Yozgat,	17	48-72
<i>F. cerealis</i>	Konya, Ankara, Yozgat, Kayseri, Nevşehir	56	57-81
<i>F. chlamydosporum</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray,	25	68-88
<i>F. culmorum</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray, Yozgat, Kırşehir, Kırıkkale	52	86-96
<i>F. equiseti</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray, Yozgat, Kırşehir, Kırıkkale	67	61-73
<i>F. graminearum</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray, Yozgat,	21	83-90
<i>F. incarnatum</i>	Konya, Ankara, Yozgat, Kırşehir	11	
<i>F. lacertarum</i>	Konya, Ankara, Nevşehir, Aksaray, Yozgat,	28	8-46
<i>F. longipes</i>	Konya, Ankara, Yozgat, Aksaray	22	23-72
<i>F. nivale</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray, Yozgat, Kırşehir, Kırıkkale	166	80-94
<i>F. oxysporum</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray, Yozgat, Kırşehir, Kırıkkale	54	1-48
<i>F. proliferatum</i>	Konya, Ankara, Eskişehir, Kayseri, Nevşehir, Yozgat	24	58-80
<i>F. redolens</i>	Kırşehir, Kırıkkale	21	65-72
<i>F. solani</i>	Konya, Ankara, Aksaray	9	14-40
<i>F. sporotrichioides</i>	Ankara, Eskişehir, Kayseri, Nevşehir, Aksaray	13	32-58
<i>F. tricinctum</i>	Konya, Ankara, Eskişehir, Nevşehir, Aksaray, Yozgat, Kırşehir, Kırıkkale	29	84-94

In consequence of the pathogenicity, it was determined that the most virulent group were respectively, 86,72-96%, 84,67-94% ve 80,67-94,67% with diseases severity *F. culmorum*, *F. tricinctum* and *F. nivale* but Aktaş *et al.* revealed that *F. verticillioides* was one of the most virulent pathogens on crowns of wheat and *F. culmorum* also was observed highly pathogenic reactions on winter wheat variety Katea A-1 under greenhouse conditions. Similarly our study, Tunali *et al.* (2008) has clearly demonstrated under greenhouse conditions that a range of *F. culmorum*, *F. pseudograminearum* and *F. graminearum* isolates are all highly pathogenic on crowns of winter, whereas other species such as *F. subglutinans*, *F. oxysporum*, *F. acuminatum*, *F. solani*, and *F. verticillioides* were weak pathogens. As with our study, Demirci & Dane (2003) examined the cause of crown and root rots on winter wheat Kirik under greenhouse conditions and found that *F. nivale* was the most virulent pathogen, while *F. acuminatum*, *F. equiseti*, *F. oxysporum*, and *F. solani* were slightly virulent in their study.



**Figure 4.** UPGMA cluster analysis obtained by sequence analysis of *Fusarium* isolates ITS region

Sixty six *Fusarium* isolates obtained from diseased wheats and selected by isolate numbers were subjected to UPMG cluster analysis in the CLC Main Workbench 7.7.3 program to assess genetic differences. Phylogenetic analysis grouped the *Fusarium* isolates into eighteen clusters (Fig. 4). UPGMA cluster analysis of rDNA sequence data separated the isolates of each particular species into unique groups based on high genetic similarity (Figure 4).

### CONCLUSIONS

*Fusarium acuminatum*, *F. avenaceum*, *F. brachygibbosum*, *F. cerealis*, *F. chlamydosporum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. incarnatum*, *F. lacertarum*, *F. longipes*, *F. nivale* (*Microdochium nivale*), *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. solani*, *F. sporotrichioides*, *F. tricinctum* can cause root rot and head blight in Central Anatolia Region. The most commonly isolated species was *F. nivale*. The most virulent isolate is *F. culmorum*. The results obtained may contribute in developing an integrated control program for *Fusarium* diseases.

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