Distribution of the 3-AcDON, 15-AcDON, and NIV Chemotypes of *Fusarium culmorum* in the North-West of Turkey

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Abstract

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Fusarium culmorum isolates originated from diseased wheat plants showing crown rot and head blight symptoms in the 2009–2010 wheat growing season in the Çanakkale, Balıkesir, and Tekirdağ Provinces in the North-West of Turkey. Fifty-six isolates were identified as *F. culmorum.* The chemotypes of *F. culmorum* known to produce the mycotoxins deoxynivalenol (DON) and its derivatives (3-AcDON and 15-AcDON) and nivalenol (NIV) were identified by PCR-based methods. Out of the 56 *F. culmorum* isolates tested with *Tri13* PCR assays, one isolate yielded an amplicon similar to the size predicted for NIV production, while 55 yielded an amplicon corresponding to the size marker for DON production. Chemotyping assays by PCR showed that the DON chemotype of *F. culmorum* was dominant among the population in all three provinces. Out of the 55 DON isolates, 16 and 39 isolates were 3-AcDON and 15-AcDON, respectively. Isolates collected from the same localities were not exclusively of a single chemotype. This is the first report demonstrating the presence and the geographic distribution of all three chemotypes on wheat spikes and crowns in Turkey.

Keywords: crown rot; head blight; trichothecenes; genetic chemotyping; mycotoxin; nivalenol; deoxynivalenol

Fusarium culmorum is one of the most dominant pathogens causing crown rot (CR) and Fusarium head blight (FHB) in cereal crops. A complex of *Fusarium* species causes Fusarium crown rot by infecting roots and crowns of winter and spring wheat. Yields can be reduced up to 30%, depending on environmental conditions and field management practices. CR is caused by many species within the genus *Fusarium*, including *F. culmorum*, *F. pseudograminearum, F. crookwellense*, and *F. graminearum* (Liddell 1985; Akinsanmi *et al.* 2004). CR is a widespread disease of wheat in Australia, South Africa, Argentina, Europe, and the USA (Burgess *et al.* 2001). *F. culmorum* is one of the dominant *Fusarium* species isolated from the crown of infected wheat plants in North-West of Turkey (Aktaş *et al*. 1999; Tunali *et al*. 2006).

FHB occurs in fields especially during suitable rainy weather that promotes the build up of enough inoculum at anthesis time. FHB can be an important problem in wheat which leads to reduced yield and can also reduce the seed quality and seed germination. FHB is of particular concern because fungal toxins (mycotoxins) that are harmful to living organisms are often produced in infected kernels (Joffe 1986). Trichothecene mycotoxins are potent inhibitors of eukaryotic protein biosynthesis making the infected wheat seed unsuitable for consumption (O'Donnell *et al*. 2000).

Based on type B trichothecene production, there are three chemotypes in *F. culmorum*. The nivalenol (NIV) chemotype produces nivalenol and acetylated derivatives. The 3-acetyl-deoxynivalenol (3-AcDON) chemotype produces deoxynivalenol

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(DON) and 3-AcDON. The 15-acetyl-deoxynivalenol (15-AcDON) chemotype produces DON and 15-AcDON (Miller *et al*. 1991). Production of DON and NIV by *F. culmorum* is known for strains from many European countries (Gang *et al*. 1998; Muthomi *et al*. 2000; Bakan *et al.* 2001; Bottalico & Perrone 2002; Waalwijk *et al.* 2003; Stepień *et al.* 2008; Baturo-Ciesniewska & Suchorzynska 2011) and the USA (Mirocha *et al.* 1994) whereas only the DON chemotype was detected in western Canada (Abramson *et al.* 2001). The *Tri13* gene was found to be the determinant for the DON-NIV switching in *Fusarium* (Lee *et al.* 2002). NIV-producers carry functional *Tri13* gene together with *Tri7* while non-functional copies of the genes are present in DON-producers.

Although type B trichotecenes differ only in means of the pattern of hydroxylation or acetylation, these changes can affect toxicity greatly (Kimura *et al.* 1998). Therefore it is important to know which chemotype(s) is dominant in the population and then risk can be evaluated for the contaminated food or feed. Chemical methods such as gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) are commonly applied along with fast and simple screening methods like ELISAs (Langseth & Rundberget 1998). However these methods are labour-intensive and require sophisticated instrumentation and skilled operators. Molecular characterisation of trichothecene mycotoxin biosynthesis pathways via PCR is a useful method with advantages over the conventional chemical methods (GC/MS and HPLC) of speed and accuracy (Chandler *et al.* 2003; Jennings *et al.* 2004). Our specific objective in this study was to determine the chemotypes of the *F. culmorum* isolates collected from three provinces in the North-West of Turkey surveyed for crown rot and Fusarium head blight during the 2009–2010 wheat growing season. Although there is no evidence that *Fusarium* infects plant tissues systemically, the isolates collected from the diseased crown have great potential for head infection (Tunali *et al.* 2006).

MATERIAL AND METHODS

Collection of the field samples of the** Fusarium culmorum **isolates. Wheat crops in the North-West of Turkey were surveyed for CR from April and FHB from May until the beginning of June in the 2009–2010 wheat growing season. Three provinces, Çanakkale, Tekirdağ, and Balıkesir, were surveyed covering a 4500 ha area.

To isolate *F. culmorum* from infected host tissue, crown and grain samples and other plant parts were surface sterilised with 1% sodium hypochlorite solution and rinsed three times with sterile distilled water. After drying in a fume hood to eliminate excess moisture, they were placed in potato dextrose agar (PDA) medium containing 100 µg/ml streptomycin sulphate and incubated at 25°C for 5–7 days. Monoconidial isolates were obtained by streaking spore suspensions onto water agar plates and single conidia were transferred onto new plates.

Identification of the** Fusarium culmorum **isolates. The isolates were examined after 7 days and selected based on pigmentation on PDA. The selected isolates were grown on carnation leaf agar (CLA) to observe the shape and size of macroconidia, nature of conidiogeneous cells, septations, absence of microconidia and chlamidospores (Leslie & Summerell 2006).

Fungal DNA extraction and PCR. The DNA extraction technique was modified from Saitoh *et al*. (2006) adding some further cleaning steps. A mycelial mass was picked with a pipette tip and put in a 2 ml Eppendorf tube. The mycelial mass was then homogenised with a micro pestle for a few seconds. One ml of lysis buffer (200mM Tris-HCl, 50mM ethylenediaminetetra acetic acid, 200mM NaCl, 1% *n*-lauroylsarcosine sodium salt, pH 8.0) were added immediately to the mycelia. The mycelial mass was dispersed in the buffer by vortexing for 10 seconds. Two µl of RNAse A and 4 µl of proteinase K (both Fermentas, Vilnius, Lithuania) was added and incubated for about 15 min at 37°C. The mixtures were centrifuged at 13 000 rpm for 5 minutes. The supernatant was removed to a clean tube containing 1 ml of chloroform. The tubes were inverted briefly before centrifugation for further 10 minutes. The supernatant (about 800 µl) was removed into a new tube containing 80 µl sodium acetate (5M) and 550 µl ice cold isopropanol, then inverted gently. The mixture was centrifuged at 13 000 rpm for 10 min following incubation on ice for several minutes. The pellet was washed twice with 70% ice cold ethanol. After drying at room temperature for 30 min, the DNA was dissolved in 100 µl of TE buffer.

PCR amplification was carried out in a 50 µl of reaction mix containing 25 µl of 2X PCR Master

Mix (0.05 units/µl TaqDNA polymerase in reaction buffer, 0.4mM of each dNTPs; Fermentas, Vilnius, Lithuania), 8 µl (25–50 ng) fungal DNA, 2.5 µl (300nM) each of primer, and 12 µl nuclease free water. A thermal cycler (Bio-RAD, Hercules, USA) was used for amplification of the specific fragment of DNA.

PCR conditions used for *F. culmorum* detection were: 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 40 s followed by a final extension of 72°C for 5 minutes. The same conditions were applied to Tri13DON and Tri13NIV with an exception that denaturation temperature was 94°C. The PCR conditions for the generic primer, Tri13P was 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 60°C for 40 s, 72°C for 40 s, then a final extension at 72°C for 6 minutes. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed under UV light in a Bio-Imaging System (Bio-RAD, Hercules, USA). All assays were repeated at least twice. The primers used to identify *F. culmorum* and its chemotypes are listed in Table 1.

RESULTS

Symptomatology of CR and FHB

F. culmorum was often isolated from the roots or stem bases of the diseased plants late in the season especially following flowering. Expansion of discolouration and browning into crown and stems were the most dominant symptoms of *F. culmorum* in wheat plants. The first symptoms of FHB caused by *F. culmorum* include a tan or brown discolouration at the base of a floret within the spikelets of the head. If the infection progressed, the diseased spikelets were light tan or bleached in colour. The infection was sometimes limited to one spikelet, but in some circumstances the fungus invaded the entire head. The base of the infected spikelets and portions of the rachis often develop a dark brown colour. Infected kernels were almost always shrivelled, white and sometimes pink and chalky in appearance.

Diagnosis of *F. culmorum*

Many fungal isolates belonging to other genera were isolated from the diseased tissues. Isolates showing typical Fusaria features were separated from the other by morphological characteristics. *Fusarium culmorum* produced initially yellow to orange pigmentation in PDA plates, however, the colonies turned to brownish red, brown, and dark brown colours. When a disk of fungal mass was transferred onto CLA, macroconidia formed abundantly in sporodochia. The macroconidia were short with 3–4 septations. They were formed from monophialides on branched conidiophores in sporodochia and to a minor extent from monophialides on the hyphae. These characteristics were similar to reports described by Leslie and Summerell (2006).

Following diagnosis of the isolates of *F. culmorum* based on morphological characteristics, PCR with species-specific primer sets (FC01) were used for molecular identification (Nicholson *et al.* 1998). All isolates described as *F. culmorum* morpho-

Table 1. Species- and chemotype-specific primers used in this study and the expected PCR product sizes

Primer	Sequence (5'-3')	Species/chemotypes	Product size (bp)
FC01F[1]	ATG GTG AAC TCG TCG TGG C	*F. culmorum*	570
FC01R	CCC TTC TTA CGC CAA TCT CG		
Tri13NIVF[2]	CCAAATCCGAAAACCGCAG	NIV	312
Tri13R	TTGAAAGCTCCAATGTCGTG		
Tri13F[2]	CATCATGAGACTTGTKCRAGTTTGGG	DON	282
Tri13DONR	GCTAGATCGATTGTTGCATTGAG		
Tri13P1[3]	CTC SAC CGC ATC GAA GAS TCT C	NIV	859
Tri13P2	GAA SGT CGC ARG ACC TTG TTT C	3-AcDON	644
		15-AcDON	589

[1]Nicholson *et al.* (1998); [2]Chandler *et al.* (2003); [3]Wang *et al.* (2008)

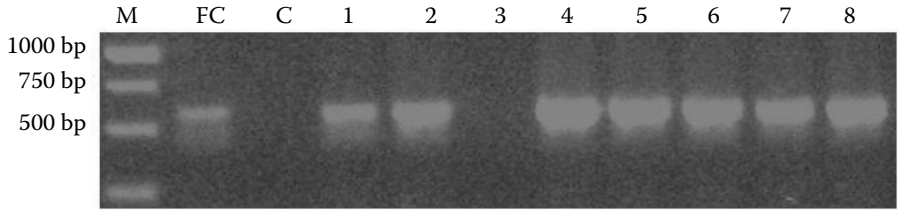

Figure 1. Amplicon of DNA from isolates of *Fusarium culmorum* associated with crown rot and head blight using the FC01 primer pair specific for *Fusarium culmorum*

M, FC, and C –1 kb ladder size marker (Fermentas, Vilnius, Lithuania); positive and negative controls of *F. culmorum* provided by P. Nicholson, Norwich, UK; lane 3 – missing sample, the remaining were *F. culmorum* isolates selected from the population

logically were positive in the PCR tests producing the expected 570 bp product (Figure 1). Among the 218 *Fusarium* spp. isolates collected during the survey, 56 were identified as *F. culmorum* by PCR. Thirty-seven were isolated from the crown or stem and 19 isolates were from infected heads.

Chemotypes of *F. culmorum*

Tri13NIV and Tri13DON primer sets were used to determine NIV and DON chemotypes of each *F. culmorum* isolate. Both assays indicated that DON and NIV chemotypes were present in the surveyed area although only one isolate was a NIV-producer. Products of 282 and 312 bp were obtained from DON and NIV producer isolates, in PCR assays using respectively the Tri13DON and Tri13NIV primers.

The Tri13P primer set detected a chemotype-specific DNA fragment with different sizes from 3-AcDON, 15-AcDON, and NIV-producers of *F. culmorum*. In single PCR reactions, 859, 644, and 589 bp products were obtained from NIV, 3-AcDON, and 15-AcDON producer isolates, respectively (Figure 2).

The proportion of NIV producers was quite small among the population. Tri13NIV assay resulted positive only with one isolate. In the Tri13P assay, only the same isolate gave a positive result. Therefore using two different primer sets confirmed that there was only one NIV producer isolate in the entire population.

Distribution of the chemotypes in surveyed area

Analysis of field location and *F. culmorum* chemotype indicated differences in the distribution of the two chemotypes (Table 2 and Figure 3). Nineteen isolates were obtained from the Çanakkale Province, including 9 isolates from crowns and 10 frominfected heads. PCR assays indicated that all these isolates were DON-producers. Among those, 4 isolates from infected crowns and 6 isolates from infected heads were the 3-AcDON chemotype, whereas 5 isolates from infected crown and 4 isolates from infected heads were the 15-AcDON chemotype.

Nineteen isolates in total were obtained from the Balıkesir Province; two were from infected

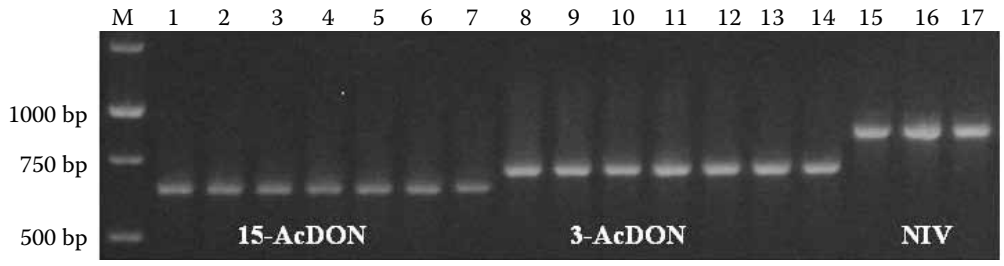

Figure 2. Amplification products using the primer set Tri13P. Three different sized fragments (583, 644, and 859 bp) were produced for 15-AcDON, 3-AcDON, and NIV producer isolates, respectively

M – 1 kb ladder; lanes 1–7 – 15-AcDON isolates; lanes 8–14 – 3-AcDON isolates; lanes 15–17 -– NIV isolates; positive controls of *F. culmorum* provided by P. Nicholson, Norwich, UK

Table 2. Origin of 56 *Fusarium culmorum* isolates from three provinces of Turkey obtained from infected wheat tissues and their chemotypes determined by PCR assays

Province	Isolates	Area	Tissue isolated		Tri13[c]	Tri13[c]	Tri13P		
			CR[a]	FHB[b]	NIV	DON	3-AcD	15-AcD	NIV
Çanakkale	CN-401	Merkez	+	–	–	+	+	–	–
	CN-807a	Lapseki	+	–	–	+	–	+	–
	CN-807b	Biga	+	–	–	+	–	+	–
	CN-502b	Biga	+	–	–	+	+	–	–
	CN-813	Biga	–	+	–	+	+	–	–
	CN-901	Eceabat	–	+	–	+	+	–	–
	CN-201a	Ezine	+	–	–	+	+	–	–
	CN-209a	Ezine	+	–	–	+	–	+	–
	CN-512	Ezine	+	–	–	+	–	+	–
	CN-606a	Ezine	+	–	–	+	–	+	–
	CN-103a	Ezine	+	–	–	+	+	–	–
	CN-103b	Eceabat	–	+	–	+	+	–	–
	CN-107a	Gelikolu	–	+	–	+	+	–	–
	CN-119	Gelikolu	–	+	–	+	+	–	–
	CN-209b	Gelikolu	–	+	–	+	+	–	–
	CN-211	Biga	–	+	–	+	–	+	–
	CN-213a	Biga	–	+	–	+	–	+	–
	CN-215a	Ezine	–	+	–	+	–	+	–
	CN-216	Ezine	–	+	–	+	–	+	–
	Total		9	10	0	19	10	9	0
Balıkesir	BL-302a	Burhaniye	+	–	–	+	–	+	–
	BL-313	Burhaniye	+	–	–	+	–	+	–
	BL-314	Bandurma	–	+	–	+	–	+	–
	BL-410a	Susureluk	+	–	–	+	–	+	–
	BL-411a	Ayvalik	+	–	–	+	–	+	–
	BL-412	Ayvalik	+	–	–	+	–	+	–
	BL-413b	Ayvalik	+	–	–	+	–	+	–
	BL-110	Ayvalik	+	–	–	+	–	+	–
	BL-204	Ayvalik	+	–	–	+	–	+	–
	BL-205	Edremit	+	–	–	+	–	+	–
	BL-304	Edremit	+	–	–	+	–	+	–
	BL-201a	Edremit	+	–	–	+	–	+	–
	BL-201b	Gönen	+	–	–	+	–	+	–
	BL-401	Gönen	+	–	–	+	–	+	–
	BL-603	Gömeç	+	–	–	+	–	+	–
	BL-704	Gömeç	–	+	–	+	–	+	–
	BL-705	Susurluk	+	–	–	+	–	+	–
	BL-905	Susurluk	+	–	–	+	–	+	–
	BL-102	Burhaniye	+	+	+	–	–	–	+
	Total		17	2	1	18	0	18	1
Tekirdağ	TK-107	Şarköy	+	–	–	+	–	+	–
	TK-512	Şarköy	+	–	–	+	–	+	–
	TK-701	Şarköy	+	–	–	+	+	–	–
	TK-200	Şarköy	+	–	–	+	–	+	–
	TK-440	Malkara	+	–	–	+	–	+	–
	TK-450	Malkara	+	–	–	+	+	–	–
	TK-830	Malkara	+	–	–	+	–	+	–
	TK-940	Malkara	+	–	–	+	–	+	–
	TK-174	Yeniköy	–	+	–	+	–	+	–
	TK-211	Yeniköy	–	+	–	+	–	+	–
	TK-226	Yeniköy	–	+	–	+	–	+	–
	TK-241	Yeniköy	–	+	–	+	–	+	–
	TK-731	Çorlu	+	–	–	+	+	–	–
	TK-732	Çorlu	+	–	–	+	+	–	–
	TK-130	Keşan	+	–	–	+	–	+	–
	TK-134	Keşan	–	+	–	+	–	+	–
	TK-550	M. Ereğli	–	+	–	+	+	–	–
	TK-760	M. Ereğli	–	+	–	+	+	–	–
	Total		11	7	–	18	6	12	–
General total			37	19	1	55	16	39	1

[a]infected wheat crown; [b]Fusarium head blight; [c]primer sets for chemotype identification as described in Table 1

heads and the remaining isolates were obtained from infected crowns. Among this population, 18 isolates were DON producers, however, only one isolate was a NIV producer. All 18 isolates were 15-AcDON producers.

Eighteen isolates were obtained from Tekirdağ, including 11 isolates from the infected crowns and 7 from infected heads. All isolates were found to be DON producers. Among the isolates isolated from the infected crowns, 4 were 3-AcDON producers, and 7 were 15-AcDON producers. Among the isolates isolated from the infected heads, 2 were 3-AcDON and five were 15-AcDON producers.

DISCUSSION

Wheat fields in three provinces in the North-West of Turkey were surveyed for CR and FHB symptoms and *F. culmorum* isolates were obtained from samples collected. *Fusarium culmorum* was diagnosed morphologically and by a PCR-based method and then the ability of the isolates to produce trichothecenes was evaluated using chemotype-specific PCR markers (Table 1). Two chemotypes of *F. culmorum* were identified, the NIV and DON chemotypes, with dominant prevalence of the latter. Among 56 isolates of *F. culmorum*, 55 isolates were DON producers, and only a single isolate was found to be a NIV producer (Table 2 and Figure 3).

Production of DON and NIV by *F. culmorum* is known for strains from many European countries and the USA (Mirocha *et al.* 1994; Muthomi *et al.* 2000; Bakan *et al.* 2001; Waalwick *et al.* 2003). Quarta *et al.* (2005) reported that although most isolates were DON producers, a few strains isolated from areas scattered among different countries in Europe were NIV producers. Jennings *et al.* (2004) also identified both DON and NIV chemotypes of *F. culmorum*, with DON chemotypes predominating overall. We also found that both chemotypes exist in the surveyed area of Turkey and the DON producer isolates prevail over the NIV isolates.

The majority of DON-producers from the regions surveyed in Turkey displayed the 15-AcDON chemotype (39 out of 56), whereas 3-AcDON chemotype was identified only in 16 isolates. Nineteen isolates were obtained from the Balikesir fields. The NIV chemotype was identified only in one field in Burhaniye, Balıkesir province. The remaining isolates, including other two isolates collected from Burhaniye, were 15-AcDON chemotype. The distribution of DON chemotypes in Çanakkale province differed greatly; among 19 isolates, 10 isolates were 3-AcDON chemotype, and 9 were 15-AcDON chemotype. The 15-AcDON chemotype prevailed over the 3-AcDON in Tekirdağ province.

The ability of DON chemotypes to produce 3-AcDON and 15-AcDON was also reported in previous research. Quarta *et al.* (2005) and Stepień *et al.* (2008) reported the 3-AcDON chemotype to be the most frequent chemotype among the *F. culmorum* isolates from Poland. Jennings *et al.* (2004) reported that in the UK, all DON chemotype isolates were 3-AcDON producers. Similarly, 3-AcDON was the only trichothecene produced by *F. culmorum* collected in Germany (Muthomi *et al.* 2000) and Tunisia (Kammoun *et al.* 2010). Results from Mediterranean countries (Logrieco *et al.* 2003) also showed that only 3-AcDON was produced by *F. culmorum* isolates recovered from cereal grains. The prevalence of 3-AcDON chemo-

Figure 3. Geographical locations of the *Fusarium culmorum* chemotypes collected from diseased wheat plants

★NIV chemotype; ●3-AcDON; ▲15-AcDON

type in Tunisian population of *F. culmorum* could have an epidemiological implication because this chemotype is known to produce more DON, to be more aggressive on hosts, and to have higher fitness than the 15-AcDON chemotype (Guo *et al.* 2008).

A previous report from Turkey showed that all 21 isolates of *F. culmorum* collected from diverse regions were of the 3-ADON chemotype (Yörük & Albayrak 2012). However having surveyed a larger area and sampled more isolates in the present research, all three chemotypes were diagnosed in the present research. To our knowledge, this is the first report demonstrating the NIV and 15-AcDON chemotypes within the population of *F. culmorum* in Turkey. Fifteen isolates collected from diseased wheat crowns were inoculated in heads of two different wheat varieties; the data revealed that all isolates were virulent and showed different levels of aggressiveness (Gencer & Mert-Türk, unpublished results). Plants with CR in fields can provide inocula during the anthesis and these inocula can then cause FHB. Therefore characterising the chemotypes of the *F. culmorum* isolates causing CR may help to predict future FHB epidemics in critical weather conditions.

Results from the current study provide a view of the overall genetic diversity of the *F. culmorum* chemotypes on wheat spikes and crowns in the North-West of Turkey. Collection of isolates from all over Turkey may provide more information on trichothecene toxin chemotyping as an aid to food safety monitoring.

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