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Population genetic structure and mycotoxin potential of the wheat crown rot and head blight pathogen *Fusarium culmorum* in Algeria

Imane Laraba[a,*], Houda Boureghda[a], Nora Abdallah[a], Oussama Bouaicha[a], Friday Obanor[b], Antonio Moretti[c], David M. Geiser[d], Hye-Seon Kim[e], Susan P. McCormick[e], Robert H. Proctor[e], Amy C. Kelly[e], Todd J. Ward[e], Kerry O'Donnell[e]

[a] *Laboratoire de phytopathologie et de biologie moléculaire, Département de botanique, Ecole Nationale Supérieure Agronomique, Algiers, Algeria*
[b] *Grains Research and Development Corporation, Canberra, Australia*
[c] *Institute of Sciences of Food Production, CNR, Via Amèndola 122/0, Bari 70126, Italy*
[d] *Department of Plant Pathology and Environmental Microbiology, Pennsylvania State University, University Park, PA 16802, USA*
[e] *U.S. Department of Agriculture, Agricultural Research Service, 1815 North University Street, Peoria, IL 61604, USA*

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ABSTRACT

Surveys for crown rot (FCR) and head blight (FHB) of Algerian wheat conducted during 2014 and 2015 revealed that *Fusarium culmorum* strains producing 3-acetyl-deoxynivalenol (3ADON) or nivalenol (NIV) were the causal agents of these important diseases. Morphological identification of the isolates (n FCR = 110, n FHB = 30) was confirmed by sequencing a portion of *TEF1*. To assess mating type idiomorph, trichothecene chemotype potential and global population structure, the Algerian strains were compared with preliminary sample of *F. culmorum* from Italy (n = 27), Australia (n = 30) and the United States (n = 28). A PCR assay for *MAT* idiomorph revealed that *MAT1-1* and *MAT1-2* strains were segregating in nearly equal proportions, except within Algeria where two-thirds of the strains were *MAT1-2*. An allele-specific PCR assay indicated that the 3ADON trichothecene genotype was predominant globally (83.8% 3ADON) and in each of the four countries sampled. In vitro toxin analyses confirmed trichothecene genotype PCR data and demonstrated that most of the strains tested (77%) produced culmorin. Global population genetic structure of 191 strains was assessed using nine microsatellite markers (SSRs). AMOVA of the clone corrected data indicated that 89% of the variation was within populations. Bayesian analysis of the SSR data identified two globally distributed, sympatric populations within which both trichothecene chemotypes and mating types were represented.

1. Introduction

Fusarium crown rot (FCR; also known as foot and root rot) and Fusarium head blight (FHB) are two of the most economically destructive diseases that affect small grain cereal production worldwide (Goswami and Kistler, 2004). Both diseases are noted for significantly reducing yields and contaminating cereals with trichothecenes and zearalenone mycotoxins, which can render them unsuitable for food or feed (Scherm et al., 2013). Trichothecenes pose a serious threat to food safety and the health of humans and other animals due to diverse toxicological effects (Wu et al., 2014). In addition, trichothecenes are acutely phytotoxic and have been shown to function as virulence factors on sensitive host plants (Proctor et al., 1995; Jansen et al., 2005).

Pathogen surveys indicate that *Fusarium culmorum* (W.G. Smith) Saccardo is the primary etiological agent of FCR in several countries in the Mediterranean and adjacent regions. This species was reported as the main FCR pathogen in Tunisia (Kammoun et al., 2009; Rebib et al., 2014), Sardinia (Balmas et al., 2015), Iran (Pouzeshimiab et al., 2014), and preliminary surveys suggest that it might be an important FCR pathogen in Algeria (Yekkour et al., 2015; Touati-Hattab et al., 2016). Nested within the B clade of trichothecene toxin-producing fusaria (Sarver et al., 2011), *F. culmorum* strains are segregating for two of the three known B type trichothecene chemotypes: deoxynivalenol (DON) + 3-acetyl-deoxynivalenol (3ADON chemotype) and nivalenol (NIV chemotype) (Scherm et al., 2013). Differences in the toxicity and bioactivity of the trichothecene toxin types, which appear to have been maintained by balancing selection, are theorized to have significant fitness effects (Ward et al., 2002). A sexual reproductive mode in this important cosmopolitan cereal pathogen is unknown, given that a

* Corresponding author at: U.S. Department of Agriculture, Agricultural Research Service, 1815 North University Street, Peoria, IL 61604-3999, USA.
E-mail address: imane.laraba@ars.usda.gov (I. Laraba).

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teleomorph has not been observed. However, it is presumed to be self-sterile or heterothallic because PCR assays for mating type idiomorph have shown that strains possess a *MAT1-1* or *MAT1-2* idiomorph (Kerényi et al., 2004; Obanor et al., 2010), and because population genetic analyses of this small-grain pathogen have revealed high genetic diversity and low population subdivision consistent with a sexually recombining species (Gargouri et al., 2003; Miedaner et al., 2013; Pouzeshimiab et al., 2014; Rebib et al., 2014).

Due to the importance of *Fusarium culmorum* induced FCR and FHB, and associated mycotoxin contamination of small-grain cereals to world agriculture, numerous molecular markers have been used to assess its genetic diversity on regional to global scales (Scherm et al., 2013 and references therein). However, for population genetic analyses (Miedaner et al., 2013; Pouzeshimiab et al., 2014; Rebib et al., 2014), microsatellites or simple sequence repeats (SSR) are the genetic marker of choice because: (1) they contain higher allelic variation at each locus due to their high mutation rate, and (2) they can be easily PCR-amplified with fluorescently labelled primers and scored on an automated capillary genetic analyzer as discreet co-dominant, selectively neutral alleles. Recognizing the need for more polymorphic markers for population genetic analyses, Giraud et al. (2002), Suga et al. (2004) and Vogelgsang et al. (2009) developed a set of SSR markers that have been used in three separate studies of *F. culmorum* that found higher genetic diversity within than between populations and low population level subdivision (Miedaner et al., 2013; Pouzeshimiab et al., 2014; Rebib et al., 2014). Because *Fusarium culmorum* appears to represent the most important FCR and FHB pathogen in Algeria (Touati-Hattab et al., 2016), the present study was conducted to characterize Algerian *F. culmorum* diversity, toxin potential, and population structure, and compare them with small collections of *F. culmorum* from Australia, Italy, and the U.S.

2. Materials and methods

2.1. Taxon sampling

We conducted FCR and FHB pathogen surveys of 500 wheat fields in 20 semiarid wheat-growing provinces of northern Algeria (Fig. 1) during 2014 and 2015. Pure cultures of the isolates were obtained by disinfecting sections of the symptomatic crown or ear tissue in 2% sodium hypochlorite solution for 5 min, rinsing them in sterile distilled water, and then drying them on sterile filter paper in a biological hood. Strains were purified by growing them on 3% water agar (Bacto, Becton Dickinson and Co.; Sparks, MD) amended with 2 mL/L of a penicillin G, streptomycin and neomycin solution (5000 units penicillin, 5 mg streptomycin and 10 mg neomycin per mL; Sigma-Aldrich P4083; St. Louis, MO). Only 3 spikes were sampled from each field, and when multiple strains of the same species were isolated from a spike, the clones were discarded. Subcultures obtained from the colony margin were single-spored on potato dextrose agar (PDA; Becton Dickinson and Co.). Of the 269 fusaria isolated, 140 were identified as *Fusarium culmorum* using morphological criteria (Leslie and Summerell, 2006), and then stored in 15% glycerol at −80 °C. The remaining isolates included 11 different *Fusarium* species, which are not considered further in the present study. To obtain a preliminary assessment of the global population genetic structure of *F. culmorum*, the Algerian isolates were compared with small collections of *F. culmorum* from Australia (n = 30), Italy (n = 27), and the U.S. (n = 28) (Table S1). Unlike the collection from Algeria, those from the other countries do not represent population-level samples.

2.2. DNA extraction

To obtain mycelium for DNA extraction, the *Fusarium culmorum* strains were grown in 25 mL yeast-malt broth (Bacto, Becton Dickinson and Co.; 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% dextrose) in 50 mL disposable polypropylene tubes at 24 °C for 3–4 days on a rotary shaker at 200 rpm. Mycelium was harvested by vacuum filtration on a #1 Whatman filter paper (GE Healthcare; Buckinghamshire, England) over a Büchner funnel, freeze-dried overnight, ground to a fine powder, and then DNA was extracted from approximately 100 mg of mycelium using a published CTAB protocol (O'Donnell et al., 1997). Total genomic DNA was suspended in sterile deionized water and stored at -20 °C when not in use.

2.3. Molecular identification by TEF1 sequencing

To confirm that the 106 strains from Algeria, and the 85 strains from the three other countries were correctly identified as *Fusarium culmorum*, a portion of the translation elongation factor 1-α (*TEF1*) gene was PCR amplified with the EF-1 x EF-2 primer pair and then sequenced with the reverse internal sequencing primer EF-22T as previously described (O'Donnell et al., 1998; Table S2). PCRs were conducted in an Applied Biosystems (ABI; Emeryville, CA) 9700 thermocycler, using the following program: 94 °C for 90 s, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 2 min, followed by a final extension at 68 °C for 5 min and ending in a 4 °C soak. Amplification products were size fractionated on 1.5% agarose gels in 1x TAE buffer, stained with ethidium bromide, and then photographed over a UV trans-illuminator (Fotodyne Inc.; Hartland, WI). After the amplicons were purified using Millipore Montage$_{96}$ filter plates (Millipore Corp., Billerica, MA), they were sequenced in a 10 μL volume containing 2 μL of a 10 pmol/μL

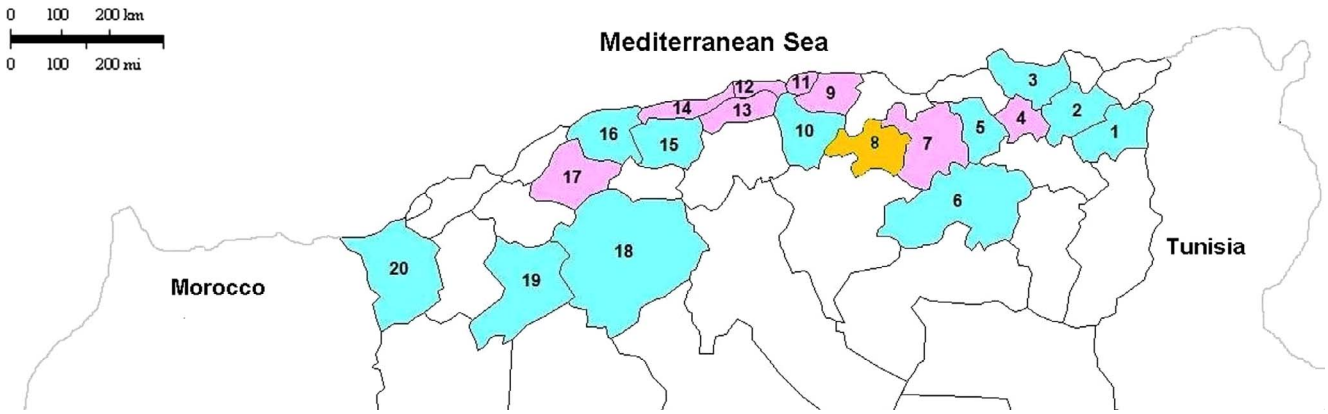

Fig. 1. Map of Algeria showing the 20 provinces where *Fusarium culmorum* was recovered from wheat symptomatic for crown rot (color coded blue), head blight (color coded pink) or both (color coded orange) during 2014 and 2015 pathogen surveys. 1, Souk Ahras; 2, Guelma; 3, Skikda; 4, Constantine; 5, Mila; 6, Batna, 7; Setif, 8; Bordj Bou Arreridj; 9, Tizi Ouzou; 10, Bouira; 11, Boumerdès; 12, Algiers; 13, Blida; 14, Tipaza; 15, Aïn Defla; 16, Chlef; 17, Relizane; 18, Tiaret; 19, Saida; 20, Tlemcen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stock of EF-22T, 2 µL of Applied Biosystems Big Dye v. 3.1, 2 µL 5x sequencing buffer, and approximately 50 ng of amplicon in an ABI 9700 thermocycler using the following cycling parameters: 96 °C for 15 s, 40 cycles of 96 °C for 15 s, 50 °C for 10 s, 60 °C for 4 min, and a 4 °C soak. After the sequencing reactions were cleaned up with BigDye XTerminator (Applied Biosystems), they were run on an ABI 3730 48-capillary automated DNA analyzer. Sequence data was edited with Sequencher v. 5.2.4 (Gene Codes Corp.; Ann Arbor, MI) and then used to query NCBI GenBank and *Fusarium MLST*, using the BLASTn tool.

2.4. Mating type idiomorph determination

A published PCR assay for mating type idiomorph determination (Kerényi et al., 2004) was used to screen the *Fusarium culmorum* isolates, using two diagnostic primers pairs: CUL-1-f x CUL-1-r for *MAT1-1* and CUL-2-f x CUL-2-r for *MAT1*-2 (Table S2). Sterile deionized water was used as a negative control. The PCR assay was run in an ABI 9700 thermocycler using the following program: 2 min initial denaturation at 95 °C, followed by 30 cycles consisting of 30 s at 94 °C, 30 s at 55 to 60 °C, 30 s to 5 min at 72 °C, a final elongation at 72 °C for 10 min, and a 4 °C soak. PCR products were size-fractionated in 1.5% agarose gels run in 1x TAE buffer, stained with ethidium bromide, visualized over a UV trans-illuminator, and sized using a 100 bp DNA ladder (Invitrogen Life Technologies; Carlsbad, CA). *Fusarium culmorum* strains NRRL 29376 and 29365, and NRRL 29381 and 23374 were used, respectively, as positive controls for the *MAT1-1* and *MAT1-2* idiomorphs.

2.5. Trichothecene chemotype determination

A trichothecene chemotype-specific multiplex PCR assay was performed as described by Ward et al. (2002), targeting *TRI3* and *TRI12* within the trichothecene biosynthetic gene cluster (Table S2). The *TRI3* multiplex included a primer common to all three chemotypes (3CON) and three chemotype-specific primers (3NA, 3D15A, and 3D3A). Similarly, the *TRI12* multiplex included a primer common to all three chemotypes (12CON) and three chemotype-specific primers (12NF, 12-15F, and 12-3F). Multiplex reactions were carried out in 10 µl volumes with 1x High Fidelity PCR buffer (Invitrogen Life Technologies), 2.5 mM $MgSO_4$, 0.2 mM of each deoxynucleoside triphosphate (Roche Diagnostic Corp.; Indianapolis, IN), 0.2 mM of each primer, 0.5 U of Platinum *Taq* DNA Polymerase (Invitrogen Life Technologies), and approximately 10 ng of genomic DNA. PCRs were conducted in an ABI 9700 thermocycler with an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 54 °C, 2 min elongation step at 68 °C, and a final soak at 4 °C. In addition to using sterile water as a negative control, the following three strains were used as a positive control for toxin chemotype: NRRL 34640 *Fusarium culmorum* for NIV, NRRL 34189 *F. culmorum* for 3ADON, and NRRL 37458 *F. graminearum* for 15ADON. Amplification products were fractionated by electrophoresis in 1% agarose gels buffered in 1x TAE buffer, stained with ethidium bromide and then sized using a 100 bp DNA ladder.

2.6. Trichothecene and culmorin toxin analysis of Fusarium culmorum

One hundred and two *Fusarium culmorum* strains from Algeria (Table S4) were screened for production of trichothecenes and the tricyclic sesquiterpene diol culmorin (McCormick et al., 2010) in liquid agmatine medium (30 g sucrose, 1.14 g agmatine, 1 g KH_2PO_4, 0.5 g MgSO4-$7H_2O$, 0.5 g KCl, 10 mg $FeSO_4$-$7H_2O$ per L) containing 2 mL per liter of a trace element solution (50 g citric acid, 50 g $ZnSO_4$-$7H_2O$, 2.5 g $CuSO_4$-$5H_2O$, 0.5 g $MnSO_4$-H_2O, 0.5 g H_3BO_3, and 0.5 g $NaMoO_4$-$2H_2O$) (Gardiner et al., 2009). Twenty mL of the chemically defined medium in 50 mL flasks were inoculated with *F. culmorum* grown on V8 medium (100 mL V8 Juice, Campbell Soup Company; Camden, NJ, 1.5 g $CaCO_3$, 20 g agar, and 900 mL distilled water), and incubated at 28 °C on a shaker at 200 rpm for 6 to 7 days. Each culture was extracted with 8 mL of ethyl acetate, dried under nitrogen in a 1 dram vial, resuspended in 1 mL ethyl acetate and then analyzed by gas chromatography-mass spectrometry (GC–MS). GC–MS analysis was performed on a 6890 gas chromatograph fitted with a HP-5MS column (30 m, 0.25 mm, 0.25 µm) and a 5973 mass detector (Agilent, Santa Clara CA). Helium was used as the carrier gas with a 20:1 split ratio and a 20 mL/min split flow. The column was held at 150 °C for one minute following injection, heated to 280 °C at 30 °C/min where it was held for 7.7 min. Compound identifications were based on GC–MS comparisons with purified standards; the level of detection was 0.25 µg toxin/mL culture.

2.7. SSR genotyping

Ten published microsatellite markers (Giraud et al., 2002; Suga et al., 2004; Vogelgsang et al., 2009) (Table S3) were used initially to assess the population genetic diversity of 191 *Fusarium culmorum* isolates. However, we discovered by mapping the SSR primers on the whole-genome sequence of *F. culmorum* strains UK99 (Urban et al., 2016) and KOD 1161, a NIV producing strain from Mila, Oued El Othmanya, Algeria, that the forward F1 and F7 primers (Giraud et al., 2002) mapped to the same locus. Therefore, the data for F7 was discarded. In addition to determining the genomic positions of the SSR markers, we also mapped the trichothecene gene cluster and mating type locus to chromosome two of UK99, using the BLASTn application in CLC Genomics Workbench (CLC bio, Qiagen, Aarhus, Denmark) (Fig. 2). Forward primers for microsatellite markers were labelled with 6-FAM, HEX or NED fluorophores (Applied Biosystems; Foster City, CA), and a GTTT PIG-tail sequence was added to the 5′ end of each reverse primer to minimize stutter bands (Wang and Chilvers, 2016). SSR amplifications were conducted in three multiplex and one uniplex reactions containing 1x PCR buffer, 2.5 mM $MgSO_4$, 2.5 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 0.2 U of Platinum *Taq* DNA Polymerase and approximately 100 ng of genomic DNA. The SSR markers were amplified in an ABI 9700 thermocycler using the following program: 2 min initial denaturation at 95 °C, followed by 25 cycles of 1 min at 95 °C, 1 min at 59 °C, 1 min at 68 °C, a final extension of 10 min at 68 °C, followed by a 4 °C soak. After the labelled amplicons were sized on an ABI 3100 genetic analyzer,

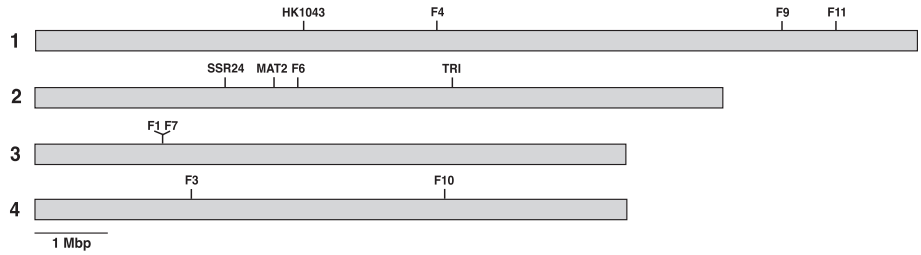

Fig. 2. Nine SSR markers, *MAT1-2*, and the trichothecene gene cluster mapped on chromosomes 1–4 of UK99 *F. culmorum* (Urban et al., 2016). SSR marker F7 (Giraud et al., 2002) was excluded from the study because it mapped to locus F1.

using GS500 ROX (Applied Biosystems) as an internal size standard, the allele data was analyzed using GeneMapper software v. 3.7 (Invitrogen Life Technologies).

2.8. Population genetic analyses

All analyses were conducted on clone-corrected data after removing nine isolates that shared identical multilocus genotypes with another isolate (Table S5). Bayesian analyses of genetic structure and population assignment using microsatellite allelic data were conducted in STRUCTURE v. 2.3.4 (Pritchard et al., 2000). Analyses were based on 100,000 Monte Carlo Markov Chain (MCMC) iterations following 25,000 burn-in iterations for each run. The number of simulated populations (K) ranged from 1 to 8, ten replicate runs were performed for each K value to estimate group assignment, and STRUCTURE HARVESTER v. A.2 (Earl and vonHoldt, 2012) was used to select the optimal model that maximized the rate of change in log likelihood values (ΔK) based on the method of Evanno et al. (2005). The SSR data was analyzed with GenAlEx v. 6.5 (Peakall and Smouse, 2012) to estimate genetic diversity based on the number of effective alleles over loci (N_e), Shannon's information Index (I), and average gene diversity over loci (H), calculated for sampling locations and genetic populations identified by STRUCTURE. In addition, an Analysis of Molecular Variance (AMOVA) was performed to assess genetic differentiation (φ_{PT}, an analogue of Wright's fixation index (F_{ST}) for haploid data) using 999 permutations of the data to assess statistical significance. Linkage disequilibrium (LD) within populations was estimated using the $\bar{r}_d$ index in *poppr* v. 1.1.2 (Kamvar et al., 2014) in R v. 3.0.3 (https://cran.r-project.org/bin/windows/base/old/3.0.3/). Statistical significance was assessed using the permutation algorithm to randomize 1000 data sets, with no linkage among markers as the null hypothesis.

3. Results

3.1. Molecular identification by TEF1 sequencing

Surveys of diseased durum and common wheat in 500 fields during 2014 and 2015 focused on the genetic diversity of *Fusarium culmorum* in the 20 main cereal growing provinces of northern Algeria (Fig. 1). Of the 269 strains from Algeria that were identified by sequencing a portion of *TEF1*, 140 were typed as *Fusarium culmorum* from 110 different fields, and these included 110 and 30 plants, respectively, symptomatic for FRC and FHB. However, only 106 of these were included in the present study because 34 strains were missing two or more SSR alleles. To confirm that the strains of *F. culmorum* from Australia (n = 30), Italy (n = 27) and the United States (n = 28) were all identified correctly, partial *TEF1* sequence data, when queried against the GenBank (https://www.ncbi.nlm.nih.gov/) and *Fusarium* MLST (http://www.cbs.knaw.nl/fusarium/) databases, revealed that all of the strains were *F. culmorum*. Analysis of the aligned sequences with Sequencher v. 5.2.4 revealed that they were 99.3% similar, differing at only 4/539 nucleotide positions (data not shown). The *F. culmorum* *TEF1* sequences generated in the present study were deposited in GenBank under accession numbers KY873384-KY873574.

3.2. Mating type determination

A PCR assay of 191 strains for *MAT* idiomorph (Kerényi et al., 2004), prior to discovering and censoring nine clones, revealed that both idiomorphs were segregating among *Fusarium culmorum* from each of the four countries sampled. *MAT1-2* strains comprised the majority in each region (Table S1): Algeria (36 *MAT1-1*:70 *MAT1-2*), Australia (12 *MAT1-1*:18 *MAT1-2*), Italy (12 *MAT1-1*:15 *MAT1-2*), and the U.S. (9 *MAT1-1*:19 *MAT1-2*). *MAT1-1* and *MAT1-2* strains were not segregating in equal proportions within Algeria (x^2 = 10.906; df = 1; P = 0.0010) where two-thirds of the strains were *MAT1-2*.

3.3. Chemotype determination

Results of a PCR assay for predicting trichothecene toxin chemotype indicated that *Fusarium culmorum* was segregating for 3ADON and NIV in the four countries sampled, but the 3ADON genotype significantly outnumbered NIV in each region (Table S1). Of the 191 *F. culmorum* strains we genotyped, 83.8% (n = 160) were typed as 3ADON and 16.2% (n = 31) as NIV. Results of the chemotype assay revealed significant (x^2 = 13.333–54.491; df = 1; P = 0.0003–0.0001) differences in trichothecene genotype frequencies in Algeria (91 3ADON:15 NIV), Australia (25 3ADON:5 NIV) and the U.S. (27 3ADON:1 NIV); however, the frequency in Italy (17 3ADON:10 NIV) was not significantly different from a 1:1 ratio (x^2 = 1.815; df = 1; P = 0.1779).

3.4. Fusarium culmorum toxin analyses

Results of the PCR assay for predicting trichothecene chemotype were evaluated by growing 102 *Fusarium culmorum* strains from Algeria in liquid agmatine medium and then analyzing them for 3ADON, acetylated NIV and the toxic tricyclic sesquiterpene diol culmorin by GC–MS. Sixty-five strains produced detectable levels of 3ADON, ranging from 8.07 to 147.09 μg/mL, 14 produced acetylated NIV; however, no trichothecenes were detected in 23 of the isolates. Our analyses also revealed that 79 isolates produced culmorin ranging from 0.21 to 475.42 μg/mL (Table S4). It is worth noting that the 65 3ADON and 14 NIV producing strains matched the results predicted by the PCR assay for trichothecene toxin chemotype.

3.5. SSR analysis of population structure and genetic diversity

All nine SSR loci used to genotype 182 *Fusarium culmorum* strains were polymorphic, with the number of alleles amplified per locus ranging from 3 at HK1043 to 35 at F10 (Table S5). Overall, a total of 135 alleles were amplified that ranged in size from 91 to 335 bp, yielding an average of 15 alleles per locus. Analyses of molecular variance revealed that 89% of the observed genetic variation was represented within sampling location (countries) and 11% between countries (Table 1).

Pairwise estimates of φ_{PT} revealed significant genetic differentiation in relation to some sampling locations (P < 0.001; Table 2); however, φ_{PT} values were relatively low and varied from 0.06 (Italy/Australia) to 0.13 (U.S./Italy). Relatively high values were obtained for Shannon's index (I) (Table 3), which ranged from 1.31 (USA) to 1.57 (Algeria). The lowest and highest average gene diversity over loci was found in the Algerian (0.60) and Australian samples (0.66), respectively. The number of effective alleles over loci (N_e) ranged from 4.0 in the U.S. to 5.35 in the Algerian samples (Table 3).

Following the recommendation of Pritchard et al. (2000), the admixture model with independent allele frequencies was used to analyze the SSR data in STRUCTURE to obtain admixture estimates with clone correction. Although simulations with five populations (K = 5) provided the highest likelihood value (Fig. 3), simulations with two populations (K = 2) yielded the greatest change in log likelihood value (ΔK = 245.90) and K = 2 appeared to capture the major genetic structure within the data. Based on the results obtained from all of the models tested, K = 2 was chosen because it captured the

Table 1
AMOVA of *Fusarium culmorum* from Algeria, Australia, Italy, and the United States after censoring clones.

Source	df	SS	MS	Est. Var.	%
Among pops	3	45.540	15.180	0.331	11%
Within pops	178	500.449	2.812	2.812	89%
Total	181	545.989		3.142	100%

Table 2
Pairwise estimates of genetic differentiation (φ_{PT}, below diagonal) and number of effective migrants (N_m, above diagonal) for *Fusarium culmorum* from the four sampling locations.

Location	Algeria	Australia	Italy	United States
Algeria	–	4.94	4.14	3.56
Australia	0.09	–	7.85	4.26
Italy	0.11	0.06	–	3.34
United States	0.12	0.10	0.13	–

Table 3
Diversity indices and trichothecene chemotype frequencies for *Fusarium culmorum* by sampling location.

Location	# isolates	N_e[a]	I[b]	H[c]	3ADON	NIV
Algeria	103	5.35	1.57	0.60	89 (86.4%)	14 (13.6%)
Australia	29	4.18	1.36	0.66	25 (86.2%)	4 (13.8%)
Italy	23	4.53	1.37	0.63	17 (73.9%)	6 (26.1%)
United States	27	4.03	1.31	0.61	26 (96.3%)	1 (3.7%)

[a] N_e, number of effective alleles over loci.
[b] I, Shannon's Information Index.
[c] H, average gene diversity over loci.

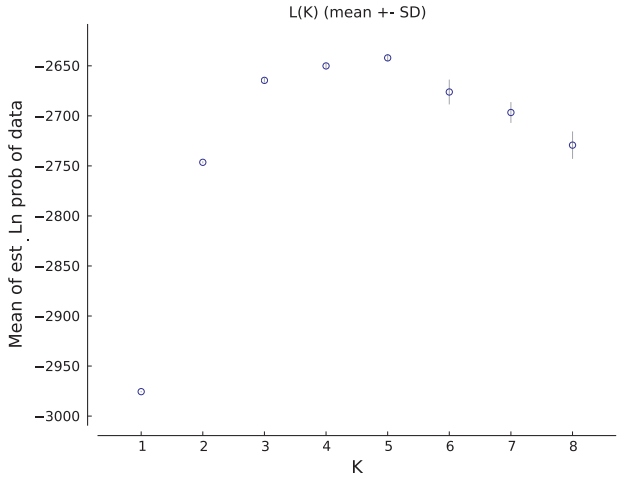

Fig. 3. Mean Ln probabilities derived from analyses based on 10 independent runs for eight different values of *K*. Runs were conducted using the admixture model with independent allele frequencies (25100adindi) in STRUCTURE (Pritchard et al., 2000). $K = 2$ appeared to capture the most data structure and provided the greatest change in log likelihood value (ΔK).

most genetic structure within the data.

Therefore, all of the STRUCTURE analyses described below (Fig. 4), with $K = 2$, utilized the admixture model with independent allele frequencies (25100adindi). With $K = 2$, 52.7% of the isolates were assigned to one genetic population (designated CU1 = color coded blue) and 47.3% to a second genetic population (designated CU2 = -color coded orange). CU1 contained all of the isolates from the U.S. ($n = 27$), the majority of the isolates from Australia ($n = 28$, 79.3%), Italy ($n = 18$, 73.9%), and approximately one-quarter of the Algerian isolates ($n = 23$, 22.3%) (Figs. 4 and 5, Table 4). By way of contrast, CU2 contained the majority of the Algerian isolates ($n = 80$, 77.7%), five isolates from Italy and one isolate from Australia (Figs. 4 and 5, Table 4). With the exception of 3ADON isolate 59 from the crown of durum wheat collected in Rouiba (Algeria), all of the Algerian isolates within CU1 were from the east of the country or possessed the NIV chemotype. Genetic differentiation ($\varphi_{PT} = 0.11$) and the number of migrants ($N_m = 3.98$) between CU1 and CU2 suggested ongoing gene flow between the two populations. Only 23 isolates (12.6%) were not assigned to one of the two genetic clusters with $q \geq 0.8$. Models with higher values of K resulted in higher numbers of weakly assigned ($q < 0.8$) isolates. Significant ($P < 0.001$) differences in trichothecene genotype frequencies were observed between the CU1 and CU2 populations, where 22 (22.9 %) of the isolates in the former were typed as NIV compared with only 3 (3.5%) of those within the latter (Table 5). Observed $\bar{r}_d$ values for the two populations indicated moderate *LD* within the CU1 ($\bar{r}_d = 0.0294$, $P = 0.002$) and CU2 ($\bar{r}_d = 0.0325$, $P = 0.001$) populations, suggesting that outcrossing may be rare. To assess whether the *LD* detected in the clone-corrected data was due to one or more pairs of loci, we calculated $\bar{r}_d$ over all nine pairs of loci (data not shown). The highest *LD* detected within CU1 occurred at the HK1043 and F1 loci, which mapped to chromosomes one and three, respectively, in the draft genome of *F. culmorum* UK99 (Fig. 2). Interestingly, when HK1043 was excluded from the analysis, the $\bar{r}_d$ value increased in both populations (CU1 $\bar{r}_d = 0.0335$, $P = 0.002$; and CU2 $\bar{r}_d = 0.0408$, $P = 0.001$).

4. Discussion

This study represents a detailed analysis of the population biology, mycotoxin potential and genetic diversity of *Fusarium culmorum* in countries on four different continents. Recent but limited pathogen surveys of these diseases in Algeria, which were focused on evaluating susceptibility of locally grown wheat cultivars to FHB (Touati-Hattab et al., 2016), and the relationship between DON toxin level accumulation in wheat and barley and disease severity (Yekkour et al., 2015), provided preliminary data suggesting that *F. culmorum* segregating for 3ADON and NIV might be the primary causal agent of FCR and FHB in Algeria. These findings raise food safety and human health concerns because, in contrast to the European Union where mycotoxin levels in food and feed are strictly regulated (EC No. 1881/2006), no standards currently exist within Algeria. The present study was focused on addressing these concerns because wheat and other small grain cereals are grown on approximately six million hectares annually in the hot semiarid Northern provinces of Algeria (Fig. 1) under conditions that likely increase their susceptibility to diseases.

While a number of PCR-based tools are available for identifying *Fusarium culmorum* strains in culture (Scherm et al., 2013 and references therein), we opted to use discrete DNA sequence data from a portion of the *TEF1* gene to obtain definitive identifications of our *F. culmorum* collection. We also used a PCR assay (Kerényi et al., 2004) to type the 191 *F. culmorum* strains for MAT idiomorph. Consistent with prior surveys that have uniformly reported this pathogen is segregating for both idiomorphs locally and globally (O'Donnell et al., 2004; Obanor et al., 2010; Rebib et al., 2014), based on limited sampling, we found that *MAT1-1* and *MAT1-2* strains were represented in nearly equal numbers in Italy, Australia and the U.S. Approximately two-thirds of the strains in Algeria were *MAT1-2*. Although a sexual cycle with a perithecial teleomorph has not been observed in *F. culmorum*, several

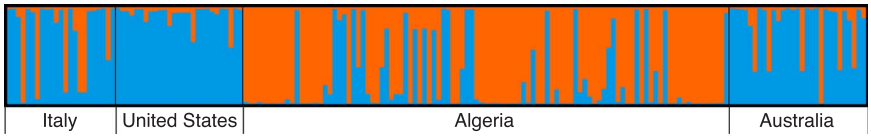

Fig. 4. Population genetic structure of 182 clone corrected strains of *Fusarium culmorum* derived from STRUCTURE analyses of 9 SSR marker loci with $K = 2$. The CU1 and CU2 populations (K) are color-coded blue and orange, respectively, and they are sorted by sampling location. Estimated K membership of each isolate, which ranged from 0 to 1, is presented on the vertical axis. Each bar represents one of the 182 isolates genotyped.

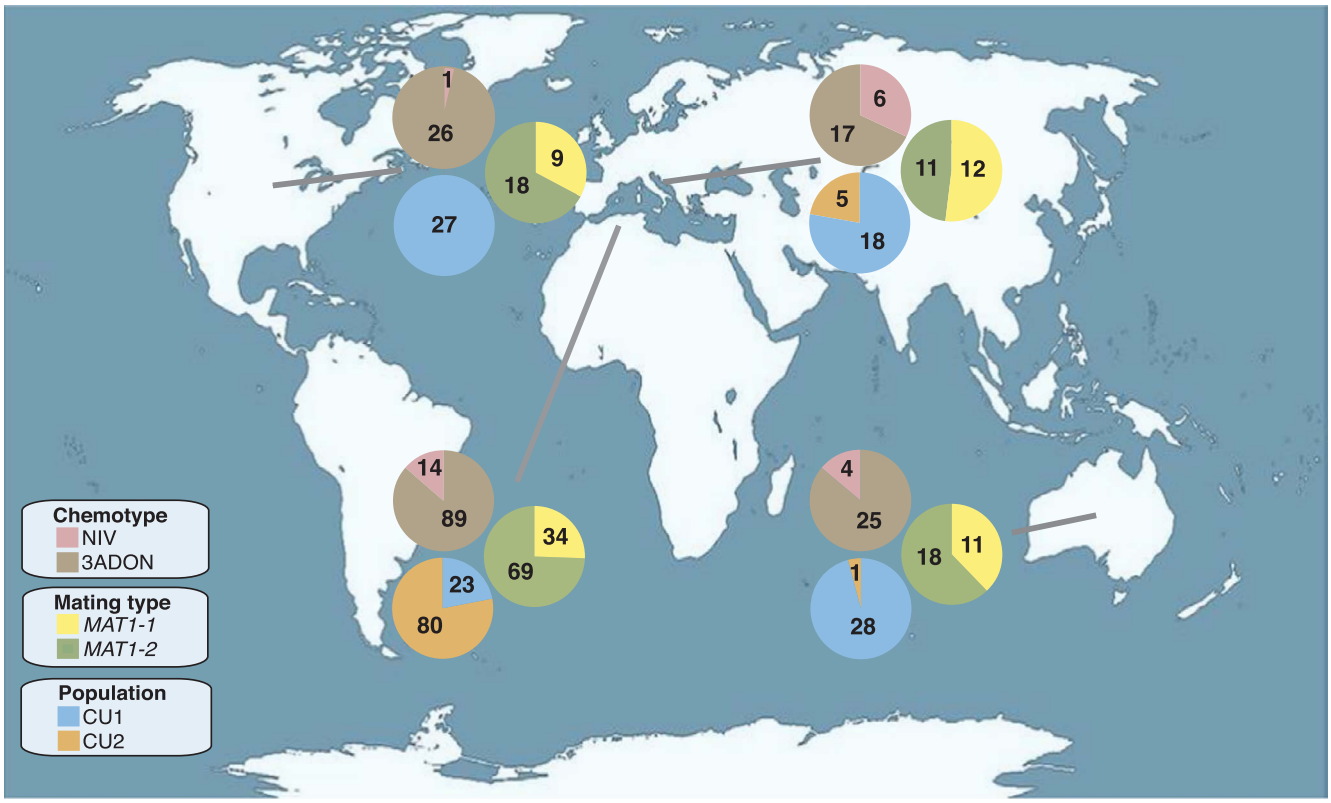

Fig. 5. Map showing number of strains of each trichothecene chemotype, mating type and CU1 and CU2 population of *Fusarium culmorum* within each of the four countries sampled. Note that the nine clones are not included in this map.

Table 4
Assignment of 182 *Fusarium culmorum* isolates to the CU1 or CU2 populations by sampling location using 9 SSR markers.[a]

Location	CU1	CU2
Algeria	23 (22.3%)	80 (77.7%)
Australia	28 (96.6%)	1 (3.4%)
Italy	18 (78.3%)	5 (21.7%)
United States	27 (100%)	0 (0%)

[a] When STRUCTURE was rerun after marker HK1043 was excluded from the analysis, CU1 and CU2 comprised 87 and 95 strains, respectively.

Table 5
Diversity indices and trichothecene genotype composition for the CU1 and CU2 populations of *Fusarium culmorum* defined by STRUCTURE (K = 2).

K[a]	Isolates	N_e[b]	I[c]	H[d]	3ADON	NIV
CU1	96	7.23	1.77	0.70	74 (77.1%)	22 (22.9%)
CU2	86	4.32	1.33	0.54	83 (96.5%)	3 (3.5%)

[a] K, population or cluster.
[b] N_e, number of effective alleles over loci.
[c] I, Shannon's Information Index.
[d] H, average gene diversity over loci.

lines of evidence suggest it might possess a cryptic sexual reproductive mode and/or parasexual cycle that remain to be discovered. RT-PCR experiments demonstrated that the *MAT1-1* and *MAT1-2* genes are expressed (Kerényi et al., 2004), in silico translations of the MAT genes indicate that they might be functional (O'Donnell et al., 2004), both idiomorphs are segregating in individual fields (Rebib et al., 2014 and present study), both idiomorphs appear to have been recombined into the genome of several strains from Turkey (Obanor et al., 2010), and several studies (Gargouri et al., 2003; Miedaner et al., 2013), including the present one, discovered a population genetic structure consistent with high levels of genetic diversity (discussed below). However, the mechanisms generating this genetic diversity remain to be elucidated.

Given the demonstrated value of PCR assays for predicting the trichothecene toxin potential of B clade fusaria (Ward et al., 2002), and concomitant toxin contamination of cereals, we used primers that target *TRI3* and *TRI12* to type our *Fusarium culmorum* strains because they have been shown to accurately predict NIV, 3ADON and 15ADON genotypes. Results of the present study are consistent with previous findings indicating *F. culmorum* is only segregating for the 3ADON and NIV chemotypes worldwide (Pasquali et al., 2010, 2016a and references therein). Our results revealed that the 3ADON chemotype accounted for over 80% of the *F. culmorum* from Algeria, Australia and the U.S. In addition, while approximately two-thirds of the 27 *F. culmorum* strains from Italy possessed the 3ADON genotype, a chi-square test (df = 1) indicated 3ADON and NIV strains were segregating in nearly equal frequencies. Several studies have reported significant differences in trichothecene genotype frequencies, and these include the predominance of 3ADON in England and Wales (Jennings et al., 2004), Turkey (Yörük and Albayrak, 2012), Sardinia (Balmas et al., 2015), Tunisia (Kammoun et al., 2010), and several European countries (Tóth et al., 2004; Quarta et al., 2005). By contrast, surveys of FHB of wheat conducted in the Netherlands during 2000 and 2001 found NIV-producing *F. culmorum* predominated (Waalwijk et al., 2003). Also an increase in the number of NIV genotypes was reported in the west and south of England (Jennings et al., 2004) and during 2007–2008 in Luxembourg to near 3ADON levels (Pasquali et al., 2010). The latter was attributed to rotation of wheat with maize. Dramatic shifts in FHB species and trichothecene genotypes over the past two decades in Europe (Waalwijk et al., 2003) and North America (Ward et al., 2008; Kelly et al., 2015) highlight the need for heightened active molecular surveillance of the FCR and FHB pathogens in support of global food security (Pasquali and Migheli, 2014; Pasquali et al., 2016a).

We tested the prediction of the PCR assay for trichothecene genotype by growing 102 *Fusarium culmorum* strains from Algeria in

an agmatine-containing medium known to induce toxins (Gardiner et al., 2009; Pasquali et al., 2016b). As previously reported (Quarta et al., 2005; Pasquali et al., 2010), we observed that *F. culmorum* strains differed significantly in the level of toxin produced in vitro, with some strains producing up to 147.09 μg/mL 3ADON, induction of acetylated NIV in 14 strains, and failure to detect trichothecenes in 23 strains. Similarly, our chemical analyses revealed significant strain diversity, based on the level of the toxic tricyclic sesquiterpene diol culmorin produced in vitro by 79 *F. culmorum* strains, which differed by three orders of magnitude (i.e., 0.21 to 475.42 μg/mL; Table S4). While culmorin has been shown to exhibit some phytotoxicity to wheat, it remains to be determined whether it plays a role in plant disease (McCormick et al., 2010) and whether it is toxic to humans and other animals. Because the toxicological data on exposure to ADON and NIV is limited (Wu et al., 2014), our results emphasize the need for future research to better understand the diverse pathophysiological impact of these toxins.

Analyses of *Fusarium culmorum* populations, including the one presented here, have uniformly reported high levels of allelic and genetic diversity as reflected in the large number of unique multilocus genotypes, low level of population subdivision, and much higher variation within than between populations (Miedaner et al., 2013; Pouzeshimiab et al., 2014; Rebib et al., 2014). We found, for example, that prior to censoring nine putative clones, 90% of the multilocus haplotypes we detected were unique; this value for *F. culmorum* has been reported as low as 78% in Tunisia (Rebib et al., 2014) and up to 95.5% in Iran (Pouzeshimiab et al., 2014). Similarly, AMOVA values for variance reported within *F. culmorum* populations have ranged from 81% in a mostly Eurasian collection (Miedaner et al., 2013) up to 96.7% in Tunisia (Rebib et al., 2014). Using 25 RAPD markers, Gargouri et al. (2003) reported 96.2% of the variance was within populations in Tunisia. It is worth noting that RFLP analysis of the IGS rDNA has been used to infer genetic diversity with *F. culmorum* (Tóth et al., 2004); however, phylogenetic analysis of this spacer region within the B clade of trichothecene toxin-producing fusaria revealed that *F. culmorum*, and several other species within this clade, appear to possess nonorthologous IGS rDNA sequences (O'Donnell, unpubl). Following the methods of Evanno et al. (2005), using the ad hoc statistic ΔK, we identified two cosmopolitan, sympatric populations within *F. culmorum* designated CU1 and CU2. Using Shannon's index to assess overall genetic variability, the values of 1.77 and 1.33 for the CU1 and CU2 populations, respectively (Table 5), are much higher than expected for a strictly clonal fungus, and these values are substantially higher than the 0.85 index value reported by Miedaner et al. (2013) for a mostly Eurasian *F. culmorum* collection. We attribute the higher Shannon indices reported in the present study, in large part, to the use of a capillary instrument, which allowed us to distinguish alleles that differed in size by only two bp. However, it is worth noting, as cautioned by Pritchard et al. (2000), that $K = 2$ might be an artifact of the Evanno method, which cannot arrive at $K = 1$. The hypothesis that $K = 1$ seems plausible because gene flow between two sympatric populations should reduce them to one over time. Therefore, future studies should investigate whether our finding of $K = 2$ could be due to *LD* across a subset of SSR markers used in this study.

The significant multilocus *LD* we detected within the *F. culmorum* CU1 and CU2 populations, using the standardized index of association $\bar{r}_d$ (Kamvar et al., 2014), contrasts with the surprisingly high level of genetic diversity and presence of both MAT idiomorphs in all populations sampled. This finding is enigmatic because neither a sexual and/or parasexual cycle have been documented in this pathogen. Prior population genetic (Miedaner et al., 2013; Rebib et al., 2014) and molecular phylogenetic (Obanor et al., 2010) studies have also suggested that the high genetic diversity might be due to a cryptic sexual cycle. We found, as previously reported by Miedaner et al. (2013) and Rebib et al. (2014) that both idiomorphs are segregating within *F. culmorum* populations. Moreover, the low φ_{PT} values that we obtained (0.06–0.13) are more in line with those reported for outcrossing, sexually reproducing species. Interestingly, the highest *LD* we detected, by calculating $\bar{r}_d$ over all nine pairs of SSR loci (data not shown), involved HK1043 and F1, but this cannot be explained by physical linkage because they are on different chromosomes (Fig. 2). It is possible that the *LD* might be due to the very low diversity of this marker, given that HK1043 only has two alleles per population and one of the alleles was only found in one isolate. Alternatively, as suggested for *Mycosphaerella graminicola* (Gurung et al., 2011), the *LD* might be due to selection for certain combinations of alleles near the SSR loci, or admixture of strains in which the allele frequencies differ significantly. Given these potentially confounding factors, future studies are needed, using genome-wide SNP data (Grünwald et al., 2016), to further investigate the global population genetic structure of *F. culmorum*.

Our analyses were greatly facilitated by published and validated SSR primer pairs for *Fusarium culmorum* (Giraud et al., 2002; Suga et al., 2004; Vogelgsang et al., 2009) and the published genome of UK99 (Urban et al., 2016). By mapping the SSR markers on the draft genome sequence of UK99, we discovered that SSR primers F1f and F7f (Giraud et al., 2002) mapped to the same location on chromosome three. Interestingly, Miedaner et al. (2013) discovered that marker F7 contributed to the high *LD*, and found by excluding the data for F7 and HK1043 that the populations appeared to be in linkage equilibrium. SSRs have enjoyed widespread use in population genetic studies because they are co-dominant and highly polymorphic, as evidenced by the 135 alleles that we detected using nine SSR loci in a total of 191 *F. culmorum* strains. The fact that we were able to discern over three times as many alleles than previously reported in population genetic analyses of *F. culmorum* may be explained in part by our sampling of countries on four different continents, use of more SSR markers, and the much greater resolving power of the ABI genetic analyzer, which allowed us to distinguish alleles that differed in size by as little as two bp (Table S5). Published studies of *F. culmorum* populations that have reported between 33 and 44 alleles included strains mostly from Eurasia (Miedaner et al., 2013), or were sampled within a country (Giraud et al., 2002). In addition, these studies only used 6 or 8 SSR loci (Pouzeshimiab et al., 2014; Rebib et al., 2014), and alleles were sized using agarose or polyacrylamide gels, with the exception of Rebib et al. (2014) where a capillary instrument was employed. It is worth mentioning that 39 strains were removed from our study because two or more of the SSR markers yielded null genotypes. Looking to the future, the availability of several *F. culmorum* genomes, especially UK99, should greatly facilitate development of more robust SSR markers (Urban et al., 2016) using a bioinformatics approach (Suga et al., 2004).

Results of the present study have raised important questions concerning the mechanisms generating the high levels of genetic variation we observed within a putatively asexually reproducing mycotoxigenic cereal pathogen of importance to world agriculture. Moreover, our results should help inform further studies directed at testing whether there is a connection between trichothecene accumulation and pathogenicity and whether 3ADON and NIV strains within the CU1 and CU2 populations have a selective advantage. In summary, our results emphasize the need to monitor trichothecene toxin levels in small grain cereals in Algeria, especially because ADON and NIV levels are not regulated currently within this and several other countries. The present study, and several others that show *F. culmorum* is a genetically diverse pathogen, should help plant disease specialists develop control strategies focused on reducing mycotoxin contamination in cereals and assist plant breeders develop cultivars with broad based resistance to FCR and FHB that are adapted to the semiarid production areas of northern Algeria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2017.04.001.

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