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Determination of the trichothecene mycotoxin chemotypes and associated geographical distribution and phylogenetic species of the *Fusarium graminearum* clade from China

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ABSTRACT

A large number of isolates from the *Fusarium graminearum* clade representing all regions in China with a known history of *Fusarium* head blight (FHB) epidemics in wheat were assayed using PCR to ascertain their trichothecene mycotoxin chemotypes and associated phylogenetic species and geographical distribution. Of the 299 isolates assayed, 231 are from *F. asiaticum* species lineage 6, which produce deoxynivalenol and 3-acetyldeoxynivalenol (3-AcDON); deoxynivalenol and 15-acetyldeoxynivalenol (15-AcDON); and nivalenol and 4-acetylnivalenol (NIV) mycotoxins, with 3-AcDON being the predominant chemotype. Ninety-five percent of this species originated from the warmer regions where the annual average temperatures were above 15 °C, based on the climate data of 30 y during 1970–1999. However, 68 isolates within *F. graminearum* species lineage 7 consisted only of 15-AcDON producers, 59 % of which were from the cooler regions where the annual average temperatures were 15 °C or lower. Identification of a new subpopulation of 15-AcDON producers revealed a molecular distinction between *F. graminearum* and *F. asiaticum* that produce 15-AcDON. An 11-bp repeat is present in *F. graminearum* within their *Tri7* gene sequences but is absent in *F. asiaticum*, which could be directly used for differentiating the two phylogenetic species of the *F. graminearum* clade.

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Introduction

Fusarium head blight (FHB) or scab of wheat and other small cereal grains caused by the *Fusarium graminearum* clade is an economically devastating disease worldwide (Windels 2000). FHB takes place both in field and during storage, producing mycotoxins in mouldy corn and wheat that are detrimental to human and animal health. Recently, FHB has reached epidemic proportions in Europe and North America, resulting in huge losses in crop revenues due to reduced yields and mycotoxin contamination of stored grains (Windels 2000). In China, epidemics of FHB occur frequently in the middle and downstream regions of the Yangtze River and in the Heilongjiang province in the northeastern region (Chen *et al.* 2000). With the changes of global climates FHB has gradually spread to the northwestern regions, covering more than ten provinces that are mainly agricultural areas in China (Chen *et al.* 2000). *Fusarium* mycotoxins are among the main fungal mycotoxin contaminations in food and livestock (Bai 1997), and some human diseases, such as Kashi neck diseases and

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oesophageal cancer, have been epidemiologically associated with consumption of trichothecences (Chen et al. 2000).

Type B trichothecenes (8-ketotrichothecenes) are the principal toxins produced by the *F. graminearum* clade. Based on the production and chemical structures of different 8-ketotrichothecenes, three trichothecene mycotoxin chemotypes were identified within the type B-trichothecene-producing *F. graminearum* clade: (1) nivalenol and 4-acetylnivalenol (NIV chemotype), (2) deoxynivalenol and 3-acetyldeoxynivalenol (3-AcDON chemotype), and (3) deoxynivalenol and 15-acetyldeoxynivalenol (15-AcDON chemotype) (Miller et al. 1991; Ward et al. 2002). The chemotypes appear to differ in geographical distribution, with both DON and NIV chemotypes reported in several countries of Africa, Asia, and Europe (Miller et al. 1991; Jennings et al. 2004b) but only the DON chemotype was reported in North America (Mirocha et al. 1989). Earlier studies by chemical analysis focused on DON detections of *F. graminearum*, and a recent study has revealed the presence of both DON and NIV chemotypes in China (Li et al. 2005). However, the acetylated derivatives of the *Fusarium* chemotypes and their associations with phylogenetic species and geographical distribution are yet to be investigated.

For analysis of the mycotoxins and their derivatives, chemical methods such as capillary gas chromatography and hplc are commonly applied (Langseth & Rundberget 1998), and quick and simple screening methods such as ELISAs have also been used (Dietrich et al. 1995). However, these methods are rather labour-intensive, expensive, and require sophisticated instrumentation and skilled operators. Molecular characterization of trichothecene mycotoxin biosynthesis pathways has brought the development of new methods for the fast analysis of the mycotoxins *via* PCR with specific primers, which have been widely used due to their advantages over the conventional methods (Bakan et al. 2002; Chandler et al. 2003; Gale et al. 2003; Jennings et al. 2004a,b; Li et al. 2005).

Our specific objectives in this study were (1) to determine the mycotoxin chemotypes of the *F. graminearum* clade from China using a series of PCR assays, (2) to reveal association of the chemotypes with their phylogenetic species and geographical distribution, and (3) to find a molecular distinction between two *Fusarium* phylogenetic species that produce 15-AcDON.

Materials and methods

Isolates of Fusarium *strains*

Diseased wheat spikes were taken in 1999 from fields that were separated by at least 3 km, and the numbers of the collected wheat ears with clear FHB symptoms were proportional to the acreage of wheat infected by the disease in the regions. These collections represent samples from all the areas in the Yangtze River valleys and other regions in China with known history of FHB epidemics in wheat, covering 12 provinces in China. Strains were obtained by single spore isolation from the diseased wheat spikes and identified by the methods described previously (Booth 1971; Nelson et al. 1984). In total 299 isolates of *F. graminearum* were used in this study and detailed information of the strains are listed in Table 1. All strains used in the study have been deposited in the Molecular Biotechnology Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China. These materials are available for public use on request.

Mycelial DNA extraction

All the *Fusarium* strains were grown on sterile glass–membrane paper over potato-dextrose agar at 23 °C for one week. The mycelia were harvested and ground to a fine powder in the presence of liquid nitrogen. The total genomic DNA was extracted as described by Nicholson et al. (1997).

Identification of DON and NIV chemotypes

A pair of generic primers ToxP1/P2 derived from the intergenic sequences between *Tri5* and *Tri6* genes was synthesized and used for the identification of DON and NIV chemotypes of *Fusarium graminearum*, generating a 300 bp fragment specific for DON producers and a 360 bp fragment from NIV chemotypes, respectively (Li et al. 2005). To verify the mycotoxin chemotypes identified by the ToxP1/P2 primers, another pair of primers GzTri7f1/r1, based on the *Tri7* sequence, was used to differentiate DON and NIV chemotypes. This set of primers produced PCR products ranging in size from 173–327 bp for DON chemotypes or a product of 161 bp for NIV producers (Lee et al. 2001).

Identification of 3-AcDON- and 15-AcDON chemotypes

Two sets of primers, Tri303F/R and Tri315F/R, developed based on the *Tri3* gene sequences, were used to further differentiate the DON chemotypes of *Fusarium graminearum* into 3-AcDON- or 15-AcDON chemotypes. *Fusarium* DON-producing isolates produce either a 3-AcDON-specific product of 586 bp with the primers Tri303F/R or a 15-AcDON specific fragment of 864 bp with the primers Tri315F/R (Jennings et al. 2004a). NIV-producing strains in *F. graminearum* do not produce any fragments with both primer sets.

To validate the chemotypes identified above, a pair of primers designed based on *Tri3* and *Tri8* intergenic sequences, MinusTri7F/R, were used to amplify a specific 483 bp fragment from *Fusarium* 3-AcDON-producing isolates only, which lack the entire *Tri7* gene and flanking regions (Ward et al. 2002; Chandler et al. 2003; Kimura et al. 2003).

SCAR analysis

All the *Fusarium* strains were subjected to SCAR (sequence characterised amplified region) analysis with a pair of primers Fg16F/R (Carter et al. 2000). A 410 bp DNA fragment specific for SCAR group I and a 497 bp fragment for SCAR group V were generated, respectively.

Analysis of lineage and phylogenetic species

Portions of three phylogenetically informative genes (*Tri101*, *reductase* and *histone H3*) from 30 strains of different chemotypes and originations were amplified and sequenced as described previously (O'Donnell et al. 2000, 2004). Among these strains, 20 strains are from phylogenetic species *Fusarium*

Table 1 – Origin of isolates of *F. graminearum* clade, SCAR groups, phylogenetic species and their mycotoxin chemotypes assayed in this study

District	Isolate[a]	Mycotoxin chemotype[b]	SCAR[c]	Phylogenetic species[d]
Huoqiu	1001 1005	3-AcDON	V	*Fusarium asiaticum*
Hefei	1002 1003 1006 1008 1018 1019	3-AcDON	V	-
	1007	NIV	V	-
Nanping	2002 2004 2005	NIV	V	*F. asiaticum*
	2008	15-AcDON	I	-
Jianyang	2012 2013 2015	NIV	V	-
Heilongjiang	3001 3002	15-AcDON	I	*F. graminearum*
Luoyang	4002 4003 4004 4005 4007 4008 4011 4012 4015 4016 4018 4019 4020 4021 4023 4024 4025	15-AcDON	I	*F. graminearum*
Zhengzhou	4006 4022	3-AcDON	V	-
Wuhan	5059 5068 5179	NIV	V	-
	5111 5133 5144 5075 5095	3-AcDON	V	-
	5167	15-AcDON	I	-
	5070 5035 5056 5157	N-15-AcDON	V	*F. asiaticum*
Qianjiang	5094 5168 5062 5085 5088	3-AcDON	V	-
	5041	15-AcDON	I	-
Jingzhou	5134 5137	3-AcDON	V	-
Jingmen	5074 5152	NIV	V	-
	5100 5039 5127 5156 5245	3-AcDON	V	-
	5096	N-15-AcDON	V	-
Huangshi	5004 5011 5071 5128 5171 5126	3-AcDON	V	*F. asiaticum*
	5048	N-15-AcDON	V	-
Enshi	5084 5090 5153 5008 5093 5117 5240	NIV	V	-
	5046 5110	3-AcDON	V	-
	5003	N-15-AcDON	V	-
Xiaogan	5116 5165	3-AcDON	V	-
Xiangfan	5097 5120 5149	NIV	V	-
	5065	3-AcDON	V	-
	5135	15-AcDON	I	-
	5005 5140	N-15-AcDON	V	-
Shiyan	5081	NIV	V	-
	5078 5087 5112 5159 5175 5176 5244	3-AcDON	V	-
	5010 5014 5025 5073 5098 5105 5119 5138 5145 5145 5161 5235	15-AcDON	I	*F. graminearum*
	5107 5148	N-15-AcDON	V	-
Huanggang	5080 5136 5168	NIV	V	*F. asiaticum*
	5109 5058 5106 5163	3-AcDON	V	*F. asiaticum*
Changsha	6008	NIV	V	-
	6004	3-AcDON	V	-
	6001 6009	N-15-AcDON	V	*F. asiaticum*
Changzhou	7017 7093	3-AcDON	V	-
Nantong	7052 7070	NIV	V	*F. asiaticum*
	7043 7055 7090 7063 7096 7048	3-AcDON	V	-
	7069	15-AcDON	I	-
Huaian	7039 7058 7092 7118 7109 7111 7034	3-AcDON	V	-
Yangzhou	7113 7072	3-AcDON	V	-
Suqian	7080	3-AcDON	V	-
Nanjing	7071 7035	NIV	V	-
	7056	3-AcDON	V	-
Zhenjiang	7057 7081	NIV	V	-
Suzhou	7106	NIV	V	-
	7107	3-AcDON	V	-
Wuxi	7089 7099 7114	3-AcDON	V	-
Yancheng	7079 7084	NIV	V	-
	7074 7075 7061 7097 7086 7110 7037 7051 7116 7101 7003 7088	3-AcDON	V	-
	7087 7060 7076	15-AcDON	I	*F. graminearum*
Xuzhou	7094 7054 7115	3-AcDON	V	-
	7032 7082 7121 7108 7001 7050	15-AcDON	I	*F. graminearum*
Taizhou	7044 7068	NIV	V	*F. asiaticum*
	7045 7007 7062 7120 7095 7103 7104 7122 7049	3-AcDON	V	*F. asiaticum*
Lianyungang	7042 7091 7122 7040 7064 7065 7105 7119	3-AcDON	V	-

(*continued on next page*)

Table 1 – (continued)

District	Isolate[a]	Mycotoxin chemotype[b]	SCAR[c]	Phylogenetic species[d]
Jiujiang	8008 8013 8015 8022	3-AcDON	V	-
Dean	8016	3-AcDON	V	-
	8006	N-15-AcDON	V	-
Ruichang	8001 8003	3-AcDON	V	-
	8007 8011 8021	15-AcDON	I	*F. graminearum*
	8004	N-15-AcDON	V	-
Shanghai	10001	15-AcDON	I	-
	10004 10007	NIV	V	*F. asiaticum*
	10005 10006 10008	3-AcDON	V	*F. asiaticum*
Danfeng	11011 11045 11064	3-AcDON	V	-
	11010 11035 11047 11051	15-AcDON	I	-
Shangluo	11006 11009 11015 11042	15-AcDON	I	-
Shangnan	11001 11022 11025 11041	3-AcDON	V	*F. asiaticum*
	11012 11018 11019 11024 11027 11029 11033 11034 11040 11049 11065	15-AcDON	I	*F. graminearum*
	11032 11043 11053	N-15-AcDON	V	*F. asiaticum*
Yaan	12001 12002 12003 12004	NIV	V	*F. asiaticum*
Fuyang	13018 13033 13041 13050 13053 13072 13076	NIV	V	*F. asiaticum*
	13012 13011 13024 13029 13030 13035 13042 13048 13049 13070 13071 13073	3-AcDON	V	-
	13023 13032 13056 13061	N-15-AcDON	V	*F. asiaticum*
Hangzhou	13028 13054	NIV	V	-
	13013 13014 13015 13016 13017 13019 13021 13022 13027 13036 13037 13038 13040 13047 13051 13052 13055 13058 13059 13060 13075	3-AcDON	V	-
	13039	N-15-AcDON	V	-

a The initial numeral of each stain code, from 1 to 13 (excluding 9), was assigned to refer to the province of strain origin. 1: Anhui; 2: Fujian; 3: Heilongjiang; 4: Henan; 5: Hubei; 6: Hunan; 7: Jiangsu; 8: Jiangxi; 10: Shanghai; 11: Shaanxi; 12: Sichuan; 13: Zhejiang. The underlined isolates were subjected to sequencing analysis of three genes (*Tri101*, *reductase*, *histone H3*).
b Chemotypes were identified by serial PCR assays as described in the text.
c SCAR types were assayed with primer set Fg16F/16R (Carter *et al.* 2000).
d The underlined isolates were identified as *F. asiaticum* or *F. graminearum* by blasting the sequences of three genes of *Tri101*, *reductase* and *histone H3* in the database. "-": not identified by sequencing of the three genes.

asiaticum (six 3-AcDON producers, eight NIV chemotypes and six new 15-AcDON chemotypes) and ten strains belong to phylogenetic species *F. graminearum* that produce 15-AcDON (Table 1, underlined strains). Lineage and phylogenetic species of *Fusarium* mycotoxin chemotypes were analysed and classified according to the methods of O'Donnell *et al.* (2000, 2004).

PCR analysis

PCR reagents used were as previously described (Li *et al.* 2005). All PCR amplifications were conducted in a thermocycler (MJ Research, Watertown, MA) with the following conditions: 95 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 50 s, 72 °C for 50 s (90 s for Tri101 gene) and a final extension at 72 °C for 6 min. PCR products with all the primers, except the primer pair GzTri7f1/r1, were separated by electrophoresis on 1.2 % agarose gels, stained with ethidium bromide and visualized under UV light in the Bio-Imaging System (Bio-Rad, Hercules, CA). PCR products amplified with the primer pair GzTri7f1/r1 were electrophoresed on 5 % polyacryamide gels followed by silver staining.

DNA sequencing

PCR products were purified using a Axyprep DNA Gel Extraction Kit (Axygene Biosciences, Union City, CA), cloned into pBluescript plasmids and sequenced in both directions with the dideoxy terminator cycle sequencing kit (Applied Biosystems PRISM, Big DyeTM Terminators v3.0, Foster City, CA) on ABI377 automated DNA sequencer.

Climate data from different regions

Based on the climate data during the 30-y period from 1970 to 1999 (http://data.cma.gov.cn/index.jsp), the annual average and highest temperatures were calculated for all regions in China where *Fusarium* strains were collected and used for association analysis of mycotoxin chemotypes with their geographical distribution.

Results

Trichothecene chemotypes of the Fusarium graminearum clade

PCR assays with the primer pair ToxP1/P2 revealed that 246 (82 %) of 299 *F. graminearum* clade isolates produced a 300 bp fragment, indicating their identities as *Fusarium* DON producers. For all the remaining 53 (18 %) isolates, a 360 bp PCR product was obtained under the same conditions and thus they were classified as NIV producers (Fig 1A) (Li *et al.* 2005).

To identify which DON derivatives were produced, all isolates were subjected to PCR assays with two pairs of primers, Tri303F/R and Tri315F/R. The results showed that with the primer pair Tri303F/R 155 (63 %) of 246 DON producers yielded a 3-AcDON-specific fragment of 586 bp (Fig 1B), and a 864 bp of 15-AcDON-specific fragment was amplified from 91 (37 %) DON producers with the primer pair Tri315F/R (Fig 1C), whereas no PCR products were seen from NIV producers (Fig 1B–C). Thus 3-AcDON is the predominant mycotoxin produced by the *F. graminearum* clade in China, accounting for 52 % of all the *Fusarium* isolates assayed.

Variations among DON chemotypes

To further characterize 3-AcDON and 15-AcDON chemotypes, two pairs of primers of MinusTri7F/R and GzTri7f1/r1 were used for PCR assays of all the isolates. The results showed that with the primer pair MinusTri7F/R, only 3-AcDON chemotypes produced a 483 bp fragment, and no product was detected from 15-AcDON or NIV chemotypes (Fig 2). Primer pair GzTri7f1/r1 generated a 161 bp fragment from all the NIV chemotypes (Fig 3) and no product from 3-AcDON producers.

PCR assays of 91 15-AcDON-producing isolates with the primer pair GzTri7f1/r1 revealed two different patterns of PCR products (Fig 3). Sixty-eight isolates yielded DNA fragments ranging in size from 205–315 bp and sequencing analysis confirmed that these isolates contained four to 14 tandem repeats of an 11-bp nucleotide stretch (CACAATATTAG) within their *Tri7* gene sequences (Lee *et al.* 2001) and no isolates contained less than four repeats. However, the remaining 23 15-AcDON producers generated only a 161 bp fragment, as did the NIV producers, indicating the absence of the 11-bp repeats within their *Tri7* sequences (Fig 3), which was verified by sequencing analyses. These results indicated that there were two classes of 15-AcDON-producing isolates of *F. graminearum* clade and a distinct new subpopulation of 15-AcDON producers different from that described so far appears to be present in *Fusarium* populations in China.

SCAR types of mycotoxin chemotypes

SCAR assays of all the strains with primer pair Fg16F/16R revealed the presence of two SCAR groups, I and V, and an association of *Fusarium* chemotypes with their SCAR groupings (Table 2). All the NIV and 3-AcDON chemotypes belong to SCAR group V. However, 15-AcDON-producing isolates vary and can be divided into SCAR groups I and V. Of the 91 15-AcDON producers, 68 with variable numbers of the 11-bp repeats (Fig 3), as previously reported (Lee *et al.* 2001), belong with SCAR group I, whereas the new subpopulation consisting of 23 isolates without the 11-bp repeats resides in SCAR group V (Table 2).

Lineage and phylogenetic species of mycotoxin chemotypes

Sequencing analyses of the *Tri101*, *reductase*, and *histone H3* gene sequences from 30 strains of NIV, 3-AcDON, 15-AcDON and the new 15-AcDON chemotypes, respectively, indicated that all the three chemotypes from SCAR group V carry the sequences of three genes characteristic of lineage 6 (O'Donnell *et al.* 2000), phylogenetic species *Fusarium asiaticum* (O'Donnell *et al.* 2004). In contrast, 15-AcDON chemotypes from SCAR group I contain *Tri101*, reductase and histone H3 gene sequences typical of lineage 7, *F. graminearum* species (O'Donnell *et al.* 2000, 2004) (Table 2). Therefore, *F. asiaticum* species, lineage 6 strains in China are able to produce 3-AcDON, 15-AcDON and NIV, among which 3-AcDON is the predominant chemotype. However, *F. graminearum* species lineage 7 strains produce only 15-AcDON and do not produce 3-AcDON or NIV (Table 2). The difference between *F. graminearum* and *F. asiaticum* that produce 15-AcDON is the presence or absence of the 11-bp

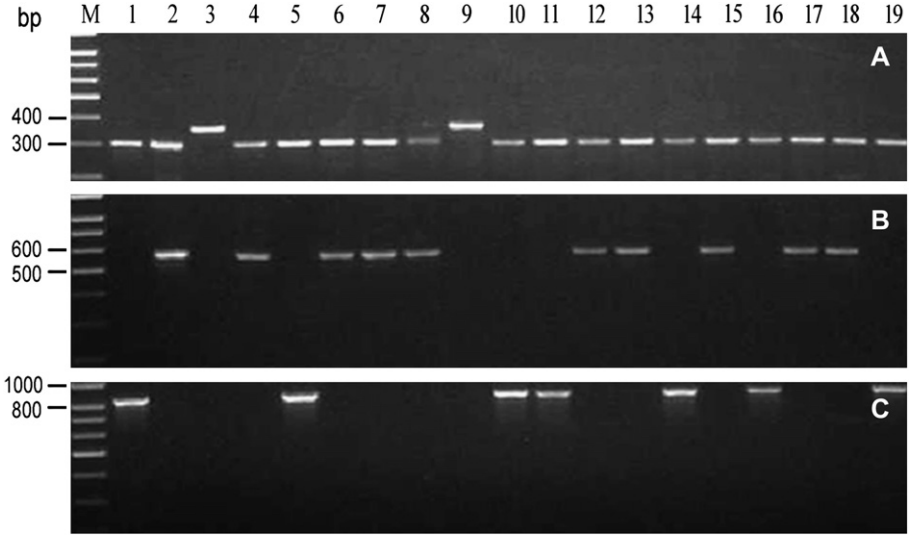

Fig 1 – PCR amplification of (A) DON and NIV chemotypes (primer set ToxP1/P2), (B) 3-AcDON chemotypes (primer set Tri303F/R) and (C) 15-AcDON chemotypes (primer set Tri315F/R) of the *Fusarium graminearum* clade. M: 100 bp ladders; lanes 1–19: 3001, 1001, 2005, 4006, 5005, 5134, 5245, 6004, 12001, 5119, 6001, 7086, 8003, 7087, 10008, 11018, 11064, 13017 and 11034.

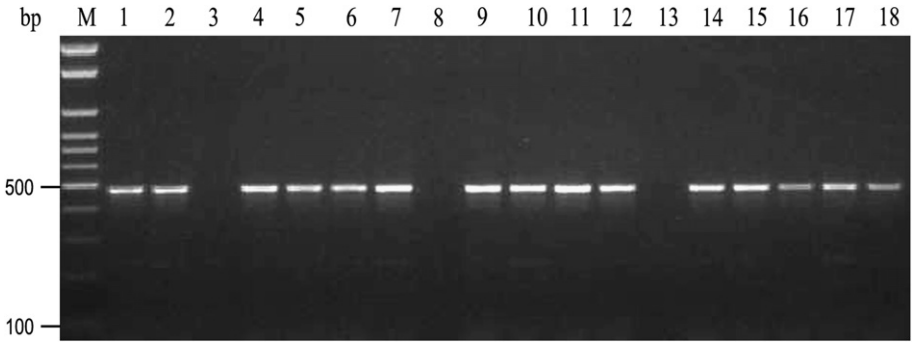

Fig 2 – PCR amplification of the 3-AcDON-, 15-AcDON- and NIV chemotypes of *Fusarium graminearum* clade with the primer set Minus Tri7F/R. M: 100 bp ladders; lanes 1, 2, 4–7, 9–12, 14–18: 3-AcDON-chemotype isolates (1001, 4006, 5134, 5245, 6004, 7086, 8003, 10008, 11064, 13017, 1018, 5128, 7003, 8016, 13075); lane 3: NIV-chemotype isolate (2005); lanes 8 and 13: 15-AcDON-chemotype isolates (5005, 11018).

repeats within their *Tri7* sequences. The 11-bp repeat is present in *F. graminearum* but absent in *F. asiaticum* (Table 2).

Geographical distributions of mycotoxin chemotypes

Geographical distributions of *Fusarium* mycotoxin chemotypes identified above are apparently associated with the annual average temperatures (or the annual highest temperatures) in the regions. Fig 4 illustrates a map with the annual average and annual highest temperatures over the 30-y period 1970 to 1999 for the regions in China where *Fusarium* isolates were collected, and the distributions of their chemotypes are indicated. Occurrence of one or more mycotoxin chemotypes in one district is represented with different signs on the map of Fig 4. In the cooler regions where the annual average temperatures were 15 °C or lower, such as in the provinces Heilongjiang, Henan and Shaanxi, DON producers are present. In contrast, NIV chemotypes and the new subpopulation of 15-AcDON producers occur mostly in warmer regions where the annual average temperatures were above 15 °C (Fig 4).

Two *Fusarium* phylogenetic species appear to have different geographical distribution patterns. Ninety-five percent of *F. aisanticum* species lineage 6 that produce 3-AcDON, 15-AcDON and NIV mycotoxins originated from warmer regions, whereas *F. graminearum* species lineage 7, which consists only of 15-AcDON chemotypes, was predominantly from cooler regions (Table 2; Fig 4).

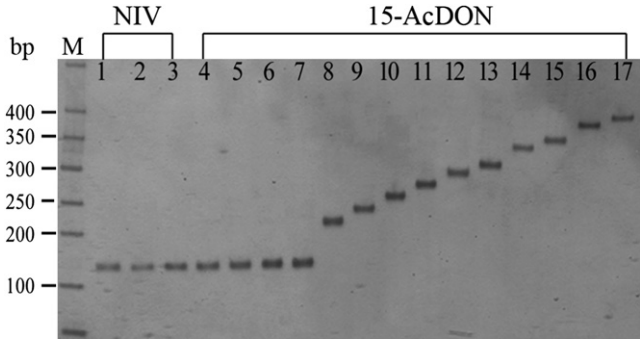

Fig 3 – PCR amplification of NIV and 15-AcDON chemotypes of the *Fusarium graminearum* clade with primer set GzTri7f1/r1. M: marker; lanes 1 to 3: NIV-chemotype isolates (2005, 5059, 12001); lanes 4 to 17: 15-AcDON-chemotype isolates (5005, 6001, 8004, 11053, 3001, 5119, 5235, 7050, 4004, 11018, 7087, 11034, 5135 and 5041). PCR products were run on a 5 % polyacrylamide gel.

Discussion

The present study provides an overall mycotoxin chemotype profile of the strains of *Fusarium graminearum* isolated from wheat in FHB-epidemic regions of China. Of the 299 strains assayed, 155 or 52 % produce 3-AcDON mycotoxins, 91 or 30 % are 15-AcDON producers, and 53 or 18 % are NIV chemotypes (Table 2). All the strains could be classified into two phylogenetically different species, *F. asianticum* (77 %) and *F. graminearum* (23 %), according to the fixed nucleotide sequences of three phylogenetically informative genes defined by O'Donnell *et al.* (2000, 2004). The two phylogenetic species are fully congruent with SCAR groupings V and I. Strains from *F. asianticum* species lineage 6 are within SCAR group V, whereas *F. graminearum* species lineage 7 are of SCAR group I (Table 2). Chandler *et al.* (2003) reported that SCAR group V strains belonged to lineage 6, and SCAR group I resided in lineage 7. Nevertheless, lineage 6 also contained strains from SCAR groups 3 and 4, and lineage 7 comprised strains from SCAR group 6. In addition, they observed that three Nepalese strains from rice (RL1, RL2 and RL3), originally classified as SCAR group I, carried a fragment sequence that was different from other SCAR sequences, and thus, they re-designated them as SCAR group I* and placed them in lineage 9 based on *Tri101* sequences. For all the strains within each SCAR group described in this study, no variants were detected when assayed in parallel with the three Nepalese strains (Qu *et al.* unpubl.). It would be interesting to see the correlation between the two phylogenetic species originated from other geographical locations and their SCAR groupings.

Table 2 – Association of mycotoxin chemotypes of *F. graminearum* clade with their *Tri7* genes, SCAR types, lineages, phylogenetic species and geographical distributions

Chemotype[a]	Isolate number	11-bp Repeat[b]	SCAR[c]	Lineage[d]	Phylogenetic species[e]	Distribution[f]	
						≤15 °C	>15 °C
NIV	53	no	V	6	*F. asiaticum*	2	51
3-AcDON	155	no	V	6	*F. asiaticum*	10	145
New 15-AcDON	23	no	V	6	*F. asiaticum*	1	22
15-AcDON	68	yes	I	7	*F. graminearum*	40	28

a Chemotypes were identified by serial PCR assays as described in the text.
b 11-bp Repeats were identified with primer set GzTri7f1/r1 (Lee *et al.* 2001).
c SCAR types were assayed with primer set Fg16F/16R (Carter *et al.* 2000).
d O'Donnell *et al.* (2000).
e O'Donnell *et al.* (2004).
f Distributions of *Fusarium* isolates were based on all the assays and Fig 4. Temperatures were derived from the annual average temperatures of the 30-y period from 1970–1999 in the regions where the *Fusarium* isolates were collected.

The trichothecene chemotypes are apparently associated with the fungal phylogenetic species and geographical distributions. *F. asiaticum* species lineage 6 comprise 3-AcDON, 15-AcDON and NIV chemotypes, 95 % of which were originated from the warmer regions. However, *F. graminearum* species lineage 7 consists only of 15-AcDON producers, the majority of which were from the cooler regions (Table 2; Fig 4). Korean strains from maize also showed an association between chemotype and phylogenetic species in that *F. asiaticum* produced NIV, whereas *F. graminearum* produced DON (Jeon

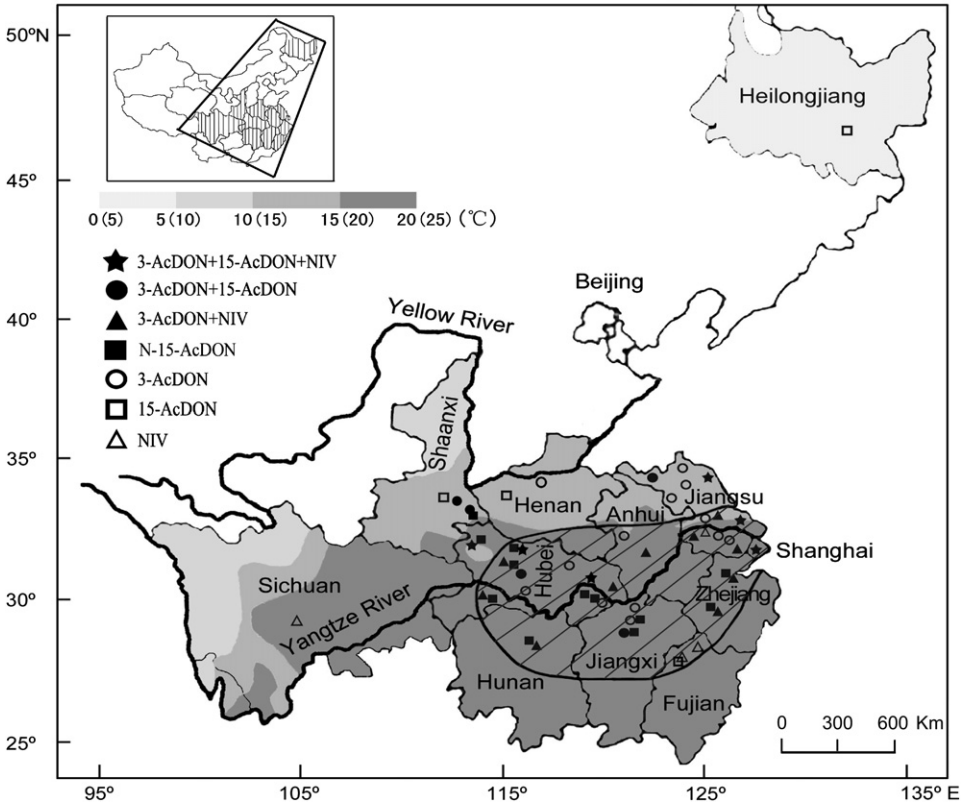

Fig 4 – Geographical distributions of mycotoxin chemotypes from *Fusarium graminearum* clade in China and their associations with regional temperatures. Temperature zones are arbitrarily divided and illustrated at 5 °C intervals starting from north to south based on the annual average temperature (or the annual highest temperature, numbers in brackets under the temperature line) data from the 30-y period of 1970–1999. Distributions of mycotoxin chemotypes are indicated on the map. Different signs designate the occurrence of one, two or three mycotoxin chemotypes in one district, as follows: black star (3-AcDON, 15-AcDOn and NIV), black circle (3-AcDON and 15-AcDON), black triangle (3-AcDON and NIV), black square (N-15-AcDON, new subpopulation of 15-AcDON), white circle (3-AcDON), white square (15-AcDON), and white triangle (NIV). The areas, along with the middle and lower reaches of the Yangtze river, with the most frequent FHB outbreaks are marked with sloping lines.

et al. 2003). Similar results were reported for the Japanese strains where *F. asiaticum* strains produced NIV, 3-AcDON and 15-AcDON. However, both 3-AcDON and 15-AcDON chemotypes were detected in *F. graminearum* species (Suga et al. 2005). The Japanese strains were isolated from wheat and barley, which is different from this study. Different hosts for the strains may cause this discrepancy between mycotoxin chemotypes from China and Japan. Furthermore, in the USA where only a single phylogenetic species *F. graminearum* lineage 7 is present, 95 % of strains produced 15-AcDON and 5 % were 3-AcDON chemotype, which was only identified in samples from two northern states, North Dakota and Minnesota (Gale et al. 2003). The current study showed that there were no 3-AcDON chemotypes in the population of Chinese *F. graminearum* species, which may reflect a geographical difference between FHB epidemic regions in China and the two states in USA or may be due to the limited numbers of strains used.

The presence of varied numbers of an 11-bp repeat within *Tri7* sequences detected with the primer pair GzTri7f1/r1 was considered to be characteristic of *F. graminearum* DON producers in comparison with NIV producers that contain a 161 bp fragment without the 11-bp repeat (Lee et al. 2001). However, when we used this pair of primers to assay several DON-producing isolates, including isolate 5005 (Table 1) that was identified as DON producer by a previous chemical analysis (Li et al. 2005), only 161 bp fragments were amplified lacking the 11-bp repeats. These observations prompted us to perform a series of PCR assays to determine mycotoxin chemotypes and to mutually validate the outcomes. All the *Fusarium* isolates were then assayed using PCR with a generic pair of primers ToxP1/P2 to differentiate DON and NIV chemotypes (Li et al. 2005). After determination of 3-AcDON producers with two pairs of primers, Tri303F/R and MinusTri7F/R (Figs 1B, 2), which have the deletion of the entire *Tri7* genes (Ward et al. 2002; Chandler et al. 2003), all the 15-AcDON-producing isolates were reassessed with the primers GzTri7f1/r1 (Fig 3), resulting in the finding of a distinct new subpopulation of 23 isolates that lacked the 11-bp repeats within their *Tri7* sequences (Fig 3). Thus, the serial PCR assays used in the current study are able to determine *Fusarium* chemotypes with greater confidence than are the individual assays previously described (Lee et al. 2001; Kim et al. 2003).

Furthermore, the present results demonstrated that the presence or absence of the 11-bp repeats within *Tri7* gene sequences in 15-AcDON producers is a hallmark for differentiating phylogenetic species *F. asiaticum* lineage 6 and *F. graminearum* lineage 7 recently defined by O'Donnell et al. (2000, 2004). 15-AcDON chemotypes carrying the 11-bp repeats belong to *F. graminearum*, whereas the same chemotypes lacking the 11-bp repeats are of *F. asiaticum* (Table 2). The Chinese species *F. graminearum* produce only 15-AcDON mycotoxins and thus the 11-bp repeats could be used to discriminate *F. graminearum* from *F. asiaticum* in the *F. graminearum* clade. *F. asiaticum* lineage 6 is the predominant species in China (Table 2) (Gale et al. 2002) and also present in other Asian countries (O'Donnell et al. 2000, 2004), whereas *F. graminearum* lineage 7 is reported in Europe, North America, and Asia (O'Donnell et al. 2000, 2004). Therefore, the molecular distinction of these two species would be of global relevance for the identification and monitoring of mycotoxins of contaminated cereal grains and the products thereof, as well as the causal pathogens.

FHB outbreak in wheat was first reported in 1936 in the middle and lower reaches of the Yangtze River in central China (Chen et al. 2000), where epidemic outbreaks occur most frequently and where 22 of the 23 15-AcDON-producing strains from *F. asiaticum* were collected that lack the 11-bp repeats within their *Tri7* sequences (Table 2, Fig 4, hashed areas). In these regions, several durable, resistant, local wheat varieties, such as Sumai 3 and Wangshuibai, were originally cultivated (Chen et al. 2000), and a number of highly aggressive *Fusarium* strains identified (Wu et al. 2005), where the annual average temperatures were above 15 °C. Under these conditions, the intimate interaction between *Fusarium* fungi and wheat plants may have created unique characteristics of *F. asiaticum* species that underlie the mycotoxin chemotypes.

Several studies have revealed that significant levels of sexual recombination occur within the populations of the *F. graminearum* clade (Bowden & Leslie 1999; O'Donnell et al. 2000; Zeller et al. 2003, 2004). Vegetative compatibility analyses revealed the presence of at least five independent *vic* loci in *F. graminearum* population in China (Hu et al. 2003), and AFLP profiles displayed a high level of genetic diversity (Qu et al. unpublished results). It is generally considered that a large number of VCGs reflect a relatively frequent recombination occurring in a population. It is probable that the abovementioned new chemotypes might be derived from natural hybrids. Further work is required to determine whether a sexual recombination is involved in the population of two *Fusarium* species in China.

The methods used in this study and the results obtained could be used for the effective determination and monitoring of *Fusarium* type B trichothecene mycotoxins at both regional and global levels, as well as for the rapid and accurate differentiation of phylogenetic species from the *F. graminearum* clade. Moreover, this study also serves as a foundation for further investigations into the molecular interaction between mycotoxin metabolism and the environment in *Fusarium*–plant systems during their co-evolution.

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