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Crops are a main driver for species diversity and the toxigenic potential of *Fusarium* isolates in maize ears in China

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RESEARCH ARTICLE

Abstract

In recent years increasing demands and the relatively low-care cultivation of the crop have resulted in an enormous expansion of the acreage of maize in China. However, particularly in China, *Fusarium* ear rot forms an important constraint to maize production. In this study, we showed that members of both the *Fusarium fujikuroi* species complex (FFSC) and the *Fusarium graminearum* species complex are the causal agents of *Fusarium* ear rot in the main maize producing areas in China. Fumonisin producing *Fusarium verticillioides* was the most prevalent species, followed by fumonisin producing *Fusarium proliferatum* and 15-acetyldeoxynivalenol producing *F. graminearum*. Both *Fusarium temperatum* and *Fusarium boothii* were identified for the first time in the colder regions in China, extending their known habitats to colder environments. Mating type analysis of the different heterothallic FFSC species, showed that both types co-occur in each sampling site suggestive of the possibility of sexual recombination. Virulence tests with *F. boothii* (from maize) and *F. graminearum* from maize or wheat showed adaptation to the host. In addition, *F. graminearum* seems to outcompete *F. boothii* in wheat-maize rotations. Based on our findings and previous studies, we conclude that wheat/maize rotation selects for *F. graminearum*, while a wheat/rice rotation selects for *F. asiaticum*. In contrast, *F. boothii* is selected when maize is cultivated without rotation. A higher occurrence of *F. temperatum* is observed on maize in colder climatological regions in China, while *Fusarium meridionale* seems restricted to mountain areas. Each of these species has their characteristic mycotoxin profile and deoxynivalenol and fumonisin are the potential threats to maize production in Northern China.

Keywords: fumonisin production, *Fusarium fujikuroi* species complex, *Fusarium graminearum* species complex, increasing maize production, trichothecene production

1. Introduction

China is the second largest maize production country in the world. In recent years, both the yield and acreage of maize increased rapidly, surpassing rice and now maize has become the largest grain crop in China (data from China's National Bureau of Statistics). However, ear rot diseases caused by *Fusarium* spp. are a serious constraint on maize production. *Fusarium* spp. not only cause a high economic loss every year in maize and small grain cereal production, but also accumulation of mycotoxins (mainly fumonisins and trichothecenes) in resulting crops (Bottalico, 1998). These mycotoxins are a significant risk to food safety and animal health, because they cause mycotoxicoses in animals and humans (Marasas, 2001; Ueno *et al.*, 1973).

Fusarium species are responsible for at least two kinds of maize ear rot. Customarily, disease caused by members

of the *Fusarium graminearum* species complex (FGSC) is known as *Gibberella* or red ear rot, and ear rot caused by the members of *Fusarium fujikuroi* species complex (FFSC) is known as *Fusarium* or pink ear rot. However, '*Fusarium*' is advocated as the sole name for a group of fungi that includes virtually all *Fusarium* species of importance in plant pathology, mycotoxicology, medicine, and basic research (Geiser *et al.*, 2013). To avoid confusion, in this report, we use *Fusarium* ear rot (FER) as the sole disease name to represent maize ear rot caused by *Fusarium* species including both members of FGSC as well as FFSC.

Members of the FGSC are some of the most frequently isolated causal agents of *Fusarium* head blight (FHB) of wheat and barley and FER of maize (Boutigny *et al.*, 2011; Ward *et al.*, 2008; Yang *et al.*, 2008). Phylogenetic species recognition, with genealogical concordance, has provided strong evidence that FGSC comprises at least 16 distinct species. On maize in South Africa only *Fusarium boothii* was found (Boutigny *et al.*, 2011). In Nepal, the predominant species on maize were *Fusarium meridionale* and *Fusarium asiaticum* (Desjardins and Proctor, 2011), while *F. meridionale* was also predominant in Argentina (Sampietro *et al.*, 2011). In South Korea, four species within the FGSC were associated with FER and *F. graminearum* was predominant (Lee *et al.*, 2012). A similar situation was also found in France and Italy, where *F. graminearum* was the main causal agents of FER (Boutigny *et al.*, 2014; Somma *et al.*, 2014). Within the FGSC there are three types of trichothecene mycotoxin producers (chemotypes): (1) nivalenol (NIV) producers and acetylated derivatives; (2) deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON); and (3) DON and 15-acetyldeoxynivalenol (15-ADON; Miller *et al.*, 1991). The vast majority of *F. graminearum* isolates reported on maize in France, Korea and China were 15-ADON producers (Boutigny *et al.*, 2014; Lee *et al.*, 2012; Ndoye *et al.*, 2012) while in Europe a small population of NIV producers was found (Boutigny *et al.*, 2014; Ndoye *et al.*, 2012; Somma *et al.*, 2014). Most *F. asiaticum* on maize in Asia produce NIV (Desjardins and Proctor, 2011; Lee *et al.*, 2012; Ndoye *et al.*, 2012), while *F. meridionale* and *F. boothii* isolates seem to be fixed for NIV and 15-ADON production, respectively. This is comparable to reports from samplings in South Africa, Nepal and Argentina (Boutigny *et al.*, 2011; Desjardins and Proctor, 2011; Sampietro *et al.*, 2011). In China, Ndoye *et al.* (2012) reported that on maize *F. asiaticum* with the NIV chemotype dominated in warmer regions while *F. graminearum* with the 15-ADON chemotype is dominant in cooler regions.

In many countries, members of the FFSC are the most important causal agent of maize ear rot. In contrast, on wheat and barley members of the FFSC are of minor importance and disease is mostly caused by members of the FGSC. Therefore, with the increase of maize cultivation the ratio of FFSC to FGSC species in fields may change. Molecular systematics has revealed that FFSC includes at least 50 distinct species, many of which are pathogens of both plants and human (Kvas *et al.*, 2009). Many FFSC species can produce fumonisins, a family of mycotoxins, causing various diseases in animals, and it has been associated epidemiologically with oesophageal cancer as well as neural tube defects in some human populations (Gelderblom *et al.*, 1988; Marasas *et al.*, 2004). In maize, *Fusarium verticillioides*, *Fusarium proliferatum* and *Fusarium subglutinans* are the most prevalent species worldwide (Adejumo *et al.*, 2007; Aguin *et al.*, 2014; Madania *et al.*, 2013; Qiu *et al.*, 2015; Torres *et al.*, 2001). The first two species are potential fumonisin producers (Logrieco *et al.*, 2002), while *F. subglutinans* is generally believed to be a fumonisin non-producer as the fumonisin biosynthetic genes are absent (Proctor *et al.*, 2004). *Fusarium temperatum* is a newly described species first found on maize in Belgium. It is closely related to *F. subglutinans*, but showed a different mycotoxin profile, especially for beauvericin production (Fumero *et al.*, 2015; Scauflaire *et al.*, 2012).

In China, most studies about species composition and population dynamics focus on *Fusarium* isolates from barley and wheat (Yang *et al.*, 2008; Zhang *et al.*, 2012). There are a few, partially conflicting, reports on maize. Ndoye *et al.* (2012) concluded that on maize the NIV chemotype of *F. asiaticum* is predominant in southern China and the 15-ADON chemotype of *F. graminearum* in northern China. However, the methodology applied by these researchers does not allow sufficient species discriminatio. Qiu *et al.* (2015) found fumonisin producing *F. verticillioides* to be the most prevalent on maize in southern provinces Jiangsu and Zhejiang, but these are no main maize producing areas. So a detailed description of all *Fusarium* species causing FER in the main maize producing areas in China is still unknown. Demonstrating which *Fusarium* spp. and their chemotypes occur in maize will help to understand the nature of recent FER outbreaks and mycotoxin contaminations, and thus is essential for developing effective strategies for preventing disease and mycotoxin contamination in agricultural products.

The objectives of this research were to study the cause of FER on maize in the major maize producing areas in China, now that maize has become the largest cereal crop. Specifically, we (1) characterised this population at the species level and studied the distribution of the different species over the various regions, (2) determined the potential mycotoxins produced by these *Fusarium* species, (3) compared the variation in pathogenicity of FGSC species from wheat and maize to analyse their host preference and (4) discuss the influence of agronomic and climatic conditions on the distribution of *Fusarium* spp. and associated mycotoxins.

2. Materials and methods

Fungal isolates

Diseased maize ears with pink or white spore mass, which were collected from 53 sampling sites in 6 provinces of China in 2013 (Figure 1), were used for isolation of *Fusarium* strains. Diseased seeds were surface sterilised in 70% ethanol for 30 s, and immediately immersed in 10% sodium hypochlorite for 90 s, after which the kernels were extensively rinsed with sterile distilled water. After drying on sterile filter paper, the seeds were placed on potato dextrose agar (PDA) plates (Chen *et al*., 2009). After 3 days of incubation at 28 °C, newly grown-out mycelium was transferred onto a fresh medium to generate uniform mycelial colonies. All *Fusarium* isolates were purified using the single-spore isolation method as previously described (Zhang *et al.,* 2012). To prevent re-isolation of the same isolate only one isolate was preserved from each maize cob after single-spore isolation. Single spore cultures were stored in 15% dimethyl sulfoxide (DMSO) at -80 °C. In order to compare the variation in pathogenicity of *Fusarium* from different hosts, we included fifteen *F. graminearum* isolates collected from wheat heads in a previous study (Zhang *et al.*, 2012) in the pathogenicity test. These isolates were collected in Hebei (n=7), Shandong (n=2), Heilongjiang (n=2), Shaanxi (n=2) and Henan (n=2) respectively.

Species determination

Fungal isolates were inoculated on PDA and incubated at 28 °C for 5 days. Mycelia were harvested, freeze-dried and ground to a fine powder using a MiniBeadbeater-96 system (BioSpec, Bartlesville, OK, USA). Genomic DNA was extracted with E.Z 96™ Fungal DNA Kit (Omega Bio-tec, Inc., Norcross, GA, USA) according to the manufacturer's instructions. After that, part of the translation elongation factor gene (*TEF-1α*, ~700 bp) was amplified and sequenced as previously described (Geiser *et al.,* 2004). The strains were identified by sequence comparison with the FUSARIUM-ID database (http://isolate.fusariumdb.org) and at GenBank (http://blast.ncbi.nlm.nih.gov).

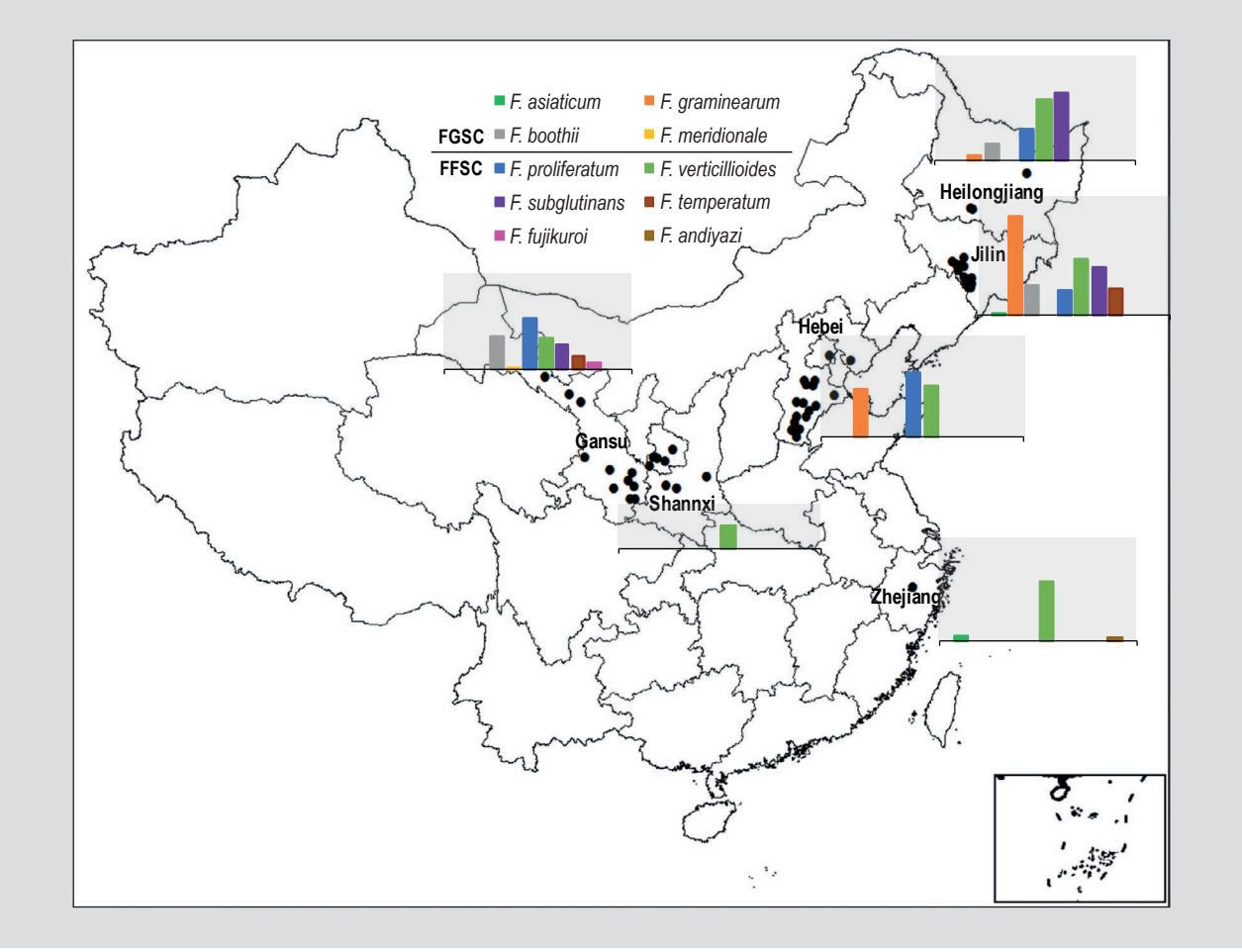

Figure 1. Map of China indicating the 175 sampling sites in 15 provinces. Coloured bars represent the numbers of isolates obtained in each province.

Phylogenetic and gene diversity analysis

Genetic diversity parameters of the *TEF-1α* gene were calculated using the program DnaSP (Librado and Rozas, 2009), including haplotypes, number of segregating sites, number of parsimony informative sites between alleles and haplotypic diversity. Phylogenetic analyses of *TEF-1α* gene were performed with MEGA5.2 for maximum likelihood analysis using default settings (Tamura *et al*., 2011). The reliability of these tree topologies was evaluated using bootstrap support with 1000 pseudo replicates of the data.

Chemotype and mating types identification

Isolates of FGSC were classified into NIV, 3-ADON, and 15-ADON chemotype by multiplex PCR using primers sets based on *Tri3* and *Tri12* gene sequences (Ward *et al.,* 2008). To assess the ability of FFSC isolates to produce fumonisins, the presence of the *FUM1* genes was checked by PCR amplification using primer sets Fum-1/Fum-2 (Baird *et al.*, 2008) and Fum-3/Fum-4 (Bluhm *et al.*, 2002). Primer sets Gfmat1a/Gfmat1b and Gfmat2c/Gfmat2d were used to identify the mating type (MAT-1 or -2) of FFSC strains (Steenkamp *et al.*, 2000). Primers used in this study are listed in Table 1.

Pathogenicity test

To compare the pathogenicity of *F. graminearum* isolates from wheat (Fg-wheat), *F. graminearum* isolates from maize (Fg-maize) and *F. boothii* isolates from maize (Fb-maize), fifteen isolates were selected randomly from each population. Medium resistant cultivar Yangmai 158 was selected for the pathogenicity on wheat. It was planted in three different blocks according to normal agronomic practices. At anthesis, ten heads in each block were inoculated by injecting 20 µl conidia suspension (10^6 conidia/ml) of each individual isolate into the floral cavity of the central floret of a spike (Zhang *et al.*, 2012). A total of 30 heads was injected by a single strain. Pathogenicity was assessed as the incidence of infected spikelets (IIS). The IIS was determined visually by counting the number of infected spikelets per head 14 days after inoculation and was expressed as percentage of total spikelets.

The pathogenicity of *Fusarium* isolates on maize stalks was tested by toothpick inoculation as described previously (Scauflaire *et al.,* 2012). Zhengdan 958, the most widely grown maize variety in China, was planted in three different blocks. The 45 strains were cultured in 15-ml tubes containing 15 triple autoclaved toothpicks and 5 ml potato dextrose broth medium (PDB; Difco, Franklin Lakes, NJ, USA), and incubated for 2 weeks at 28 °C in the dark. One day before plant inoculation, the toothpicks were removed from the tubes and air-dried in a sterile bench. Five 7-week-old maize plants per block were selected and the stalks were inoculated at approximately 10 cm above ground by insertion of toothpicks inoculated with single isolate. The toothpick was cut at the stalk surface and the stalk was sealed with Parafilm™ (Bemis NA, Neenah, WI, USA). After 11 days of growth the plants were harvested and the stalks were cut longitudinally with a sterile knife flanking the inoculation point. The length of the necrotic region in the stalk was measured. A total of 15 replicates

Table 1. List of polymerase chain reaction (PCR) primers used in this study.

Primer	Sequence (5' to 3')	Target (PCR fragment length)	Reference
EF1	ATGGGTAAGGA(A/G)GACAAGAC	*TEF-1α*, ~700 bp	Geiser *et al.,* 2004
EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT		
3CON	TGGCAAAGACTGGTTCAC	*Tri3*	Ward *et al.,* 2008
3NA	GTGCACAGAATATACGAGC	840 bp NIV	
3D15A	ACTGACCCAAGCTGCCATC	610 bp 15-ADON	
3D3A	CGCATTGGCTAACACATG	243 bp 3-ADON	
12CON	CATGAGCATGGTGATGTC	*Tri12*	Ward *et al.,* 2008
12NF	TCTCCTCGTTGTATCTGG	840 bp NIV	
12-15F	TACAGCGGTCGCAACTTC	670 bp 15-ADON	
12-3F	CTTTGGCAAGCCCGTGCA	410 bp 3-ADON	
Fum-1	GTCCTACGCGATACATCCCACCACAAT	*FUM1*, 419 bp	Baird *et al.,* 2008
Fum-2	GATCAAGCTCGGGGCCGTCGTTCATAG		
Fum-3	GTCGAGTTGTTGACCACTGCG	*FUM1*, 845 bp	Bluhm *et al.,* 2002
Fum-4	CGTATCGTCAGCATGATGTAGC		
Gfmat1a	GTTCATCAAAGGGCAAGCG	*MAT-1*, ~200 bp	Steenkamp *et al.,* 2000
Gfmat1b	TAAGCGCCCTCTTAACGCCTTC		
Gfmat2c	AGCGTCATTATTCGATCAAG	*MAT-2*, ~800 bp	
Gfmat2d	CTACGTTGAGAGCTGTACAG		

were performed for each strain. The data analysis was done with the statistical software SAS v8 (SAS Institute Inc., Cary, NC, USA).

3. Results

Fusarium species associated with maize ear rot

A total of 395 isolates was collected from 53 sampling sites in six provinces in 2013 (Table 2, Figure 1). The detailed information of species composition in each sampling site is summarised in Table 3. Heilongjiang and Jilin Province are in northeast China and have a very cold and dry climate (annual average temperature is 3~5 °C, and the minimum temperature in winter is about -30~-40 °C). The planting system results in one harvest per year. Hebei Province is in the North China Plain, where the annual average temperature is 10~12 °C. Maize-wheat rotation alternating per year dominates this region. The provinces Shaanxi and Gansu are in northwest China, the south part of this region, where our samples were collected is a little warmer than Hebei. In the south part of Gansu, wheat and maize are sometimes grown in the same area and crop rotation occurs within the same year. These five provinces are important maize producing areas in China producing roughly 40% of the nation's output. Furthermore, we collected diseased ears from a breeding nursery in Dongyang County in Zhejiang Province in Eastern China and south of the Yangtze River, where the acreage of maize is very small. This region is warm and humid; the annual average temperature is about 17 °C.

Based on the results of *TEF-1α* gene sequence alignments against the FUSARIUM-ID and GenBank databases, a total of ten different *Fusarium* species was detected, which could be classified into two species complexes: FFSC (n=283, 71.65%) and FGSC (n=112, 28.35%) (Table 2). Six of the identified species belonged to FFSC, of which *F. verticillioides* was the dominant one that was found in all six provinces with a total of 120 isolates (30.38%). We identified 76 *F. proliferatum* isolates (19.24%) in five provinces. The sister species *F. subglutinans* and *F. temperatum* co-occurred in both northeast and northwest China with a number of 63 (15.95%) and 20 (5.06%) isolates respectively. The remaining 4 strains within FFSC were identified as *Fusarium fujikuroi* (n=3, 0.76%) and *Fusarium andiyazi* (n=1, 0.25%). Among the 112 members of the FGSC, four species were identified including *F. graminearum* (n=70, 17.72%), *F. boothii* (n=38, 9.62%), *F. asiaticum* (n=3, 0.76%) and *F. meridionale* (n=1, 0.25%). The species composition in each province was quite distinct: *F. subglutinans* dominated the most northern province Heilongjiang and the isolation frequency of *F. graminearum* was the highest in the neighbouring province Jilin, *F. proliferatum* was predominant in the provinces Hebei and Gansu and almost all isolates in Shaanxi and Zhejiang, were *F. verticillioides* (Figure 1).

Comparing only the provinces with larger sampled populations (n>50), we found that *F. graminearum* was absent in Gansu, where *F. boothii* was the predominant FGSC species, while *F. boothii* was not isolated in Hebei province, where *F. graminearum* was the sole FGSC species isolated (Table 2).

Table 2. Geographical origin, numbers and species of *Fusarium* populations from maize.

	Sampling sites	Numbers	*Fusarium graminearum* species complex				*Fusarium fujikuroi* species complex					
			F. asiaticum	*F. graminearum*	*F. boothii*	*F. meridionale*	*F. proliferatum*	*F. verticillioides*	*F. subglutinans*	*F. temperatum*	*F. fujikuroi*	*F. andiyazi*
Heilongjiang	3	69	0	2	6	0	12	23	26	0	0	0
Jilin	12	142	1	49	15	0	12	28	24	13	0	0
Gansu	17	84	0	0	17	1	27	16	13	7	3	0
Shaanxi	3	10	0	0	0	0	0	10	0	0	0	0
Hebei	17	64	0	19	0	0	25	20	0	0	0	0
Zhejiang	1[a]	26	2	0	0	0	0	23	0	0	0	1
Total	53	395	3	70	38	1	76	120	63	20	3	1
Percentage			0.76	17.72	9.62	0.25	19.24	30.38	15.95	5.06	0.76	0.25

[a] This site is a nursery for maize breeding while Zhejiang is not a maize-producing province.

Table 3. The distribution of *Fusarium* isolates in sampling sites.

	Sampling sites	Latitude	Longitude	*F. asiaticum*	*F. boothii*	*F. graminearum*	*F. meridionale*	*F. andiyazi*	*F. fujikuroi*	*F. proliferatum*	*F. subglutinans*	*F. temperatum*	*F. verticillioides*	Total	FGSC	FFSC
Heilongjiang	Yumisuo	45°41'17.18'	126°37'11.91'		6	2				7	15		11	41	8	61
	Zhibaosuo	45°43'43.10'	126°30'51.37'							2	11		4	17		
	Jiamusi	46°46'41.71'	130°25'44.74'							3			8	11		
Jilin	Kaoshan	43°22'28.20'	125°01'59.78'			1				3	9	3		16	65	77
	Yitong	43°18'21.17'	125°22'31.51'							2	7	3		12		
	Fucai	43°12'32.88'	125°29'17.81'										7	7		
	Changchun	43°37'38.20'	125°35'12.15'			1				2				3		
	Meihekou	42°38'31.21'	125°50'36.54'			8				3	6	4	2	23		
	Jinhua	42°22'20.11'	125°46'23.03'		4	5					1	3		13		
	Tonghua	42°13'49.73'	125°40'53.47'	1	2	5							1	9		
	Hongmeizhen	42°19'04.87'	125°32'01.07'		5	9							2	16		
	Dongfeng	42°29'40.24'	125°26'52.74'										9	9		
	Dongliao	42°45'47.98'	125°22'27.39'			11								11		
	Jianan	43°05'51.74'	125°05'10.72'		4	5				2	1			12		
	Gongzhuling	43°30'43.52'	124°48'38.24'			4							7	11		
Gansu	Longxi	34°59'10.94'	104°28'43.12'								1			1	18	66
	Zhangye	38°55'16.49'	100°30'3.42'							1				1		
	Wuwei	37°55'50.13'	102°39'7.32'										5	5		
	Zhenyuan	35°29'45.41'	107°30'2.75'		2					1			2	5		
	Qingcheng	36° 1'8.42'	107°52'34.54'							3				3		
	Yongchang	38°14'42.83'	101°59'18.90'								2			2		
	Linxia	35°28'12.97'	103° 3'44.23'									4		4		
	Qincheng	34°19'16.39'	105°49'20.25'		1					1	4			6		
	Liuping	34°53'47.27'	105°43'33.41'								2	3		5		
	Paosha	33°43'22.22'	105°40'41.78'						3				1	4		
	Hongchuan	33°44'51.41'	105°54'14.41'				1			2				3		
	Caofeng	35°37'19.76'	106°50'43.81'							5	1			6		
	Huating	35°14'8.86'	106°37'29.40'		1					1	2		1	5		
	Jikou	34°33'16.76'	105°29'25.60'		3					1	1			5		
	Lixian	34°10'8.42'	104°46'31.82'		2									2		
	Xincheng	35°38'20.54'	106°55'32.35'		5					11				16		
	Pingyuan	35°37'1.79'	107°2'5.75'		3					1			7	11		
Shaanxi	Yangling	34°16'18.82'	108°6'34.66'										3	3	0	10
	Baoji	34°23'23.90'	107°32'13.16'										4	4		
	Weinan	34°47'4.35'	109°46'9.36'										3	3		
Hebei	Langfang	40°00'40.02'	116°54'55.44'							4			6	10	19	45
	Longyao	37°21'15.52'	114°48'43.36'			1								1		
	Mancheng	38°56'49.06'	115°22'0.30'			2				2				4		
	Renxian	37°05'55.07'	114°41'37.68'										2	2		
	Yongnnian	36°47'18.70'	114°31'21.56'			3				4				7		
	Jizhou	37°34'52.14'	115°32'44.27'			1							1	2		
	Gaocheng	37°59'58.52'	114°52'12.26'			2				1			1	4		
	Quzhou	36°46'42.28'	114°54'55.38'			1				1				2		
	Nangong	37°21'34.31'	115°20'22.11'			1							2	3		
	Tangshan	39°41'8.37'	118°06'51.16'			1				3				4		
	Chengan	36°26'53.04'	114°43'25.85'			2								2		
	Xinji	37°57'6.20'	115°15'9.73'										3	3		
	Cangxian	38°12'42.69'	117°00'32.20'			1				5				6		
	Gaoyang	38°41'40.54'	115°48'45.78'										2	2		
	Wuyi	37°46'46.97'	115°54'4.64'							3			3	6		
	Anxin	38°56'2.01'	115°57'13.48'			1								1		
	Qingyuan	38°46'17.50'	115°26'54.28'			3				2				5		
Zhejiang	Dongyang	29°17'31.86'	120°13'7.61'	2				1					23	26	2	24
			Total	3	38	70	1	1	3	76	63	20	120	395	112	283
			Ratio (%)	0.76	9.62	17.72	0.25	0.25	0.76	19.24	15.95	5.06	30.38		28.35	71.65

Phylogenetic analysis

Based on the partial *TEF-1α* sequences, a total of 38 haplotypes was obtained. The dominant species identified in this study showed the highest diversity in haplotype: 14 haplotypes for *F. verticillioides*, 8 for *F. graminearum* and 6 for *F. proliferatum*. Low diversity was found in *F. boothii* (2 haplotypes), *F. subglutinans* (3 haplotypes) and *F. temperatum* (2 haplotypes). A summary of the polymorphism and diversity is presented in Table 4. The sequences of *F. verticillioides* strains showed the highest value of haplotypic diversity (0.862) indicating the highest level of polymorphism. *F. boothii* revealed the lowest diversity, the sequences of most isolates (37/38) were the same haplotype as the holotype of the species, the South African strain CBS 316.73/ NRRL26916) and only one SNP was detected between the two haplotypes resulting in a unique new genotype.

Molecular phylogenetic analysis was performed to reveal the evolutionary relationship of the isolates together with several reference strains of FGSC and FFSC and an isolate of *F. solani* as outgroup. Bootstrap analyses clearly classified all haplotypes into two clades coalescing with either FFSC or FGSC. Within each species complex, the haplotypes clustered into different subclades with reference isolates (Figure 2).

Ability of mycotoxin production

We identified the trichothecene chemotype of isolates within FGSC based on PCR reactions targeted at the *Tri3* and *Tri12* gene (Ward *et al.*, 2008). All *F. graminearum* and *F. boothii* isolates were characterised as 15-ADON producers, while the *F. asiaticum* and *F. meridionale* isolates had the NIV chemotype.

To assess the ability of isolates within FFSC to produce fumonisin, the presence of the *FUM1* gene was checked by PCR amplification using two different primer sets: Fum-1/Fum-2 and Fum-3/Fum-4. All of the 120 *F. verticillioides* isolates were positive with both sets of primers. This indicates that all *F. verticillioides* isolates possess the *FUM1* gene and have the potential to produce fumonisins. All *F. proliferatum* (n=76) and *F. fujikuroi* (n=3) isolates amplified the expected 420 bp fragment of the *FUM1* gene using Fum-1/Fum-2. However, no amplicon was observed using primers Fum-3/Fum-4 due to 1 or more single nucleotide polymorphisms in the primer regions in these species. Finally, all the *F. subglutinans* and *F. temperatum* isolates lacked the *FUM1* gene and are presumably unable to produce fumonisins.

Mating type determination

Species in the FFSC are considered heterothallic with strains having one of the two mating types MAT1-1 or MAT1-2. Regardless of the presence of the mating types for some species no sexual stage is known and hence these are presumed asexual. The populations of *F. verticillioides, F. proliferatum* and *F. subglutinans* all showed both mating type idiomorphs and combining all isolates the proportion of MAT1-1 and MAT1-2 idiomorphs within each species did not significantly differ from equal rations expected for sexual populations (Chi^2-tests, P=0.23, P=0.09 and P=0.31, respectively) (Supplementary Table S1).

Pathogenicity test

F. graminearum is a main pathogen of both wheat and maize worldwide. In contrast, *F. boothii* although phylogenetically related to *F. graminearum*, has never been isolated from wheat. In order to compare their host preference and variation in pathogenicity, we tested the pathogenicity of three populations on wheat and maize stalks: *F. graminearum* isolates from wheat (Fg-wheat), *F. graminearum* from maize (Fg-maize) and *F. boothii* from maize (Fb-maize). Fifteen strains were selected randomly from each population. The IIS of Fg-wheat was significantly higher (P<0.05) than Fg-maize, while Fb-maize showed the

Table 4. Global polymorphism of the nucleotide alignments of sequences of *TEF-1α* gene of *Fusarium* species including more than 20 isolates.

	Species	Sample size	Number of segregating sites	Number of haplotypes	Number of parsimony informative site between alleles	Haplotypic diversity
FGSC	*F. graminearum*	70	10	8	6	0.665
	F. boothii	38	1	2	0	0.053
FFSC	*F. verticillioides*	120	14	14	12	0.862
	F. proliferatum	76	11	6	9	0.363
	F. subglutinans	63	12	3	12	0.258
	F. temperatum	20	4	2	4	0.479

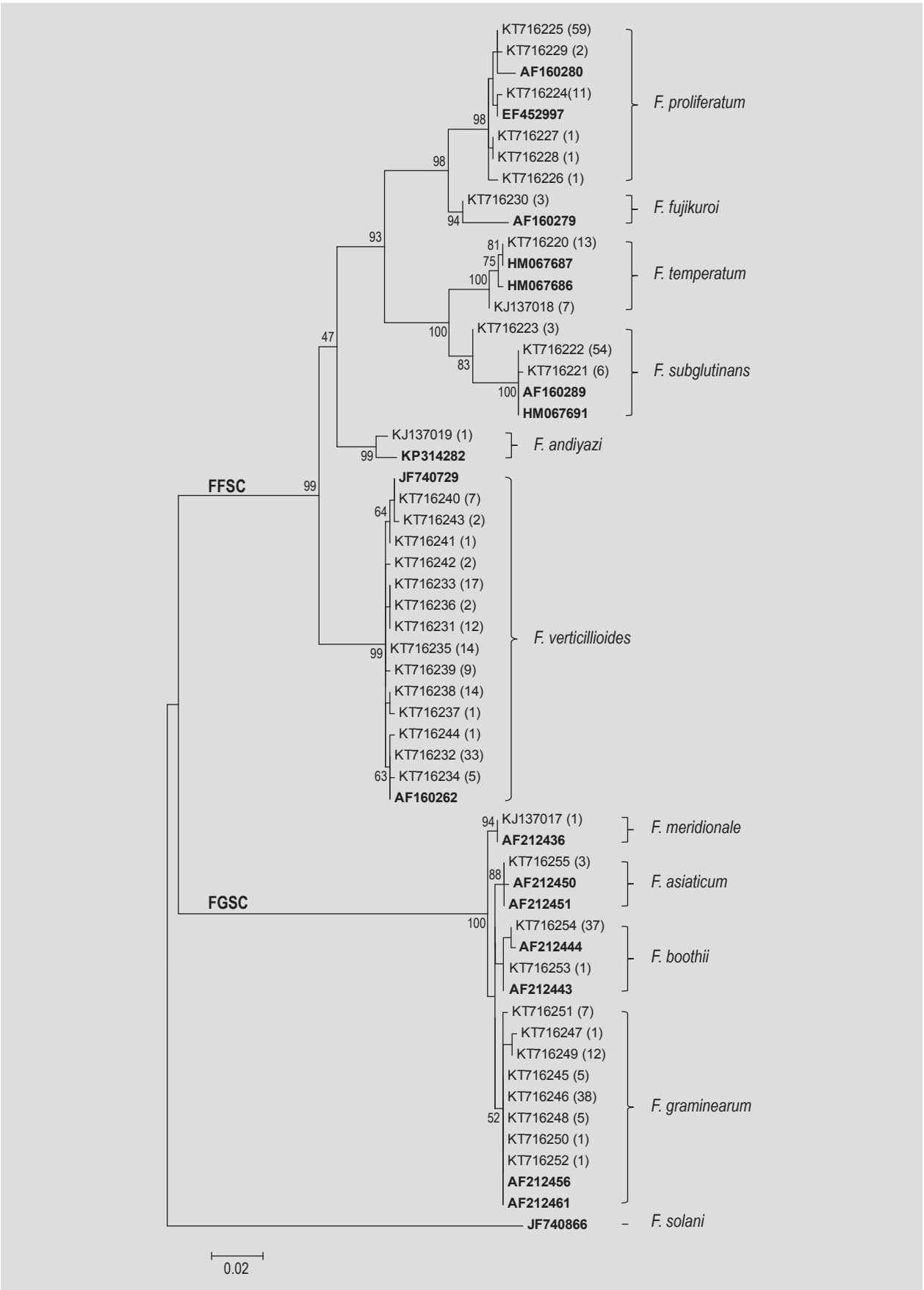

Figure 2. Phylogenetic tree inferred from *TEF-1α* sequences by maximum likelihood method with program MEGA5.2. The numbers above internodes represent bootstrap support based on 1000 pseudoreplicates. The GenBank accession numbers in bold are reference sequences published previously.

lowest pathogenicity. This indicated there is difference in the pathogenicity towards wheat between isolates collected from wheat and maize even if they belong to the same species.

In the pathogenicity assays on maize no specialisation was observed Although Fb-maize and Fg-wheat populations showed the largest and smallest average lesion length on maize stalks respectively, no significant difference (P=0.75) was observed between the three populations (Table 5). Isolates within a single population can vary considerably from one another (Figure 3).

4. Discussion

FER is a devastating disease of maize all over the world, where mycotoxin accumulation depends on the etiological agents. Particularly in China, FER forms an important constraint on maize production. To take the appropriate agronomic measures, population dynamic studies on the pathogens are essential. Therefore, investigating the *Fusarium* species and their mycotoxins associated with FER in maize is indispensable for sustainable agriculture in China. There have been a few reports focusing on particular regions in China (Ndoye *et al.*, 2012; Qiu *et al.*, 2015; Zhang *et al.*, 2013b). Recently, Fu *et al.* (2015) investigated the *Fusarium* species associated with maize kernels from nine provinces in China based on a large collection (n=2,321). Their study focused on the natural occurrence of *Fusarium* on maize including non-pathogenic species and latent infections with *Fusarium*. These researchers collected maize ears at random and no visible signs of mould contamination were observed in most of them. Here, we focused on the pathogen of Fusarium ear rot and all sampled ears showed clear symptoms of FER with pink or white spore masses. Only one isolate from each maize cob after single-spore isolation was preserved, preventing re-isolation of the same isolate. Because of differences in the experimental approach, distinctly different species profiles may be expected between the two studies. Fu *et al.* identified nine *Fusarium* species from non-symptomatic ears. Six of them were also found in this study, indicating that these species are capable of infecting maize latently, but they can also cause disease under conducive conditions. In both studies *F. verticillioides* and *F. graminearum* were the predominant species. However, we also found *F. proliferatum* (19.24%) and *F. subglutinans* (15.95%) to be frequently associated with FER, which occurred only sporadically on seemingly healthy maize ears (Fu *et al.,* 2015). *F. boothii* – absent in Fu's study done in 2012 – was isolated in three provinces with an overall proportion of 9.62%. This may indicate that *F. boothii* infects maize with a short latent period before symptoms occur or became much more prevalent in recent years.

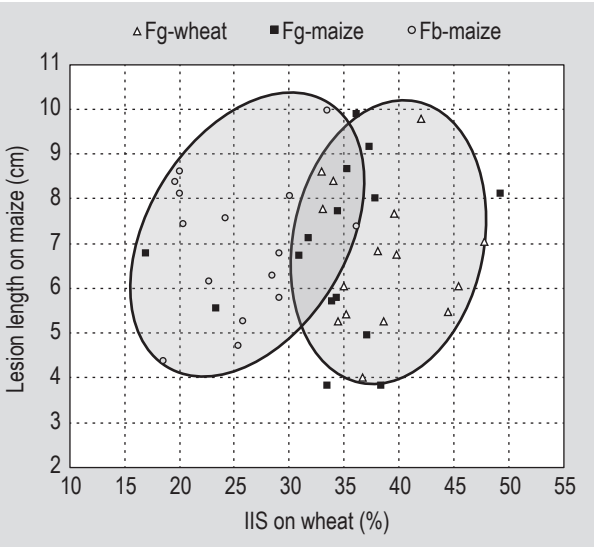

Figure 3. Pathogenicity of *Fusarium graminearum* strains isolated from wheat (Fg-wheat, triangles), *F. graminearum* strains isolated from maize (Fg-maize, diamonds) and *Fusarium boothii* strains isolated from maize (Fb-maize, circles) on wheat (as incidence of infected spikelets; IIS) and on maize (as length of lesions in maize stalks).

Table 5. The incidence of infected spikelets on wheat and lesion length on maize stalks of three population.

Population[1]	Means of IIS[2,3]	Means of lesion length (cm)[3]
Fb-maize	25.39c	6.99a
Fg-maize	33.82b	6.81a
Fg-wheat	38.42a	6.69a

[1] Fb-maize and Fg-maize are *Fusarium boothii* and *Fusarium graminearum* isolates from maize, Fg-wheat are *Fusarium graminearum* isolates from wheat.
[2] IIS is the incidence of infected spikelets. For statistical analysis, IIS data were arcsine transformed.
[3] ANOVA analysis has been performed with Duncan's multiple range test as post-hoc test and values within a column followed by different letters are significantly different at P<0.05.

In total, we identified ten *Fusarium* species in the main maize producing areas in China. *F. verticillioides* is the dominant species that occurred in all provinces. This is in agreement with previous studies in eastern China (Qiu *et al.*, 2015; Zhang *et al.*, 2013b). *F. verticillioides* is also the most commonly isolated fungus from maize across the world (Aguin *et al.*, 2014; Madania *et al.*, 2013; Torres *et al.*, 2001), while also *F. proliferatum* and *F. subglutinans* are commonly associated with FER. We observed a similar trend in China, where the ratio of *F. subglutinans* to *F. proliferatum* proved highest in Heilongjiang and Jilin in the most northern and coldest region in China, while in the more southern provinces Gansu and Hebei, no *F.*

subglutinans was isolated. These results support the notice that *F. subglutinans* prefers a cooler temperature (Boutigny *et al.*, 2012; Goertz *et al.*, 2010; Logrieco *et al.*, 2002).

F. temperatum is a newly recorded species found first in 2011 in Belgium (Scauflaire *et al.*, 2011). Recently, it was reported in many countries, including Spain (Varela *et al.*, 2013), Poland (Czembor *et al.*, 2014), Argentina (Fumero *et al.*, 2015), and Korea (Shin *et al.*, 2014). In China, *F. temperatum* was first reported in Guizhou and Hubei Province (Wang *et al.*, 2014), and even more recent in Sichuan and Yunnan (Fu *et al.*, 2015). These are all mountain regions with a warm climate. Previous reports suggested that *F. temperatum* prefers warmer regions compared to *F. subglutinans* (Fumero *et al.*, 2015; Moretti *et al.*, 2008; Scauflaire *et al.*, 2011). However, in this study, we also collected *F. temperatum* in Jilin, northern China, where the annual average temperatures are 3~5 °C and to our knowledge this is the coldest region where *F. temperatum* was isolated so far. *F. temperatum* co-occurred with its sister species *F. subglutinans*.

F. graminearum is another of the dominant species on maize ears worldwide (Boutigny *et al.*, 2014; Lee *et al.*, 2012). In our collection, 17.7% of the isolates were identified as *F. graminearum*, slightly less than the incidence of *F. proliferatum*. This is in agreement with previous studies for northern China (Ndoye *et al.*, 2012) but distinct from what was reported for northeast China (Ndoye *et al.*, 2012). Based on PCR diagnostics, Ndoye *et al.* identified 70% of the isolates collected in the northeast of China as *F. asiaticum,* while 30% of the isolates were identified as *F. graminearum*. In this study, most isolates within FGSC in the Northeast were *F. graminearum* (n=51, 70%), followed by 21 *F. boothii* (29%) and only one *F. asiaticum* (1%) was identified.

F. boothii was identified in both northwest and northeast of China. This species has not been found on maize in China in previous reports maybe due to the detection methods (Ndoye *et al.*, 2012; Qiu and Shi, 2014; Qiu *et al.*, 2015). *F. boothii* has been identified in many countries including Ethiopia (O'Donnell *et al.*, 2008), Argentina (Sampietro *et al.*, 2010), Kenya (Wagacha *et al.*, 2010), South Africa (Boutigny *et al.*, 2011), Nepal (Desjardins and Proctor, 2011), Mexico (Malihipour *et al.*, 2012), and Korea (Lee *et al.*, 2012). All of these regions are warm compared to the northeast of China. This indicates that *F. boothii* not only occurs in warm regions, but is capable to survive even -30 °C in winter.

Climate is usually thought to be an important driver of species distribution. In Asia, temperature is frequently used to explain *F. graminearum* as the predominant species of FGSC in northern areas, while *F. asiaticum* dominates southern areas (Qu *et al.*, 2008; Suga *et al.*, 2008). However, our previous study did not corroborate this finding. In contrast, a strong association between crops and pathogen species was observed: *F. asiaticum* dominated in rice-wheat rotation areas while *F. graminearum* is linked to maize-wheat rotation (Zhang *et al.*, 2012). Similar relations to host-preference were observed in the USA (Gale *et al.*, 2011), South Korea (Lee *et al.*, 2012) and Brazil (Gomes *et al.*, 2015). Also increased production of maize and/or climatic changes may have resulted in the shift from *F. culmorum* to *F. graminearum* in the Netherlands (Waalwijk *et al.*, 2003). *F. boothii* has a strong association with maize and ear rot. However, it has rarely been found on wheat, with only small populations in Ethiopia (n=9, 29%) (O'Donnell *et al.*, 2008) and Mexico (n=7, 47%) (Malihipour *et al.*, 2012). Many reports on the *Fusarium* composition on wheat and barley are available for China, yet *F. boothii* has never been retrieved (Qiu and Shi, 2014; Yang *et al.*, 2008; Zhang *et al.*, 2010, 2013a). In this study, *F. boothii* was found widely distributed in both northwest and northeast China including 12 sampling sites (Table 3). Approximately half of them were isolated in the provinces Heilongjiang and Jilin, where farmers only grow maize every year. The other *F. boothii* strains were collected from the central and south parts in Gansu. In this area, wheat-maize rotation was customary, but in the most recent decade, the acreage of wheat has decreased sharply and maize became the dominant crop (data from China's National Bureau of Statistics). No *F. boothii* strains were found in Hebei and Shaanxi, where the traditional wheat-maize rotation in one year is customary. Based on this, we propose that *F. boothii* may have maize as preferred host and wheat, which is rotated with maize, restricts its infection cycle. Similar conditions are encountered in South Korea (Lee *et al.,* 2012), where farmers only grow maize and *F. boothii* was commonly detected. It is also interesting that all FGSC isolates (n=17) in Gansu were *F. boothii* and no *F. graminearum* was found. This is reminiscent to the situation in South Africa where a large and unique *F. boothii* population is associated with FER (Boutigny *et al.*, 2011). The cropping system is also similar between Gansu and South Africa, occasionally barley or wheat are rotated with maize over multiple years. In this cropping system, maize is grown continuously if we consider a regional scale, so *F. boothii* can complete all parts of its life cycle on maize. But in Hebei, wheat-maize rotation is strict for two harvests per year. In the wheat season maize is absent in this large region. If wheat is an essential part of the life cycle, *F. boothii* will have to compete with *F. graminearum* on wheat. Alternatively, *F. boothii* may be growing solely on maize and survive on crop debris or in soil during the wheat cultivation. This may result in a small population that is difficult to detect due to its size.

To validate if differentiation in host-specific pathogenicity influences the distribution of *F. boothii*, we tested the pathogenicity of three populations on wheat and maize stalk respectively: *F. graminearum* isolates from wheat (Fg-wheat), *F. graminearum* from maize (Fg-maize) and *F.*

boothii from maize (Fb-maize). These populations showed significant differences in pathogenicity on wheat with the Fg-wheat population as the most aggressive, followed by the Fg-maize population and the Fb-maize being the least pathogenic. This result is in agreement with a previous report in Mexico (Malihipour *et al.*, 2012). Remarkably, in pathogenicity on maize no significant differences were found among these three populations. The toothpick inoculation procedure used to test the pathogenicity on maize stalks may not directly reflect the ability of this species to cause FER. In field conditions, maize ear rot and stalk rot often coincide (Bottalico, 1998; Picot *et al.*, 2012). Stalk rot is easy to score by lesion length and artificial inoculation on maize cobs may not be a good representation for the natural route of infection.

F. boothii showed extremely low haplotypic diversity of *TEF-1α* gene. Only two haplotypes were found and most isolates (37/38) were fixed for one. This indicates that the structure of *F. boothii* populations is much more clonal than that of the other encountered species. We hypothesise that this may represent either a new migrant population with limited genetic variation or an asexually propagating population under a strong selective pressure. Both options may apply for *F. boothii* and more research is required to provide conclusive answers.

In this study only one strain of *F. meridionale* was isolated (0.25%), originating from Hongchuan on the border between Gansu and Sichuan Province. *F. meridionale* was reported in Sichuan, Yunnan and Hubei on wheat and barley, but similar to our study, it was typically found at low frequencies (Wang *et al.*, 2010; Yang *et al.*, 2008; Zhang *et al.*, 2012). This contrasts with reports where *F. meridionale* was the predominant species on maize in Nepal and Northern Argentina (Desjardins and Proctor, 2011; Sampietro *et al.*, 2012). However, small populations of *F. meridionale* were also reported in Brazil (1.1%), Korea (1.2%) and South Africa (3.6%) (wheat 3.6%, maize roots 14%). It seems that *F. meridionale* is preferentially found on maize, but may be affected by other factors. Recently, a larger *F. meridionale* population was identified on wheat in Brazil thought to be related to the two harvests of maize in one year (Del Ponte *et al.*, 2015). We also found all the sites where *F. meridionale* were reported are mountain regions.

Species within FGSC typically produce type B trichothecenes and different chemotypes may affect species or population ecology, because the corresponding mycotoxins differ in toxicity and bioactivity (Kimura *et al.*, 1998). Our previous study also indicated that chemotype differences have a significant impact on pathogen fitness (Zhang *et al.*, 2012). Recently a number of displacement events appear to find their origin in the inadvertent introduction of new genotypes into new regions: 3-ADON *F. graminearum* in Canada (Ward *et al.*, 2008); 3-ADON *F. asiaticum* in Eastern China (Zhang *et al.*, 2012); 15-ADON *F. graminearum* in Uruguay (Umpierrez-Failache *et al.*, 2013); and NIV-producing *F. asiaticum* in the southern United States (Gale *et al.*, 2011). These genotypes appear to have selective advantages and recently overcame significant barriers due to changes in agricultural practices or environmental conditions. In this study, all *F. graminearum* isolates on maize had the 15-ADON chemotype, which is in agreement with previous reports (Ndoye *et al.*, 2012; Qiu and Shi, 2014). In addition to this, another 15-ADON producing species, *F. boothii,* was identified and the occurrence of this species may increase with the expansion of maize cultivation in China. In our study, only four NIV producers (three *F. asiaticum* and one *F. meridionale*) were found. This is in contrast to Ndoye' study, where 70% of the isolates in northeast China in 2009 were NIV producers (*F. asiaticum*). This change may reflect a regional difference or a chemotype/species shift in recent years. Similar results were reported from Switzerland, where both dimensional and temporal variation of *Fusarium* composition on maize was observed (Dorn *et al.*, 2009, 2011). Temporal variation events were also reported on wheat (Waalwijk *et al.*, 2003; Ward *et al.*, 2008). This study was performed on a collection from a single sampling year. Therefore, continuous sampling is needed to distinguish potential trends.

Members of the FFSC are capable of producing fumonisins and the main producers are *F. verticillioides* and *F. proliferatum*. Several other FFSC members also harbour the entire fumonisin gene cluster involved in the biosynthesis of this secondary metabolite and some were shown to produce varying amounts of fumonisins (Proctor *et al.*, 2013). A PCR assay with two sets of primers revealed that all *F. verticillioides* possessed the *FUM1* gene. This result is concordant with other studies that established *F. verticillioides* as a potent fumonisin producer (Proctor *et al.*, 2006). Also *F. proliferatum* and *F. fujikuroi* are fumonisin producers and *F. andiyazi* was shown to produce only trace amounts of fumonisin (Leslie *et al.*, 2005). *F. subglutinans* is considered to be a fumonisin non-producing species (Nelson *et al.*, 1992) due to the absence of the cluster of fumonisin biosynthetic genes (*FUM* cluster) (Proctor *et al.*, 2004). We found all *F. subglutinans* were negative for both primer sets. It is controversial whether *F. temperatum* is a fumonisin producer. In Belgium, Scauflaire *et al.* (2012) found that one out of eleven isolates produced low levels of FB_1. In Argentina, higher ratio of *F. temperatum* isolates (10/38) were identified as low fumonisin producers (max. 130 µg/g) in comparison with the main fumonisin producers (Fumero *et al.*, 2015). However, all ten *F. temperatum* strains isolated in southern China were able to produce both FB_1 and FB_2 (Wang *et al.*, 2014). In the present study, twenty *F. temperatum* strains were all negative to two sets of primers, indicating the possible absence of the *FUM1* gene. However, inconsistencies between PCR and LC-MS assay had also been reported (Covarelli *et al.*, 2012). This may reflect

mutations of the *FUM* cluster or alteration(s) in the control of *FUM* gene expression.

Although many reports on occurrence and prevalence of *Fusarium* species exist (reviewed by Van der Lee *et al.*, 2015) and population structures are found to be changing over time, the drivers for this phenomenon are still largely unknown. Both climate and cropping systems have been suggested to play an important role, but comparative biological studies are scarce. However, based on our findings and data from literature we conclude that a cropping system with wheat/maize rotation selects for *F. graminearum*, while a wheat/rice rotation selects for *F. asiaticum*. In contrast, *F. boothii* is selected when maize is cultivated without rotation. The pathogens in wheat and barley crops are predominantly *F. graminearum* as well as *F. asiaticum*. When maize is grown under colder climatological conditions in China, a higher occurrence of *F. temperatum* is observed while *F. meridionale* seems restricted to mountain areas. Each of these species has their characteristic mycotoxin profile, thus, if we can predict which species will be prevalent, then we also can predict the resulting mycotoxin contamination.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/WMJ2015.2004.

Table S1. The distribution of different mating type idiomorphs of *Fusarium verticillioides*, *Fusarium proliferatum* and *Fusarium subglutinans*.

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