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Molecular characterization of the *Fusarium graminearum* species complex in Eastern China

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Abstract Members of the *Fusarium graminearum* species complex (FGSC) cause Fusarium head blight in small cereal grains all over the world. To determine the species and trichothecene chemotype composition and population structure of FGSC in Jiangsu and Anhui provinces, an area where epidemics occur regularly, 891 isolates were collected in two consecutive years (2011 and 2012) and characterized with species- and chemotype-specific polymerase chain reaction. Of the 891 isolates typed, 83 were *F. graminearum sensu stricto* (s. str.) and 808 were *F. asiaticum.* All 83 *F. graminearum* s. str. isolates were of a 3ADON (26.51 %) or 15ADON (73.49 %) type, while *F. asiaticum* isolates included 696 3ADON producers, 46 15ADON producers, and 66 NIV producers. Eight variable number tandem repeat (VNTR) markers were tested on a representative 384 *F. asiaticum* isolates from 55 sampling sites. VNTR analysis showed high gene diversity and genotypic diversity but low linkage disequilibrium in both populations Fg2011 and Fg2012 grouped based on the year of collection. Low genetic differentiation (F_{ST}= 0.026) and high gene flow (N_m= 15.13) was observed between the two populations and among subpopulations within the same population (N_m= 3.53 to 48.37), indicating that few influence of temporal and spatial variations on population differentiation in this area. Similar result was obtained from 3ADON, 15ADON and NIV populations or carbendazim resistant and sensitive populations, indicating that chemotype of *Fusarium* isolates and carbendazim application had minor influence on population subdivision.

Keywords *Fusarium graminearum* · Population genetics · VNTR · Carbendazim resistance

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Introduction

Fusarium head blight (FHB) or scab is a significant fungal disease of wheat, barley and other small cereal grains all over the world (Jennings et al. 2004; Parry et al. 1995). In China, FHB has been a serious problem in wheat-growing regions since 1936, when the first severe outbreak was recorded (Xu and Chen 1993). During the past 10 years, epidemic outbreaks have become more frequent, severe

and widespread in China. The recent epidemics happened in 2003, 2008, 2010 and 2012 resulting in huge losses in yield and quality of crops. The disease frequently occurs in the middle and downstream regions of the Yangtze River, including Jiangsu, Zhejiang, and Anhui provinces 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 and southwestern regions and central parts of China, covering more than ten provinces that are mainly agricultural areas in China (Chen et al. 2000).

In many regions of the world, FHB is mainly caused by members of *Fusarium graminearum* species complex (FGSC), consisting of at least 16 phylogenetically distinct species (O'Donnell et al. 2000, 2004, 2008; Sarver et al. 2011; Starkey et al. 2007). Most species appear to be restricted to specific geographic regions (Xu and Nicholson 2009). In North America and Europe, *F. graminearum* sensu stricto is dominant according to the investigation of *Fusarium* (s. str.) spp. composition and population structure (O'Donnell et al. 2000, 2004; Starkey et al. 2007). *F. asiaticum* and *F. graminearum* s. str. were found to be the predominant etiological agents of FHB in Asia, including Japan and Korea, although their distributions vary depending on the sampling sites (Gale et al. 2002; Suga et al. 2008; Lee et al. 2009, 2012). In China, the majority of *F. graminearum* s. str. isolates were found in cooler northern regions and *F. asiaticum* mainly existed in the warmer wheat-growing regions where FHB epidemics occur most frequently (Qu et al. 2008).

Mycotoxins are secondary metabolites produced by filamentous fungi (Edwards et al. 2002) and trichothecenes are the most common mycotoxins found in cereals (Desjardins 2006). DON is associated with feed refusal, vomiting and suppressed immune functions, and NIV is more toxic to humans and domestic animals than DON (Ryu et al. 1988). Strains of FGSC usually produce one of the three trichothecene profiles: (i) deoxynivalenol and 3-acetyldeoxynivalenol (3ADON chemotype), (ii) deoxynivalenol and 15-acetyldeoxynivalenol (15ADON chemotype), or (iii) nivalenol, its acetylated derivatives and low levels of DON (NIV chemotype) (Ward et al. 2002). Population subdivision based on the trichothecene genotypes of *F. graminearum* prevalent in both the northern (Gale et al. 2007) and southern (Gale et al. 2011) USA has been inferred from population structure analysis. In Japan, high population differentiation and low gene flow values observed between NIV and 3ADON producers suggested that population subdivision in *F. asiaticum* may be correlated with trichothecene type (Karugia et al. 2009a, b).

Understanding the genetic profiles of pathogen populations may provide insights into the epidemiology and evolutionary potential of FGSC and could lead to improved strategies for controlling this pathogen (Zeller et al. 2003). Molecular markers are valuable tools in population studies because they allow tracking and tracing of genetic variation. Various genetic marker systems such as random amplified polymorphic DNA (RAPD), sequence related amplified polymorphism (SRAP), restriction fragment length polymorphism (RFLP), simple-sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) have been employed over the past few years to analyze the population genetic structure, reproductive behavior and biogeographic structure of FGSC (Liu et al. 2002; Carter et al. 2002; Fernando et al. 2006; Gale et al. 2002; Zeller et al. 2003; Suga et al. 2008; Mishra et al. 2004; Lee et al. 2012). VNTR markers have been developed for FGSC and have been effectively used for population analyses recent years (Zhang et al. 2010; Puri et al. 2012; Burlakoti et al. 2011; Ward et al. 2008; Karugia et al. 2009a, b).

Due to the typical humid and warm climate during the flowering stage of wheat, scab broke out seriously in Jiangsu and Anhui provinces, an traditional FHB epidemic area located in lower reaches of Yangtse river. Results of wheat mycotoxin detection from yearly surveys in these regions had showed DON and NIV content generally exceeded the standard. Zearalenone with high concentrations and fumonisin-producing strains were also detected in some regions (unpublished data). All these results indicate that *Fusarium* population genetic structure which reflects toxin producing ability is changing. We hypothesize that temporal and spatial variations can influence the genetic exchange among *Fusarium* populations in Jiangsu and Anhui provinces, leading to switches in the frequencies of isolates associated with different trichothecene-specific genetic markers, 891 isolates collected in this region in 2011 and 2012 were characterized in the further research. The objectives of this study were to: (I) investigate species composition, (II) determine trichothecene types, (III) analyze genetic structure of FHB pathogens in two consecutive years and (IV) assess the reaction of *Fusarium* isolates to carbendazim.

Materials and methods

Isolate collection, purification and DNA extraction

Wheat spikes showing typical symptoms of FHB were collected near harvest time in 2011 and 2012 from approximately 60 counties in Anhui and Jiangsu provinces (about 242600 km^2), China (Fig. 1). The sample size in each sampling site was provided in Table S1. Individual seed from wheat heads was dislodged and surface sterilized before plating on potato dextrose agar (PDA) as previously described (Zhang et al. 2012). Conidia produced by mung bean broth (MBB) were spread on PDA and a single conidium was isolated. Isolates were maintained on PDA and kept at 4 °C for short-term storage and at -80 °C for long-term storage. DNA was extracted with a cetyltrimethylammonium bromide (2%CTAB) method (Leslie and Summerell 2006).

Identification of *F. graminearum* and trichothecene genotype

DNA from all isolates were amplified by PCR with the Fg16F/R primers which produce polymorphic products (400–500 bp) with DNA from members of the *F. graminearum* species complex (Nicholson et al. 1998).

Single and multiplex PCR assays were performed for detection of trichothecene genotypes. Chemotypes of the FGSC isolates were determined using the specific primer pair described by Li et al. (2005). Another two primer sets, Tri303F/Tri303R and Tri315F/Tri315R,

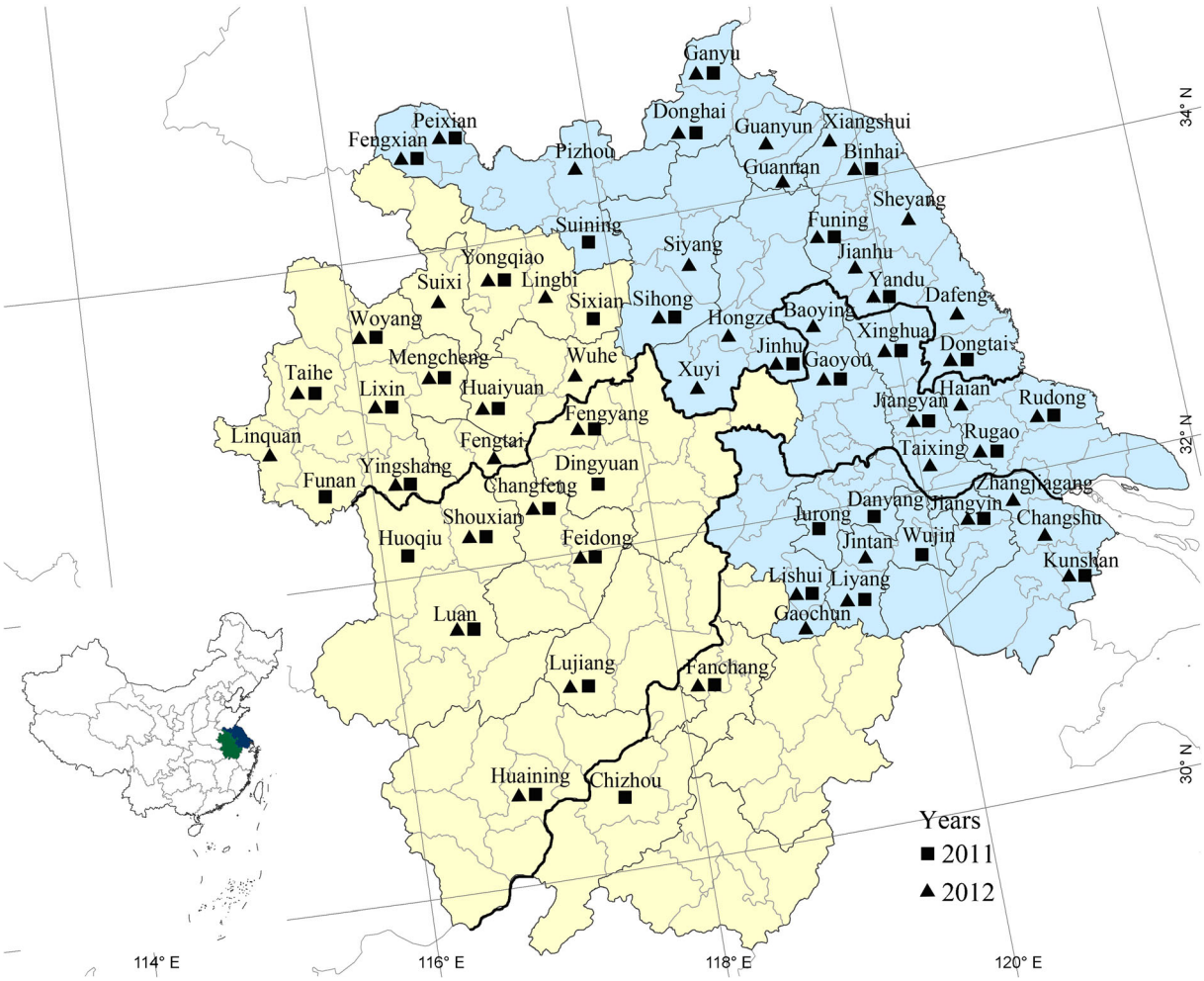

Fig. 1 Geographical map of Jiangsu and Anhui provinces (*upper right*) indicating the sampling sites, located in the eastern area of China (*lower left*). The thick black line indicates division into northern, central and southern wheat growing regions

which target the Tri3 gene (Jennings et al. 2004), were used to further characterize DON chemotypes of the *F. graminearum* s. str. complex as 3ADON or 15ADON. Multiplex PCR assays developed by Wang et al. (2012) were performed with primer pairs based on sequences of Tri11 gene sequences, a key enzyme in the pathway leading to T-2, DON, 3ADON, 15ADON, and NIV biosynthesis in *Fusarium* species (Alexander et al. 1998). They produced a 279 bp fragment for the 15ADON chemotype, a 334 bp fragment for the 3ADON chemotype and a 497 bp for the NIV chemotype.

In vitro carbendazim sensitivity

The sensitivity of all strains to carbendazim was measured as described (Qiu et al. 2011). Carbendazim-sensitive strains could not grow on PDA plates containing 1.4 μg/mL carbendazim, while carbendazim-resistant strains grew normally on this medium.

Generation of VNTR allele data

Genetic variation at eight variable number tandem repeat (VNTR) loci (Suga et al. 2004) was used to assess population structure and diversity. The forward primer of each marker was labeled with either fluorescent 6-FAM (HK630, HK1043, HK1059, and HK957) or PEX (HK917, HK1073, HK913, HK967) and PCR was performed as described by Suga et al (2004). Reaction products were scored in line with a GS500 ROX (Applied Biosystems) internal size standard using an ABI3100 Genetic Analyzer with GENEMAPPER 3.7 software (Applied Biosystems).

Population genetic analyses

GENALEX 6.5 (Peakall and Smouse 2006) was used to perform analysis of molecular variance (AMOVA), pairwise gene flow (*Nm*), population differentiation or fixation index (*Fst*), allele frequencies, percentage polymorphic loci, gene diversity (*H*) (Nei 1973, 1978), Nei's unbiased genetic distance (*D*) (Nei 1978), Nei's unbiased genetic identity (*I*) (Nei 1978). Allele frequency was determined for each allele for a single locus. H is a function of the number and frequencies of alleles at each locus. AMOVA was performed to test hierarchical partition of genetic variation between two populations and individuals within the whole population (Excoffier et al. 1992). The gene flow was calculated by $Nm=(1-Fst)/2Fst$), where N is the effective population size and m is the migration rate per generation. The statistical significance of pairwise Fst was tested by 1,000 permutations. The fixation index (F_{ST}) is the standardized measure of population differentiation based on genetic polymorphism and shows the genetical differences in populations. Multilocus 1.3 software (Agapow and Burt 2001) was used to calculate multilocus genotype (*G*), genotype diversity (*GD*), and multilocus linkage disequilibrium (*LD*). *GD* is defined as the probability that two individuals taken at random have different genotypes and was calculated by $(n/n-1)\ (1-\Sigma pi2)$, where *pi* is the frequency of the *i*th genotype and *n* is the number of individuals sampled (Agapow and Burt 2001). The *LD* measures the nonrandom association of alleles at different gene loci in a population. The test of significance was determined by using 1,000 randomizations in all populations and subpopulations.

Results

Species composition

A total of 891 isolates from two provinces were acquired and identified. In the PCR reaction with the Fg16F/R primers, all isolates produced either a specific fragment, confirming that they belong to the *F. graminearum* species complex. According to six types of *Fusarium* spp. by Carter et al. (2002), 83 isolates yielding a single type 1 (0.41 kb) product were identified as *F. graminearum* s. str., while 808 isolates yielding a single type 5 (0.49 kb) product were identified as *F. asiaticum*. *F. asiaticum* was predominant in this area; the frequency in southern and central region was similar (99 % and 99.23 %, respectively). Compared with those two regions, the proportion of *F. graminearum* s. str. strains increased and that was 9.52 % in the northern region.

Trichothecene chemotypes determination

Trichothecene types were determined using the single and multiplex PCR, and consistent results were obtained in more than 99 % of all isolates. Among 891 isolates, 718 (81 %) of them were of 3ADON type while 107 (12 %) and 66 (7 %) isolates were of 15ADON and NIV types, respectively. The frequency of 3ADON producers in the newer population (Fg2012) reduced about 10 %,

followed by the increase of the proportion of 15ADON and NIV producers.

The NIV chemotype was not observed among the 83 strains of *F. graminearum* s. str., while the 3ADON and 15ADON chemotypes were observed among 26.51 % and 73.49 % of *F. graminearum* s. str. strains, respectively (Fig. 2a). In *F. asiaticum*, all three trichothecene types were identified, with 696 (86.14 %) being of the 3ADON type, 46 (5.69 %) and 66 (8.17 %) being of the 15ADON and NIV type, respectively (Fig. 2b).

Isolates were further divided into three subpopulations (northern, central, and southern) according to the location of collections to analyze trichothecene chemotype. 3ADON chemotype was found mostly in this area, especially in south and center (Table 1). Frequencies of 15ADON and NIV chemotypes were highest in north and decreased from north to south (Table 1).

In vitro carbendazim resistance

PDA plates containing 1.4 μg/mL carbendazim were used to test all 891 isolates for their sensitivity to this fungicide. 130 (14.59 %) isolates were determined to be naturally resistant to carbendazim, and these isolates widely distributed in this area. Only 3 isolates of the 130 isolates belong to *F. graminearum* s. str., indicating that *F. asiaticum* could be more tolerant to carbendazim.

Carbendazim resistant isolates in Jiangsu province were further analyzed as 108 of 130 isolates were obtained in this area. We found the number of carbendazim resistant isolates in the central and northern region was higher than that in the southern region.

The distribution frequency of *F. asiaticum* isolates in Jiangsu province with the 3ADON, 15ADON and NIV markers was further analyzed according to the phenotype to carbendazim. The $\chi 2$ value was highly significant ($\chi 2=13.73; P<0.01$) for the distribution of genetic markers for 3ADON, 15ADON and NIV between carbendazim-resistant and-sensitive isolates. However, 15ADON chemotype was not observed in the carbendazim-resistant population. No significant difference ($\chi^2=2.06; P>0.05$) in the frequencies of the DON (3ADON+15ADON) and NIV markers was observed, if 417 isolates were divided into these two chemotypes.

Population genetic analysis with VNTR markers

The 8 VNTR markers used in this study were highly polymorphic and the numbers of alleles in each locus was varied from 6 to 23 (Table 2). The allele sizes were 124 to 33 bp (Table 2). Allele frequencies of each locus were 0.003 to 0.69. Gene diversity (*H*) also differed among the 8 VNTR markers, with HK967 being the highest (0.863). Marker HK1059 had the lowest *H* (0.449) (Table 2. All 384 isolates of *F. asiaticum* analyzed from the combined older and newer populations were distinct genotypes, indicating that there was 100 % genotypic diversity (Table 3). High *H* was observed in both populations ($H=0.63$ to 0.69) and in three subpopulations (northern, central, and southern) of each population ($H=0.53$ to 0.68). Very low linkage disequilibrium ($LD=0.02$ to 0.08, $P<0.01$) was observed in both populations and all subpopulations (Table 3).

The genetic distance was low ($D=0.053$) and genetic identity was high ($I=0.949$) between the two populations (Fg2011 and Fg2012) grouped according to the years of collection. The hierarchical AMOVA showed that genetic variation between the populations accounted for only 3 % while most of the genetic

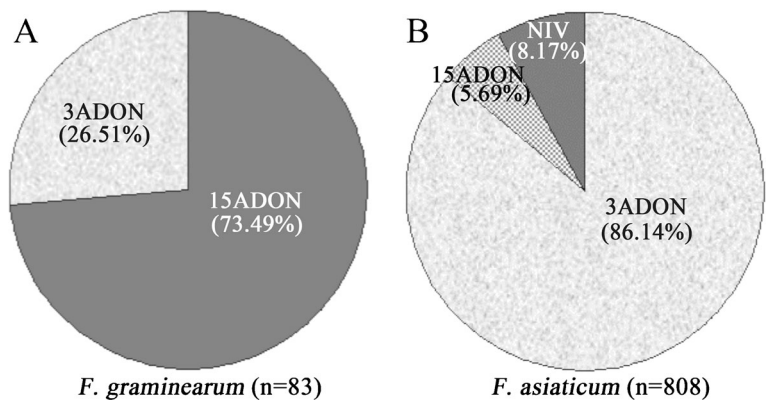

Fig. 2 Trichothecene chemotype compositions of *F. graminearum* s. str. (**a**) and *F. asiaticum* (**b**) strains. The chemotype of *F. graminearum* s. str. ($n=83$) and *F. asiaticum* ($n=808$) strains was determined by single and multiplex polymerase chain reaction

Table 1 Frequency of distribution of *Fusarium* isolates with 3ADON, 15ADON and NIV types in populations Fg2011 and Fg2012 in Jiangsu and Anhui provinces determined by trichothecene biosynthesis-based polymerase chain reaction assays

Region	Species	Fg2011				Fg2012			
		n	3ADON	15ADON	NIV	*n*	3ADON	15ADON	NIV
Northern region	*F. asiaticum*	145	123(84.83 %)	14(9.65 %)	8(5.52 %)	235	173(73.62 %)	27(11.49 %)	35(14.89 %)
	F. graminearum s. str.	13	4(30.77)	9(69.23 %)	0	46	3(6.52 %)	43(93.48 %)	0
Central region	*F. asiaticum*	113	108(95.58 %)	3(2.65 %)	2(1.78 %)	144	136(94.45 %)	1(0.69 %)	7(4.86 %)
	F. graminearum s. str.	9	5(55.56 %)	4(44.44 %)	0	6	3(50 %)	3(50 %)	0
Southern region	*F. asiaticum*	64	59(92.19 %)	0	5(7.81 %)	107	97(90.66 %)	1(0.93 %)	9(8.41 %)
	F. graminearum s. str.	6	5(83.33 %)	1(16.67 %)	0	3	2(66.67 %)	1(33.33 %)	0
Total	*F. asiaticum*	322	290(90.06 %)	17(5.28 %)	15(4.66 %)	486	406(83.54 %)	29(5.97 %)	51(10.49 %)
	F. graminearum s. str.	28	14(50 %)	14(50 %)	0	55	8(14.55 %)	47(85.45 %)	0

variation (97 %) was from individuals within population. Low genetic differentiation (*Fst*=0.026) and high gene flow (*Nm*=15.13) was observed between Fg2011 and Fg2012, indicating that a low level of genetic differentiation occurred in *F. asiaticum* populations from wheat in this region. The overall genetic identity was also high (0.94) between the two populations. Within each population, high values of *I* (0.82 to 0.99) and *Nm* (3.54 to 29.97) were observed among subpopulations from northern, central, and southern regions (Table 4), suggesting that spatial variation has a very low effect on population subdivision in *F. asiaticum*. Hierarchical AMOVA revealed low genetic variation between the two populations (3 %) and between subpopulations within the population (4 %) (Table 5). In contrast, genetic variation among the isolates within subpopulation was high (93 %) (Table 5).

When the isolates were grouped into 3ADON, 15ADON and NIV populations, the overall estimate of *Nm* was high (12.68 to 34.43) and F_{ST} was low (0.02 to 0.03), confirming low genetic differentiation between three populations (Table 6). AMOVA showed 2 % genetic variation between subpopulations within the population.

When the isolates were grouped into carbendazim resistant and sensitive populations, subsequent analyses determined the level of population subdivision between them. AMOVA determined that only 3.39 % of the observed variation was caused by variation among populations. The majority of variation therefore was caused

Table 2 Number of alleles, allele size range, allele frequencies and gene diversity of eight variable number tandem repeat (VNTR) markers used to analyze the 384 *F. asiaticum* isolates collected from Jiangsu and Anhui provinces

VNTR marker	Location[a]	Number of alleles	Allele size range	Allele frequencies	Gene diversity[b]	
					2011	2012
HK630	Ch4/Ct371/112,325	10	200~266	0.003~0.586	0.662	0.709
HK913	Ch1/Ct73/664	11	185~236	0.003~0.69	0.68	0.731
HK917	Ch1/Ct82/2,471	10	212~236	0.016~0.404	0.564	0.587
HK957	Ch1/Ct91/16,055	16	166~307	0.003~0.175	0.712	0.579
HK967	Ch3/Ct196/164,228	6	200~214	0.365~0.513	0.847	0.863
HK1043	Ch1/Ct52/41,839	10	195~300	0.005~0.609	0.735	0.726
HK1059	Ch3/Ct196/164,228	16	185~292	0.003~0.482	0.449	0.572
HK1073	Ch4/Ct398/70,812	23	124~240	0.003~0.497	0.62	0.503
Mean		12.75			0.659	0.659

[a] Marker location in the genome was indicated by chromosome (Ch) number/contig (Ct) number/position in contig

[b] Nei's gene diversity (*H*) within populations was calculated using GenAlEx 6.5

Table 3 Genetic diversity and multilocus linkage disequilibrium analysis of *F. asiaticum* isolates from the newer population (2011) and the older population (2012)

Population	n[a]	*G*[b]	*GD*[c]	*H*[d]	*LD*[e]
Fg2011					
Northern	63	63	1	0.55	0.07**
Central	48	48	1	0.65	0.02**
Southern	81	81	1	0.66	0.08**
Subtotal	192	192			
Fg2012					
Northern	54	54	1	0.66	0.07**
Central	48	48	1	0.53	0.03**
Southern	90	90	1	0.68	0.08**
Subtotal	192	192			
Total	384	384			

[a] Number of isolates used for VNTR analysis

[b] Number of distinct genotypes

[c] enetic diversity (*GD*) within population was calculated using software MULTILOCUS with the algorithm $GD=(n/n-1)(1-\Sigma pi2)$, where *pi* is the frequency of the *i*th genotype and *n* is the number of individuals sampled

[d] Nei's gene diversity (*H*) within populations was calculated using GenAlEx 6.5

[e] Measure of multilocus linkage disequilibrium (*LD*); **=significant at $P<0.01$

by variation among isolates within populations. AMOVA showed 2 % genetic variation between subpopulations within the population. Low pairwise F_{ST} (0.018) and effective number of migrants ($Nm=27.43$) also confirmed the high levels of gene flow between all field population pairs.

Discussion

A pair of primers Fg16F/R was used to SCAR (sequence characterized amplified region) analysis with (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. Zhang et al. (2007) reported that all the strains could be classified into two phylogenetically different species, *F. asiaticum* and *F. graminearum* s. str., according to the fixed nucleotide sequences of three phylogenetically informative genes (*Tri101*, *reductase* and *histone H3*). The two phylogenetic species are fully congruent with SCAR groupings V and I. Strains from *F. asiaticum* species lineage 6 are within SCAR group V, whereas *F. graminearum* species lineage 7 are of SCAR group I. Chandler et al. (2003) also reported that SCAR group V strains belonged to lineage 6, and SCAR group I resided in lineage 7. Because many previous reports indicate thes two species are predominant in China, it is fast and simple to use Fg16F/R for population analysis in our region.

Our results indicated that *F. asiaticum* is the primary pathogenic fungus prevalent in the FHB disease nursery. This is consistent with previous studies, which showed that *F. asiaticum* is the predominant and primary pathogen of FHB in Asian countries (China, Korea, Nepal, and Japan) and Brazil, while *F. graminearum* s. str. is widely distributed (O'Donnell et al. 2004). Qu et al. (2008) reported that the vast majority of *F. asiaticum* isolates were collected from warmer regions, where the annual average temperature is 15 °C or higher. Karugia et al. (2009a, b) indicated that most isolates (179 out of 184) sampled in a small experimental field in Japan classified

Table 4 Pairwise comparisons of gene flow (*Nm*, above diagonal) and genetic identity (below diagonal) of *F. asiaticum* subpopulations from wheat in Jiangsu and Anhui provinces

Population	Fg2011			Fg2012		
	Northern	Central	Southern	Northern	Central	Southern
Fg2011,Northern	…	16.48	6.37	6.46	3.53	6.22
Fg2011,Central	0.96	…	17.43	20.45	6.22	15.76
Fg2011,Southern	0.88	0.94	…	37.91	6.66	40.59
Fg2012,Northern	0.88	0.95	0.97	…	22.18	48.37
Fg2012,Central	0.82	0.89	0.89	0.97	…	9.44
Fg2012,Southern	0.88	0.93	0.98	0.99	0.93	…

[a] Gene flow (*Nm*) was calculated as $Nm=0.5[(1/F_{ST})-1]$ using GENALEX 6.5, where F_{ST} was calculated as the proportion of the variance among populations relative to the total variance. Probability of obtaining equal or lower F_{ST} value was determined by 1,000 randomizations by permuting individuals among populations. Genetic identity=Nei's unbiased genetic identity

Table 5 Analysis of molecular variance for *Gibberella zeae* populations of from wheat in Jiangsu and Anhui provinces

Hierarchical analysis[a]	df[b]	Estimated variance	Variation(%)	Phi[c]	*P* value[d]
Between population(PhiRT)	1	0.071	3	0.03	0.001
Between subpopulations within population(PhiRT)	5	0.116	4	0.05	0.001
Individuals within subpopulations(PhiPT)	377	2.561	93	0.08	0.001

[a] Variance was partitioned into two populations Fg2011 and Fg2012, among three subpopulations (northern, central, and southern) within population, and individuals within three subpopulations of Fg2011 and Fg2012

[b] Degree of freedom

[c] PhiRT was calculated as the proportion of the variance among groups, relative to the total variance. PhiPR was calculated as the proportion of variance among subpopulations within population, relative to the variance among and within subpopulations. PhiPT was calculated as proportion of variance among populations and subpopulations of individuals, relative to the total variance

[d] Probability of obtaining equal or lower Phi value was determined by 1,000 random permutations

as *F. asiaticum*. However, some reports indicated temperature may not be the only factor affected the distribution of *Fusarium* species. Zhang et al. (2012) reported *F. graminearum* s. str. isolates in the Southwest region significantly more than in Yangtze River region; nevertheless, the average temperature of Southwest region was even a little higher. This result indicates that temperature may not be the critical factor in the distribution of the *Fusarium* species and that other, yet unknown factors affected their distribution. It has previously been recognized that the *F. graminearum* clade species composition appears to be location and, perhaps, host dependent (Lee et al. 2009; O'Donnell et al. 2000). Maize residues and rice straw seem to be critical for overwintering of the pathogen. In Korea, a predominance of lineage 6 of *F. graminearum* (equivalent to *F. asiaticum*) over lineage 7 of *F. graminearum* (equivalent to *F. graminearum* s. str.) was found in all of the 249 isolates collected from rice field. In southern USA, the occurrence of *F. asiaticum* neatly overlaps with rice-growing areas in Louisiana (Gale et al. 2011). They hypothesize that *F. asiaticum* has a host preference and specificity to rice, and perithecium production typically favors rice straws under warmer conditions. Zhang et al. (2012) collected the acreage data of rice, wheat and maize, and found a strong association between the occurrence of *F. asiaticum* and the predominant crops. *F. asiaticum* were not detected in northern China where rice is not rotated with wheat in the same field. In the region along the middle and lower valleys of Yangtze River, rice acreages were higher, *F. graminearum* s. str. isolates were obtained in only a few sampling site. Therefore, *F. asiaticum* may be adapted to higher temperatures and rice compared with *F. graminearum* s. str., although there are some other possible explanations including relative humidity, cropping practices, etc.

Table 6 Pairwise comparisons of gene flow (*Nm*, above diagonal) and genetic identity (below diagonal) of *F. asiaticum* 3ADON, 15ADON and NIV populations from wheat in Jiangsu and Anhui provinces

Population	3ADON	15ADON	NIV
3ADON	…	12.68	33.36
15ADON	0.92	…	34.43
NIV	0.98	0.97	…

[a] Gene flow (*Nm*) was calculated as $Nm=0.5[(1/F_{ST})-1]$ using GENALEX 6.5, where F_{ST} was calculated as the proportion of the variance among populations relative to the total variance. Probability of obtaining equal or lower F_{ST} value was determined by 1,000 randomizations by permuting individuals among populations. Genetic identity = Nei's unbiased genetic identity

The trichothecene chemotype composition was shown to be significantly different between *F. graminearum* s. str. and *F. asiaticum* isolates (Fig. 2). The NIV chemotype was not detected among the 83 *F. graminearum* s. str. strains, while 8.17 % of the *F. asiaticum* strains had the NIV chemotype. NIV type strains of *F. graminearum* s. str. have not been detected in Japan (Suga et al. 2008), but previous global surveys identified all three trichothecene types (NIV, 3ADON, 15ADON) in both *F. asiaticum* and *F. graminearum* s. str. (Suga et al. 2008; Ward et al. 2002). Trichothecene chemotype composition also appears to be correlated with geographic origin. Puri et al. (2012) found all three trichothecene types were found in the same place in southeastern China for the first time, and the percentage of NIV producers was high (79 %). Karugia et al. (2009a,

b) analyzed 208 isolates of *F. asiaticum* collected from four locations in Zhejiang province and showed that NIV and 3ADON isolates accounted for 42.3 and 57.7 %, respectively, but no 15 ADON isolates were detected. Zhang et al. (2007) identified 77 % of 299 isolates from 12 provinces along the middle and lower reaches of Yangtze River and found as *F. asiaticum*. Of these *F. asiaticum* isolates, 67 % were of 3ADON type and 23 % were NIV type but only 10 % were 15ADON isolates. All *F. graminearum* isolates collected in the northern region of China had 15ADON chemotype, while 80 % of the *F. asiaticum* isolates from southwest China were NIV makers and along the middle and lower valleys of the Yangtze River, 83 % of the *F. asiaticum* isolates were of the 3ADON type (Zhang et al. 2012). In the present study, 3ADON type covered the large proportion of 808 *F. asiaticum* isolates (696 out of 808); while 66 isolates were of NIV type and 46 isolates were of 15ADON type. In *F. graminearum* s. str., 73.49 % strains synthesized 15ADON and 26.51 % strains synthesized 3ADON. Several researchers have reported that isolates with the 3ADON marker are dominant in warm regions and that isolates with the 15ADON marker are found mainly in cool regions (Ji et al. 2007). Here, most 15ADON producers were detected in the northern region where the average temperature was lower. The different frequency of three trichothecene chemotypes could reflect an ecological gradient in China that directly influences chemotype distributions. However, differences in the distribution of DON and NIV types might also be explained in terms of the different geographical distributions of *F. graminearum* s. str. and *F. asiaticum*, which differ in their chemotype composition (Fig. 2).

The change of 3ADON chemotype frequency was observed in China and North America. Ward et al. (2008) documented that 3ADON chemotype frequency increased more than 14-fold between 1998 and 2004 in western Canada. The frequencies of isolates with the 3ADON type in the newer population (2010) were 11-fold higher that among isolates in the older population (1997 to 2000) in North Dakota and Minnesota from (Burlakoti et al. 2011). Zhang et al. (2010) reported increase in 3ADON isolates replacing isolates with the 15ADON isolates in southern China. Yang et al. (2008) also reported that *F. asiaticum* isolates with DON markers have been replacing the traditional isolates with NIV markers in barley-growing areas in China. Zhang et al. (2007) demonstrated that 53.1 % of the *F. asiaticum* isolates obtained in the middle valley of Yangtze River in 1999 were of 3ADON type. The ratio of 3ADON maker increased to 87.1 % in 2005 in the same region. However, in this study, there was a slight decrease in 3ADON producer percentage from 2011 to 2012 followed by a little raise in 15ADON and NIV producer. There was no clear evidence for the accurate explanation of rapid change in trichothecene-producing isolates in crops up to now. Some authors suggested that selection is driving the rapid spread of the pathogen population that is more toxigenic and potentially more vigorous (Ward et al. 2008). They also demonstrated that isolates from DON populations produced more trichothecene and had higher growth rates and that may be a factor in the change. Though several researchers connected the shift in species or chemotypes with cropping patterns and climatic factors or selection forces such as use of resistant wheat cultivars and fungicides (Ward et al. 2008), various studies showed no significant difference of these factors in the distribution frequency of isolates with DON and NIV markers (Burlakoti et al. 2008; Yang et al. 2008). It is difficult to speculate on the cause of the rapid change in the frequency of trichothecene-specific isolates without extensive further studies.

The population genetics study revealed that population subdivision was not observed between the population Fg2011 and Fg2012 based on the year of collection. This conclusion is supported by the high genetic identity (97 %), high gene flow (Nm=15.13), and very low genetic differentiation (Fst=0.026) between Fg2011 and Fg2012. Similarly, minimal influence of space on population differentiation was observed, as indicated by high pairwise *Nm* (3.53 to 48.37) values among three subpopulations (northern, central, and southern) within each population. Gale et al. (2002) found *F. asiaticum* populations collected from four fields located in Zhejiang province are apparently part of a geographically larger population, as a high gene flow (Nm=7 to 30) and low *Fst* (0.01 to 0.07) between all field population pairs. All 179 isolates of *F. asiaticum* collected in two successive years from a small wheat field in Japan were classified as part of a geographically larger population and the authors failed to reveal distinct genetic differentiation between the populations sampled during the 2 years (Karugia et al. 2009a, b). Zeller et al. (2003) also observed a large number of migrants (approximately 70) and high genetic identity (99 %) among *F. graminearum* isolates sampled in two small fields located in North Dakota and Kansas, respectively.

There was no significant genetic differentiation between the 159 *F. asiaticum* isolates located at southeastern China in two consecutive years (2008 and 2009) as genetic differentiation was low *Fst* (0.032) and high gene flow (15.13) (Puri et al. 2012). These results suggest that a pathogen population from a small, isolated disease nursery may keep stable for some period of time. Wright (1951) stated that those populations having ≥4.0 *Nm* values were considered to be part of a large single population. Our result indicated that all subpopulations within a population are part of a single large population in this region. Thus, information from a local population may not be applicable to a larger population across China or vice versa. Population studies on field, regional, and global scales are still necessary to have a better understanding of genetic variation and population structure of the pathogen.

However, population subdivisions based on trichothecene type, geographic difference, and temperature gradient from a larger geographic area have been reported (Gale et al. 2007; Karugia et al. 2009a, b; Starkey et al. 2007; Ward et al. 2002; Yang et al. 2008; Zhang et al. 2007, 2010) Although the frequency of isolates with 3ADON markers had increased rapidly in the middle and lower reaches of the Yangtze River region (Zhang et al. 2010), our results indicated that frequency distribution of trichothecene-specific *Fusarium* isolates had no effect on population differentiation in this smaller region, while some previous findings reported that population subdivision was due to chemotype differences (Gale et al. 2007; Ward et al. 2008). Zhang et al. (2012) found a biased gene flow from 3ADON to NIV and 3ADON producers are replacing NIV producers by recombination. Here, when overall isolates were grouped into 3ADON, 15ADON and NIV populations, low F_{ST} and high *Nm* and *I* values were observed within three populations, which showed they were in one random mating population and isolates with three different type are in the fusion process Table 6.

In the present study, we analyzed populations of *F. graminearum* from four commercial wheat fields located in eastern China along the Yangtze River. FHB was especially prevalent here as moist conditions and moderate temperatures prevail during flowering. Carbendazim has been extensively used to control FHB for decades in China before the first carbendazim-resistant strain was detected in the field in Zhejiang Province, China, in 1992 (Zhou et al. 1994). Since then, some resistant isolates have also been identified in Zhejiang Province and vicinity, such as Jiangsu Province. Results from yearly surveys in these regions have shown that the proportion of resistant isolates in the population is increasing. While their frequency in the population was below 5 % before 2003, resistant isolates accounted for 15.26 % of screened isolates in 2010 (Zhang et al. 2009; Shao et al. 2011).

An initial objective of this study was to compare the population genetic structure of fungicide resistant isolates with sensitive isolates. The concentration of 1.4 μg/ml was chose as the cut-off for detecting resistant field isolates in vitro (Zhou and Wang 2001). 130 of 891 (14.59 %) were resistant as resistant isolates grew normally on the medium containing carbendazim at 1.4 μg/ml and only 3 isolates (2.31 %) were identified as *F. graminearum* s. str.. Results were in agreement with the previous study in this region (Shao et al. 2011), which showed that the percentage of carbendazim resistance isolates was higher in *F. asiaticum* obtained in central and northern region of Jiangsu province. It can be inferred that increasing use of carbendazim in the central and northern region aggrandized the selection pressure and accelerate the occurrence of resistance. Wang et al. (2010) documented that colonies of *F. asiaticum* developed normally at a carbendazim concentration of 5.0 μg /ml, indicating that *F. asiaticum* was highly tolerant to carbendazim. Carbendazim resistance often develops in many plant pathogenic fungi after these fungicides have been used for as few as 2–3 years (Bollen and Scholten 1971; Schroeder and Provvidenti 1969). The first case of carbendazim resistant isolate was found in 1992, until then, this fungicide was used to control FHB in China for more than 20 years. Sexual reproduction takes place once per year under field conditions (Chen and Zhou 2009), which means the emergence of carbendazim resistance of *F. graminearum* was not occurred like other fungus. These results suggested

Table 7 Frequency of distribution of *F. asiaticum* isolates with 3ADON, 15ADON and NIV types in carbendazim-resistant and -sensitive populations in Jiangsu province determined by trichothecene biosynthesis-based polymerase chain reaction assays

Population	n	3ADON	15ADON	NIV
R	106	99(93.4 %)		7(6.6 %)
S	311	246(79.1 %)	29(9.32 %)	36(11.58 %)
Total	417	345(82.73 %)	29(6.96 %)	43(10.31 %)

that carbendazim may be still efficient for controlling FHB in Northern China. However, species identification is necessary before the application of carbendazim due to the existence of *F. asiaticum*, which is easily resistant to carbendazim.

The population genetics study revealed that two populations grouped based on the sensitivity to carbendazim was genetically similar. It seems that the application of fungicide was not responsible for the rapid spread of *Fusarium* isolates. In general, the frequencies of isolates with the 3ADON marker were higher in both populations. Our results showed no significant difference in the frequencies of isolates with DON marker and isolates with NIV marker between the resistant and sensitive populations (Table 7). We hypothesize that carbendazim resistance gene may have little or no influence on variation between isolates with DON marker and isolates with NIV marker in this region. It is worthwhile to note that only two trichothecene chemotypes were collected in carbendazim-resistant isolates, further analysis should be carried out in the isolates with a lager amount and obtained in a lager region.

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