Population Genetic Analyses of *Fusarium asiaticum* Populations from Barley Suggest a Recent Shift Favoring 3ADON Producers in Southern China

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ABSTRACT

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Fusarium asiaticum is the predominant causal agent of Fusarium head blight (FHB) in southern China. The genetic diversity was assessed by analyzing 448 single-spore *F. asiaticum* isolates from 18 sampling sites that were 10 to 2,000 km apart, using seven highly informative variable number of tandem repeat (VNTR) markers. This analysis showed a significant degree of population subdivision ($P < 0.001$) among populations from upper, middle, and lower valleys of the Yangtze River, with little gene flow ($N_m = 1.210$). We observed a strong association between

this genetic population subdivision and the mycotoxin produced. Our results show that the dramatic cline in trichothecene chemotypes may be explained by a recent and significant invasion of 3-acetyldeoxynivalenol (3ADON) producers in FHB pathogen composition in the middle valley. Using Bayesian statistics, we found a biased gene flow from 3ADON to nivalenol (NIV) populations. In addition, we observed significant genetic differentiation and linkage disequilibrium between NIV- and 3ADON-producing isolates at the same sampling sites. The impact of the changed agronomy and trade of cereal commodities on the spread of the new *Fusarium* population and the consequent increase of FHB observed in southern China are discussed.

Additional keywords: genetic variation.

Fusarium head blight (FHB) or scab is an economically devastating disease of wheat, barley, and other small cereal grains all over the world (16,36). The disease is a growing threat to the world's food supply due to recent outbreaks in Asia, Europe, and South America (14). In addition to quantitative yield losses, harvested grain sustains qualitative problems of contamination with mycotoxins such as nivalenol (NIV), deoxynivalenol (DON), and several acetylated derivatives of NIV and DON (20). These trichothecenes pose a significant risk to food safety and animal health because they inhibit eukaryotic protein synthesis and modify immune function (14,31).

In China, the first outbreak of FHB was reported in 1936, when the percentage of scabbed spikes was as high as 95% in cv. Jinda 2905 in Xuancheng County, Anhui Province (38). Since then, the disease occurred frequently in the middle and downstream regions of the Yangtze River, including the provinces of Jiangsu, Zhejiang, Anhui, and Hubei, and in the northeastern region, including Heilongjiang Province. During the last few years, an increasing number of wheat-growing regions throughout the country,

including the provinces of Henan, Shaanxi, Ningxia, Gansu, Qinghai, Hebei, Shandong, and Sichuan, have been affected by the disease, resulting in extensive yield losses in wheat and barley. This indicates that FHB epidemics are becoming more frequent, more severe, and more widespread in China.

FHB is caused by a complex of *Fusarium graminearum* species, which consists of at least 12 phylogenetically distinct species, most of which cannot be distinguished morphologically (23–25,29). Previous studies indicated that Chinese strains were mainly divided into two groups (e.g., *F. graminearum* sensu stricto and *F. asiaticum*) (26,39). *F. graminearum* sensu stricto mainly existed in the cooler wheat regions where the annual average temperature was $\leq 15^\circ\text{C}$. The vast majority of *F. asiaticum* isolates were found in the warmer regions, where the annual average temperature is $> 15^\circ\text{C}$ and where FHB epidemics occur most frequently (26). Yang et al. (39) screened a large number of FHB isolates collected from barley in 23 counties of seven provinces and two municipalities along the Yangtze River in southern China. *F. asiaticum* was the predominant species in all regions whereas, in the upper valleys of the Yangtze River, *F. graminearum* sensu stricto and *F. meridionale* were also found.

For population studies, random amplified DNA polymorphism (RAPD) and amplified fragment length polymorphism (AFLP) have been used to analyze the genetic variation and biogeographic structure of *F. graminearum* complex on wheat in China (12,26). However, there is no report about the genetic structure of *F. asiaticum*, the predominant pathogen on barley in China. Compared with RAPD and AFLP, variable number of tandem repeat (VNTR) markers are more desirable for population genetic

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*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains three tables showing the results of seven VNTR markers on 448 *F. asiaticum* isolates, the reference strains used to validate the newly designed chemotype-specific PCR primers, and Nei's unbiased genetic distance based on the VNTR data and geographical distance for the *F. asiaticum* strains from 18 sampling sites.

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analysis due to the simplicity of collecting accurate polymorphic data and due to their co-dominance (30). Generally, developing VNTR markers is costly and cumbersome; therefore, few VNTR markers have been reported for *F. graminearum* (13). However, the whole-genome sequence of *F. graminearum* strain PH-1 (NRRL 31084) made it convenient to design new VNTRs (30,32).

Type B trichothecene-producing species of the FHB complex consist of members of the *F. graminearum* species complex (FGSC) and five closely related species. These species have the tendency to produce different, strain-specific trichothecenes (19,25): NIV and acetylated derivatives (NIV chemotype), DON and primarily 3-acetyldeoxynivalenol (3ADON chemotype), or DON and primarily 15-acetyldeoxynivalenol (15ADON chemotype). These chemotypes may affect species or population ecology because oxygenation or acetylation is known to alter the toxicity and bioactivity of trichothecenes (2,17). It has been hypothesized that trichothecene chemotype polymorphisms are maintained by balancing selection, suggesting that chemotype differences have fitness consequences (18,34). According to sequences of some vital genes in the trichothecene biosynthesis pathway, a number of polymerase chain reaction (PCR) assays have been developed that allow the detection and differentiation of 3ADON, 15ADON, and NIV chemotypes among type B trichothecene-producing species (6,16,33). In previous studies, all three trichothecene chemotypes have been found in *F. asiaticum* in China (41). Recently, we reported that the distribution of type B trichothecene producers on barley showed a dramatic gradient along the Yangtze River (39). DON producers dominate the lower valleys while NIV producers are dominant in the upper valleys. Therefore, we hypothesize that DON-producing isolates were replacing NIV producers from east to west (39). However, additional evidence is needed to test this hypothesis. Ward et al. (35) demonstrated that the population structure of *F. graminearum* in Canada was strongly associated with chemotype. How-

ever, the association between trichothecene chemotype and population subdivision among *F. asiaticum* from China is still unknown.

In this study, we (i) investigated the population structure of *F. asiaticum* collected from the upper, middle, and lower reaches of the Yangtze River using VNTR markers and (ii) developed a effective set of diagnostic primers based on single-nucleotide polymorphisms (SNPs) among three different chemotype isolates and determined the distribution of two acetylated derivatives of DON (15ADON and 3ADON) in southern China. Collectively, the results of this study provide critical information on the structure and distribution of genotypes and the mycotoxin diversity within *F. asiaticum*, which is needed to develop effective FHB management practices in the field.

MATERIALS AND METHODS

Biological materials. The 448 single-spore strains were isolated from barley spikes with FHB symptoms, which were collected from 18 counties in seven provinces and two municipalities along the Yangtze River (Fig. 1). The details of the isolate, such as the number of strains tested in each county, were summarized in Table 1. Methods for culturing strains, DNA isolation, and identification at species level by species-specific PCR have been described previously (39).

VNTR analysis. Genotyping of the isolates was performed by VNTR-PCR with seven pairs of VNTR primers (*e*-Xtra) designed by Suga et al. (30). The reaction mixture was 20 μ l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTP, 1 μ M of each primer, 0.5 unit of *Taq* polymerase (Takara, Japan), and 10 ng of genomic DNA. PCR was carried out in a Mastercycler gradient (Eppendorf, Germany), using the following cycling parameters: 94°C for 1 min; then, 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and a final extension at

Fig. 1. Map of China indicating the sampling sites along the Yangtze River (bold line). Numbers indicate the counties and correspond with the numbering in Table 1. Major cities are also indicated.

72°C for 5 min. Then, the samples were cooled to 4°C until recovery. The PCR products were separated in 6% polyacrylamide denaturing gels. Before sample loading, the PCR products were mixed with equal amounts of loading buffer (Takara) and denatured at 95°C for 5 min. After electrophoresis, fragments were visualized using the silver-staining procedure.

Because *F. asiaticum* is homothallic and can also propagate asexually, we applied clone correction to reduce overrepresentation of individual genotypes derived from location-specific clonality (7). Isolates collected from the same sampling site with an identical VNTR profile were considered to be clones and, in these cases, only one representative was taken for the clone-corrected analysis. All clonal isolates, based on sampling site and VNTR markers, also had the same chemotype. Some isolates obtained from different sampling sites with the same VNTR genotype had different chemotypes. Five isolates in Maliang and three isolates in Shayang with an identical VNTR genotype had different chemotypes (15ADON and 3ADON, respectively). Consequently, isolates obtained from different sampling sites with the same genotype were not treated as clones. After clone correction, the total number of isolates was 282, and the number of isolates before and after clone correction in each sampling site is summarized in Table 1. All further analyses were done with the clone-corrected data.

Data generated from the seven VNTR loci were combined to identify a multilocus genotype (*G*). Genotype diversity (*GD*) and multilocus linkage disequilibrium (*LD*) were estimated by Multi-locus 1.3 (1). *GD*, the probability that two individual strains taken at random have different genotypes, was calculated by $(n/n - 1)(1 - \sum p_i^2)$, where p_i is the frequency of the *i*th genotype and *n* is the number of individuals sampled (1). 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. Gene diversity (*H*) and Nei's unbiased genetic distance (22) were calculated in the program POPGEN version 1.32 (available at <http://www.ualberta.ca/~fyeh/>). The correlation analysis between genetic distance and geographical distance of strains from 18 sampling sites was carried out using Mantel test procedure installed in IBD 1.52 (3).

The analysis of molecular variance (AMOVA) and the determination of pairwise population genetic differentiation (*F_{st}*) and gene flow (*N_m*) were calculated by Arlequin 3.1 (10).

AMOVA was performed to estimate the variance components for the VNTR genotype, partitioning the total genetic diversity into that occurring among and within populations. Pairwise *F_{st}* distance estimates were based on the number of different alleles; the statistical significance of pairwise *F_{st}* estimates was assessed using a permutation test with 1,000 permutations. Exact tests of population differentiation (15) were conducted using the program GENEPOP 4.0 (27). Analysis of admixture was performed using the Bayesian method implemented in the computer program BAPS 5.2 (8).

Design of chemotype-specific PCR primers. Primers were designed with the aid of the Primer Premier 5 Program (PREMIER Biosoft International, Canada). The *trill* gene sequences of 13 B-FHB strains, including 5 NIV producers (NRRL13383, NRRL13818, NRRL28585, NRRL13393, and NRRL13721), 3 15ADON producers (NRRL6394, NRRL5883, and NRRL26916) and 5 3ADON producers (NRRL6101, NRRL26156, NRRL2903, NRRL28062, and NRRL3288), were compared through multiple sequence alignments. SNPs between strains of different chemotype were translated into PCR amplicons of different lengths (Fig. 2). This allowed designing one set of primers—N11 (5'-CTTGTCAGGCGGCACAGTAG-3'), 15D11 (5'-AAGTATGGTCCAGTTGTCCGTATT-3'), 3D11 (5'-GCAA GTCTGGCGAGGCC-3'), and 11R (5'-TCAAAGGCCAGAGCA ACCC-3')—that generate a 643-bp fragment from NIV-producing strains, a 424-bp fragment from 3ADON producers, and a 342-bp fragment from 15ADON producers, respectively.

Validation of the chemotype-specific PCR assay was performed using 34 strains, including 11 FGSC members and 4 closely related B-trichothecene lineage species (*F. lunulosporum*, *F. cerealis*, *F. culmorum*, and *F. pseudograminearum*) (*e*-Xtra). Chemotype identities of 17 NRRL strains were published (34), and the mycotoxin chemotypes of the 17 Chinese isolates collected around China were determined by gas chromatography/mass spectrometry as described by Mirocha et al. (21). According to the size of the amplicons, all of the reference strains were distinguished into different trichothecene chemotypes (Fig. 2). These results were completely congruent with the chemical analyses, indicating the high efficiency and reliability for this multi-PCR detection of three trichothecene chemotypes from B-FHB pathogens.

Determination of the *F. asiaticum* chemotype. PCR reactions were carried out in 20-μl volumes containing 100 ng of fungal

TABLE 1. *Fusarium asiaticum* isolated (*n* = 448) isolated from 18 counties in seven provinces and two municipalities (indicated by numbered circles in Figure 1)

Region, province ^a	Site no.	County	Isolates	Clone-corrected	Latitude	Longitude	Altitude (m)
Upper							
Yunnan	1	Yuxi	20	2	24°21'	102°32'	1,639
Sichuan	2	Mianzhu	42	30	31°19'	104°12'	593
	3	Mianyang	19	11	31°28'	104°46'	453
	4	Xinsheng	45	34	30°55'	105°02'	451
Chongqing	5	Tongnan	9	4	30°13'	105°47'	263
	6	Yongchuan	22	12	29°21'	105°53'	301
Middle							
Hubei	7	Shayang	30	18	30°41'	112°28'	56
	8	Maliang	29	14	30°52'	112°31'	52
	9	Jingzhou	22	13	30°35'	112°19'	36
	10	Wuchang	31	21	30°19'	114°07'	20
	11	Huanggang	7	4	30°26'	114°47'	24
Lower							
Anhui	12	Huaiyuan	24	14	32°57'	117°11'	9
Zhejiang	13	Hangzhou	47	33	30°15'	120°10'	17
	14	Jiaying	27	20	30°45'	120°44'	6
Jiangsu	15	Nanjing	16	14	32°03'	118°46'	2
Shanghai	16	Yongfeng	30	23	31°06'	121°68'	4
Fujian	17	Putian	6	3	25°26'	119°00'	9
	18	Fuzhou	22	12	26°03'	119°18'	5
Total	...	...	448	282	...	...	...

^a Region of Yangtze River and province or municipality.

DNA. The reaction mixture consisted of 0.25 mM each dATP, dCTP, dGTP, and dTTP and 1 unit of *Taq* DNA polymerase in 1× PCR buffer with 1.5 mM MgCl₂, with the following primer concentrations: 0.3 μM N11, 0.1 μM 15D11, 0.15 μM 3D11, and 0.3 μM 11R. The cycling conditions consisted of 94°C for 3 min; 30 cycles of denaturation (94°C) for 40 s, annealing (69°C) for 60 s, and extension (72°C) for 60 s; followed by a final extension of 72°C for 10 min. Then, the samples were cooled to 4°C until recovery. PCR products were separated by electrophoresis through 1.5% agarose gels. Gels were stained with ethidium bromide at 1 μg/ml and photographed under UV light.

RESULTS

Genetic structure of *F. asiaticum* populations. With the seven VNTR markers, we detected 64 different alleles, and the number of alleles at each locus varied between 5 and 14. Among the 448 *F. asiaticum* isolates analyzed, 256 distinct genotypes were identified. The large number of alleles and genotypes demonstrate a high level of genetic diversity in our Chinese collection. All strains were classified into three populations according to their geographic origin along the Yangtze River. The upper

population contained 54 alleles, while the middle and lower populations had 30 and 41 alleles, respectively (*e*-Xtra). *G* (multi-locus genotype) was 88, 60, and 108 in upper, middle, and lower populations, respectively. *GD* values (0.991, 0.981, and 0.982, respectively) were high in all populations. Gene diversity (*H*) of the three populations ranged from 0.49 (lower) and 0.55 (middle)

TABLE 2. Genetic diversity (*GD*) and multilocus linkage disequilibrium (*LD*) analysis of *Fusarium asiaticum* populations from the upper, middle, and lower valley of the Yangtze River

Population	n ^a	G ^b	GD ^c	H ^d	LD ^e
Upper	157	88	0.991	0.62	0.092**
Middle	119	60	0.981	0.55	0.132**
Lower	172	108	0.982	0.49	0.013
Total	448	256	...	...	...

^a Sample size (*n*).
^b Number of distinct genotypes (*G*).
^c *GD* within population was calculated as $GD = (n/n - 1)(1 - \sum p_i^2)$, where *p_i* is the frequency of the *i*th genotype, and *n* is the number of individuals sampled (1).
^d Gene diversity (*H*).
^e Measure of multilocus LD; ** indicates significant at *P* < 0.01.

Fig. 2. A, Schematic representation of the multi-polymerase chain reaction targeted on *tri11*, to determine the chemotype of type B trichothecene producers. **B,** Amplifications using primers N11, 15D11, 3D11, and 11R were performed on a reference set of *Fusarium* spp. Lane M, 100-bp ladder marker; lanes 1 to 8, nivalenol (NIV) producers NRRL13818 (*Fusarium asiaticum*), NRRL25797 (*F. mesoamericanum*), NRRL29297 (*F. cortaderiae*), NRRL38380 (*F. gerlachii*), NRRL28721 (*F. meridionale* × *F. asiaticum*), NRRL28723 (*F. meridionale*), NRRL13393 (*F. lunulosporum*), and NRRL13721 (*F. cerealis*); lanes 9 to 12, 15-acetyldeoxynivalenol (15ADON) producers NRRL38208 (*F. vorosii*), NRRL46722 (*F. aethiopicum*), NRRL5883 (*F. graminearum*), and NRRL 29020 (*F. boothii*); lanes 13 to 17, 3-acetyldeoxynivalenol (3ADON) producers NRRL6101 (*F. asiaticum*), NRRL31281 (*F. brasiliicum*), NRRL2903 (*F. austroamericanum*), NRRL3288 (*F. culmorum*), and NRRL28338 (*F. pseudograminearum*). Chinese isolates are depicted in the bottom panel: lanes 18 to 20, NIV producers 13033, 7071, and 7069; lanes 21 to 25, 15ADON producers 7076, 3002, 5226, 4021, and 5167; lanes 26 to 34, 3ADON producers 13082, 13081, 5039, 4022, 7107, 7092, 11036, 1009, and 8003.

to 0.65 (upper) (Table 2). The LD values were very low in all populations and varied from 0.013 (lower) via 0.092 (upper) to 0.132 (middle) (Table 2). The LD values of upper and middle populations were both significant ($P < 0.01$).

The AMOVA revealed significant genetic variation (26.8%) among upper, middle, and lower populations ($F_{st} = 0.247$, $P < 0.001$), while 73.2% of the variation was observed among individuals within populations; the overall level of gene flow among populations was low ($N_m = 1.210$). Pairwise population genetic differentiation (F_{st}) and gene flow (N_m) across three populations were 0.055 to 0.238 and 1.601 to 8.591, respectively, and the values of genetic differentiation were all statistically significant ($P < 0.001$). The upper and lower population pair showed the highest F_{st} and lowest N_m (Table 3).

More detailed information was obtained at the county level (Table 4). The gene diversity was highest in the mountainous county of Xinsheng in the west ($H = 0.592$) and the lowest in Putian at sea level in the east ($H = 0.191$). The gene diversity of counties in upper reaches was significantly higher than counties in the lower valley (t test, $P < 0.05$), indicating higher genetic diversity in mountain regions. The LD value was highest in Jingzhou ($LD = 0.128$), and all counties with significant LD value were in the upper and middle reaches.

Pairwise estimates of genetic distance (e -Xtra) between sampling sites ranged from 0.008 (Hangzhou/Jiaxing) to 0.208

TABLE 3. Pairwise comparisons of gene flow (N_m) (above diagonal) and genetic differentiation (F_{st}) (below diagonal) among populations of *Fusarium asiaticum* from the upper, middle, and lower valley of the Yangtze River^a

Population	Upper	Middle	Lower
Upper	...	1.558	1.023
Middle	0.243**	...	6.853
Lower	0.308**	0.068**	...

^a Asterisks (**) indicate significant at $P < 0.001$.

TABLE 4. Gene diversity and linkage disequilibrium (LD) in all 18 sampling sites

Region, site ^a	Counties	H^b	LD ^c	F_{st}^d
Upper				
1	Yuxi ^e	0.243	0.014	...
2	Mianzhu	0.574	0.102**	...
3	Mianyang ^f	0.513	0.052	...
4	Xinsheng	0.592	0.122**	0.342**
5	Tongnan ^e	0.368	0.066	...
6	Yongchuan ^f	0.526	0.073**	...
Middle				
7	Shayang ^f	0.485	0.128**	0.471**
8	Maliang ^f	0.418	0.089**	...
9	Jingzhou ^f	0.512	0.188**	0.234**
10	Wuchang	0.476	0.054	...
11	Huanggang ^e	0.291	0.008	...
Lower				
12	Huaiyuan ^f	0.295	0.058	0.244**
13	Hangzhou	0.376	0.052	0.225**
14	Jiaxing	0.504	0.041	0.231**
15	Nanjing ^f	0.289	0.004	...
16	Yongfeng	0.331	0.012	0.141**
17	Putian ^e	0.191	0.003	...
18	Fuzhou ^f	0.307	0.016	0.387**

^a Region of Yangtze River and site number.

^b Gene diversity (H).

^c Asterisks (**) indicate significant at $P < 0.001$.

^d Genetic differentiation (F_{st}) was calculated between nivalenol (NIV) and 3-acetyldeoxynivalenol (3ADON) populations in seven co-occurred counties, except Fuzhou. The F_{st} of Fuzhou was calculated between 15-acetyldeoxynivalenol and 3ADON populations.

^e F_{st} and LD of this sampling site were based on a very low number of genotypes (<5 genotypes). This data is for reference only.

^f F_{st} and LD of this sampling site were based on <20 genotypes. This data is for reference only.

(Yuxi/Putian). Based on the matrix, the Mantel test revealed that there was a highly significant positive correlation ($r = 0.5147$, $P < 0.001$) between estimates of genetic distances (22) and geographical distances between populations (Fig. 3). Significance was assessed by 1,000 randomizations.

Chemotype diversity. A PCR assay based on the *tril1* gene was conducted to determine the chemotype of each *F. asiaticum* isolate. The assay indicated that NIV, 3ADON, and 15ADON producers were present among the 282 clone-corrected isolates tested; 109 isolates yielded a NIV-specific PCR fragment, 159 isolates had a 3ADON-specific fragment, and a small set ($n = 14$) of strains produced a 15ADON-specific fragment. The geographical distribution of chemotypes showed a dramatic gradient; the NIV chemotype was remarkably more frequent in upper valleys and the 3ADON chemotype was predominant in the middle and lower reaches of the Yangtze River (Fig. 4). In all, >86% of strains collected from upper valleys of the Yangtze River were NIV producers, while 79% isolates obtained from middle and lower valleys were DON producers (3ADON and 15ADON). 3ADON producers were predominant in the middle and lower valleys of the Yangtze River but not in Fujian Province. The number of 15ADON producers as a percentage of the total DON producers was high in Fujian (Fig. 4).

In order to evaluate the genetic variation of different populations in different areas along the Yangtze River, samples were divided into nine groups based on geographic origin and trichothecene chemotype. These groups consisted of 3ADON, 15ADON, and NIV producers from upper reaches (3U, 15U, and NU); 3ADON, 15ADON, and NIV producers from middle reaches (3M, 15M, and NM); and 3ADON, 15ADON, and NIV producers from lower reaches (3L, 15L, and NL). Pairwise F_{st} values showed significant differentiation between NIV and 3ADON populations (NU-3M, $F_{st} = 0.257$, $P < 0.001$; NU-3L, $F_{st} = 0.312$, $P < 0.001$) which were predominant in different areas. A small but statistically significant degree of genetic differentiation was also observed between 3ADON populations from middle and lower reaches (3M-3L, $F_{st} = 0.058$, $P < 0.001$). F_{st} values among populations within the upper (NU-3U, $F_{st} = 0.282$, $P < 0.001$; NU-15U, $F_{st} = 0.269$, $P < 0.001$), middle (3M-15M, $F_{st} = 0.211$, $P < 0.001$; 3M-NM, $F_{st} = 0.248$, $P < 0.001$), and

Fig. 3. Correlation between calculated genetic distance and geographic distance among 18 sampling points ($r = 0.5808$, $P < 0.001$).

lower (3L-15L, $F_{st} = 0.291$, $P < 0.001$; 3L-NL, $F_{st} = 0.194$, $P < 0.001$) reaches were all high. The LD value of NU, 3M, and 3L was 0.048 (NU), 0.078 (3M), and 0.008 (3L), which was lower than the value of populations including the whole isolates in each area (upper, middle, and lower reaches), respectively (Table 2).

A Bayesian clustering method was used to investigate admixture between the three dominant populations (NU, 3M, and 3L) in each area. Admixture probabilities >95% were obtained for 24 of the 227 isolates genotyped (Table 5), and analyses of population structure after removal of the 24 admixed isolates produced results similar to those described above; there was also significant pairwise F_{st} (Table 6) and a similar LD value (NU = 0.045, 3M = 0.069, and 3L = 0.009). We found that 9 of the 80 (11.3%) NIV strains had significant admixture probabilities ($P < 0.001$). Among these nine strains, Bayesian posterior mean estimates for the proportion of the genome derived from the 3M or 3L populations was 44 to 98% (Table 5), and the proportion from 3M was significantly ($P < 0.005$) more than from 3L. Only 3 of 147 (2.0%) 3ADON strains had significant ($P < 0.001$) admixture probabilities with some proportion of their genomes derived from the NU population. NIV isolates with 3ADON genetic material were significantly (exact test; $P < 0.001$) more common than 3ADON isolates with NIV genetic material. The remaining admixed isolates were composed of genetic material from the 3M and 3L populations, which likely share a high level of background ground relatedness given their close genetic relationship ($F_{st} = 0.054$ when all isolates are included in the analysis).

In order to get more detailed information about the genetic variation and potential migrants of the pathogen, population genetic differentiation (F_{st}) between 3ADON and NIV producers and multilocus LD in the same sampling site were estimated. This

analysis focused on seven counties where 3ADON and NIV producing isolates co-occurred. The result revealed significant genetic differentiation ($P < 0.001$) between the two chemotype populations existing in each sampling site; F_{st} was from 0.141 (Yongfeng) to 0.471 (Shayang) and the LD value of Xinsheng, Shayang, and Jingzhou populations was significant ($P < 0.001$), although it was low in other four low valley counties (Table 4). This result showed a trend that the genetic differentiation and multilocus LD increased from east to west along the Yangtze River. We also found that, in each region of the Yangtze River, the LD of counties was relatively high in counties where NIV and 3ADON producers co-occurred, indicating that the differences between NIV and 3ADON populations contribute significantly to the LD in the same sampling site.

DISCUSSION

The low LD estimates detected in all counties suggest that frequent outcrossing occurred in all three populations along the Yangtze River. This is in agreement with the previous reports on other members of the FGSC such as *F. graminearum* of which low LD values were found in populations in the United States (28,40). However, no study until now could present a direct evidence of sexual mating or crossing in the field. In contrast, clear LD was observed in a single interbreeding population of *F. graminearum* collected from wheat in Zhenjiang province using restriction fragment length polymorphism (RFLP) markers (12). We found no significant LD values for Zhejiang whereas Gale et al. (12) did observe significant LD values. This revealed that the original high LD found may have been decreased over time (2000 to 2005) due to outcrossing. We assume that local significant LD

Fig. 4. Distribution of 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), and nivalenol (NIV) chemotypes among all strains in 18 counties, which are arranged from the upper valleys to the lower valleys of the Yangtze River. Numbers at the top indicated the number of isolates in each county.

values indicate continuous gene flow from migrants, resulting in local gametic disequilibria discussed in more detail below. When these migrants are absorbed in the gene pool, the LD values are reduced, and this may explain the differences in LD values found over time and space in the different *Fusarium* populations.

AMOVA showed that genetic variation within the upper, middle, and lower populations was higher than among these populations, which may be due to the prevalence of sexual reproduction, population size, or high levels of migration. Previous studies (5,11,12) have found similar results for *F. graminearum* populations from wheat, and sexual recombination in the field may result in the formation of novel genotypes (4). The results of the Mantel test suggested restricted gene flow among sampling sites and were at least partly consistent with the model of isolation by distance (37). Also, Zeller et al. (40) found significant negative correlations between population identity and distance of different *Gibberella zeae* populations in the United States. In our case, the Mantel test revealed significant correlation ($r = 0.5147$, $P < 0.001$) between genetic distances and geographical distances. Previously, Zeller et al. reported a significant negative correlation ($r = -0.591$, $P < 0.001$). It is clear that these correlations will only be shallow, if detected at all, because they do not take other factors that are important for migration into account, such as geographical barriers or prevailing winds. Moreover, Dusabenyagasani et al. (9) also performed RAPD genotyping on 72 *F. graminearum* strains collected from Canada in 1999, and found very little population differentiation with distance. The genetic variation among the upper, middle, and lower populations was significant ($P < 0.001$), although its value is lower. It demonstrated that these regions contain three different populations, with limited genetic exchange among populations. We also observed that *Fst* between upper and middle populations was much higher than between middle and lower populations. This phenomenon reflected the presence of geographic barriers such as high mountain ridges adding to the isolation by distance as discussed above.

In previous studies of these samples, we have found that the geographical distribution of chemotypes showed a dramatic

gradient along the Yangtze River and we hypothesized that isolates from the mountain-rich provinces represented the old FHB population and that NIV-producing isolates may have dominated Chinese FHB populations in the past but are currently being replaced by DON producers (39). In this study, we tested this hypothesis by combining genotyping analysis using “neutral” VNTR markers with the chemotype of the isolates.

After testing 448 *F. asiaticum* isolates with the newly designed chemotype-specific primers, the DON producers were further differentiated into 3ADON or 15ADON chemotypes. This demonstrates that 3ADON and not 15ADON predominates in the middle and lower valleys of the Yangtze River. This geographic gradient in trichothecene chemotype frequency was similar to the recently reported shift in the *F. graminearum* population in North American. Ward et al. (35) observed a pronounced longitudinal cline of the trichothecene chemotype frequency distribution in Canada, in which 3ADON producers were most frequent in eastern Canada and decreased in frequency in each province sampled from east to west across Canada. They also documented a recent and significant shift in FHB pathogen composition by demonstrating that the 3ADON chemotype frequency in western Canada increased >14-fold between 1998 and 2004. The change of 3ADON chemotype frequency was also observed in China. Zhang et al. (41) reported the trichothecene chemotypes of *F. graminearum* SCAR group 5 (now called *F. asiaticum*) collected in 1999 from China. In their study, 53.1% of all isolates obtained from the middle valley of the Yangtze River were 3ADON producers. However, in the present study, we observed a ratio of 3ADON producers in the middle valley of 87.1%. This indicated that the 3ADON producers increased significantly (exact test, $P < 0.001$) in the middle reaches in the 6 years between 1999 and 2005.

We also tested the direction of the gene flow using Bayesian analysis. Pairwise *Fst* distance values showed significant differentiation between NIV and 3ADON groups (NU-3M, *Fst* = 0.227, $P < 0.001$; NU-3L, *Fst* = 0.256, $P < 0.001$), indicating that groups defined based on trichothecene chemotype represented distinct populations, even where isolates from these groups co-occur (Table 4). Bayesian analysis revealed a biased gene flow from 3ADON populations in the middle and lower reaches to the NIV population in the upper valley, which may determine the migration direction of *F. asiaticum*. 3ADON isolates from middle and lower reaches showed a close genetic relationship (*Fst* = 0.053), which is likely due to recent migration of 3ADON isolates. To further verify this conjecture, we performed LD analysis. The LD value of populations including 3ADON, NIV, and 15ADON isolates in each area were all higher than the dominant chemotype populations (NU, 3M, and 3L, respectively), indicating there has been insufficient time for random mating to break down LD created by migrants. The situation of counties where different chemotype producers co-occurred provided more detailed evidence about it. Both the genetic differentiation between 3ADON- and 15ADON-producing isolates and multi-locus LD increased from east to west along the Yangtze River. Consistent with the 3ADON isolates migrating from east to west, the genetic differentiation and LD was larger in the west. In addition, all counties with significant LD value belonging to

TABLE 5. Isolates with significant admixture probabilities from Bayesian analysis^a

No.	Source population	Percent genome represented by each population		
		NU	3M	3L
05023	NU	0.15	0.84	0.01
05034	NU	0.45	0.55	0.00
05035	NU	0.33	0.62	0.05
05048	NU	0.45	0.55	0.00
05061	NU	0.33	0.00	0.67
05063	NU	0.06	0.01	0.93
05070	NU	0.22	0.72	0.06
05081	NU	0.56	0.44	0.00
05084	NU	0.02	0.24	0.74
05105	3M	0.67	0.33	0.00
05108	3M	0.00	0.54	0.46
05113	3M	0.48	0.52	0.00
05115	3M	0.00	0.26	0.74
05122	3M	0.00	0.40	0.60
05135	3M	0.06	0.16	0.78
05142	3M	0.00	0.00	1.00
05145	3M	0.00	0.45	0.55
05172	3M	0.00	0.41	0.59
05173	3L	0.00	0.65	0.35
05198	3L	0.00	0.90	0.10
05205	3L	0.08	0.65	0.35
05206	3L	0.00	1.00	0.00
05212	3L	0.00	0.48	0.52
05245	3L	0.00	0.81	0.19

^a NU = nivalenol population from the upper reaches of the Yangtze River, 3M = 3-acetyldeoxynivalenol (3ADON) population from the middle reaches, and 3L = 3ADON population from the lower reaches.

TABLE 6. Pairwise genetic differentiation (above diagonal) and permutation test probability (below diagonal) estimates with admixed isolates excluded

Population ^a	NU	3M	3L
NU	...	0.343	0.339
3M	<0.001	...	0.104
3L	<0.001	<0.001	...

^a NU = nivalenol population from the upper reaches of the Yangtze River, 3M = 3-acetyldeoxynivalenol (3ADON) population from the middle reaches, and 3L = 3ADON population from the lower reaches.

upper and middle reaches provided additional evidence of this. Based on (i) the cline in trichothecene chemotypes, (ii) a recent and significant shift of 3ADON producers in FHB pathogen composition in the middle valley, (iii) the bias gene flow from 3ADON to NIV populations, and (iv) the substantial genetic differentiation and LD between sympatric NIV and 3ADON populations along the Yangtze River, we conclude that a 3ADON population from eastern China has only recently spread west. This spread may have caused the increased problems of FHB on barely and wheat observed in the last decade. Ward et al. (35) reported that *F. graminearum* isolates from 3ADON populations produced significantly more trichothecene and had significantly higher fecundity and growth rates. This may also be true for *F. asiaticum*, and this selective advantage may have driven the change to a pathogen population which is more toxigenic and potentially more vigorous. To further validate and explain the hypothesis, monitoring the shifts of genetic variation and trichothecene chemotype composition of the pathogen along the Yangtze River and detecting the biological characteristics of different populations are necessary in the future.

This study showed that *F. asiaticum* isolates that produce 3ADON are spreading from the lower and central valleys of the Yangtze River. Although this spread may be delayed by physical barriers such as mountain ridges, we expect that the driving force articulated by the capacity to produce different type B trichothecenes will cause a further spread of 3ADON producers in the future. These results have important implications for the control of scab and the reduction of trichothecene contamination of cereals in southern China. Our findings highlight the potential for the exchange of adaptations between FHB populations via recombination and emphasize the need to consider pathogen variation in addition to host variation in FHB management. Specifically, the pathogen variation in the severe disease epidemic area of China is required to be considered in the development of cereal cultivars with broad-based resistance to the pathogens.

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