Molecular Characterization of Fusarium Head Blight Pathogens Sampled from a Naturally Infected Disease Nursery Used for Wheat Breeding Programs in China

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Abstract

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Fusarium head blight (FHB) is an important disease of wheat and barley worldwide. The disease is primarily caused by members of the *Fusarium graminearum* species complex, consisting of at least 14 phylogenetically distinct species. To determine the population structure of the FHB pathogens in a naturally infected disease nursery located at Jianyang, Fujian province, China, 160 isolates of the *F. graminearum* complex were recovered from symptomatic wheat spike samples collected in two consecutive years (2008 and 2009) and characterized using species- and chemotype-specific polymerase chain reaction as well as variable number tandem repeat (VNTR) markers. All isolates analyzed were identified as *F. asiaticum* except for one isolate, which was identified as *F. avenaceum*. Among the 159 *F. asiaticum* isolates, 126 (79%) isolates were of the nivalenol (NIV) type while 29 (18%) isolates were of the 15-acetyl deoxynivalenol type and only 4 (3%)

isolates were of the 3-acetyl deoxynivalenol type. The 10 VNTR markers revealed 124 distinct haplotypes and 76 polymorphic alleles across the whole population. The two subpopulations (FA-08 and FA-09) grouped based on the year of collection exhibited low genetic differentiation ($F_{st} = 0.032$) and high gene flow ($N_m = 15.13$). However, a significant genetic differentiation was found within the NIV-type isolates as revealed by the Structure software. The pairwise linkage disequilibrium tests did not support the hypothesis of random mating in the population because half (48.8%) of the locus pairs showed a linkage disequilibrium ($P > 0.05$). Our results suggest that FHB in this nursery was caused by a genetically homogenous and non-random mating population of *F. asiaticum* in 2008 and 2009, which consisted of all three tricothecene types with various levels of aggressiveness.

Fusarium head blight (FHB), or scab, is a major disease of wheat and barley worldwide. The disease causes significant losses in yield and quality of the two crops (23,46). In North America, more than \$3 billion of losses were estimated after the FHB epidemics during the 1990s (23). Frequent disease outbreaks also have been reported from Asia, Europe, and South America in recent years, posing a potential threat to the world's grain production and food supply (17). In China, the first FHB outbreak occurred in 1936 in Anhui province but epidemics were sporadic for almost a half century after that year (47). Since 1985, the disease has become more frequent and widespread in major wheat-growing areas of eastern and central China, especially along the lower and middle reaches of the Yangtze River Valley as well as the northeastern and central parts of China (3,35).

Several *Fusarium* spp. can cause FHB but the predominant pathogens vary with regions and may change over time. In many regions of the world, FHB is mainly caused by members of the *Fusarium graminearum* species complex, consisting of at least 14 phylogenetic species (28,29,38,50). In China, *F. graminearum* sensu lato was found in 94.5% of the samples recovered from 20 provinces along the Yangtze River Valley and 17 other *Fusarium* spp. were also isolated (43). Global studies of *Fusarium* spp. composition and population structure showed that *F. graminearum* sensu stricto, one member of the *F. graminearum* complex, is dominant in North America (28,29,38), although other species were also found in some other regions of the United States (15). In China, *F. asiaticum* and *F. graminearum* sensu stricto are the dominant species to initiate FHB in wheat (14,35,54) and barley (48,53,55), with *F. asiaticum* being more common in the southern China and *F.*

graminearum sensu stricto being more frequent in the north (35). *F. asiaticum* and *F. graminearum* were also found to be the predominant etiological agents of FHB in Japan, although their distributions vary depending on the regions (19,20,40).

Population subdivision of *F. graminearum* correlates to geographical differences and tricothecene types worldwide (14,16,45,48). Previously, Karugia et al. (19) found 42.3% of isolates were of nivalenol (NIV) type and 57.7% were of 3-acetyl deoxynivalenol (3ADON) type but no 15-acetyl deoxynivalenol (15ADON)-type isolates were identified among 208 isolates collected from Zhejiang province in China. However, a recent study of 448 *F. asiaticum* isolates collected from barley at 18 sampling sites along the Yangtze River Valley identified all three tricothecene types (NIV = 109, 3ADON = 159, and 15ADON = 14) (53). They also found significant genetic differentiation among populations originated from upper, middle, and lower regions of Yangtze River. Earlier studies on population genetics of *F. graminearum* indicated a relatively homogenous population in the United States (51,52), but more recent studies revealed genetically divergent populations in the Northern Great Plains of the United States and Canada (16,34,45).

Although population genetics of FHB pathogens have been studied extensively on a global to regional scale (16,35,45,48,52, 53,55), information on the species composition, tricothecene types, and genetic structure of the pathogen population from a smaller area or experimental unit is relatively limited. In Jianyang, Fujian province of China, where the weather is consistently warm and humid during and after wheat anthesis, FHB develops very well under natural infection conditions every year (3,56). For this reason, a scab nursery was established in the early 1970s and has been used for screening breeding lines or other materials of wheat for FHB resistance ever since. However, the pathogen profile of the nursery is not known. Studies of the genetic structure of pathogen populations not only may provide important information regarding the epidemiology and evolutionary potential of the *F. graminearum* complex but also could lead to improved strategies for controlling this pathogen (51). We attempted to answer several questions, in-

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cluding (i) how many species of the *F. graminearum* complex caused FHB in the nursery, (ii) what were the number and relative frequency of trichothecene types, and (iii) was there genetic differentiation in the pathogen population? Therefore, the major objectives of our study were to determine (i) species composition, (ii) trichothecene types, and (iii) genetic structure of FHB pathogens from this small (approximately 500 m²) disease nursery under natural infection.

Materials and Methods

Fungal isolates. Symptomatic wheat heads were sampled from an FHB nursery (10 by 50 m² in size) located at Jianyang, Fujian province, China (Fig. 1), in two consecutive years (2008 and 2009). In 2008, wheat spikes showing FHB symptoms were randomly collected from representative plots of the nursery. In 2009, two to three scabby heads were sampled from each of 54 spots evenly distributed in the nursery, with a distance of 5 to 10 m between two neighboring spots. Fungal isolation, growth, and storage were done as described by Puri and Zhong (34). Each isolate was named after JY (Jianyang, the nursery location) followed by 08 or 09 (collection year) and serial numbers. For example, JY08-001 represents the isolate collected from Jianyang in 2008 with a serial number 001.

DNA extraction, *Fusarium* spp. identification, and trichothecene type determination. For DNA isolation, mycelia were har-

vested from a single spore-derived culture of each fungal isolate grown on cellophane layered over half-strength potato dextrose agar for 6 to 7 days. DNA was extracted using the FastDNA Kit and FastPrep instrument (MP Biomedical) according to the manufacturer's instructions, and then quantified using a NanoDrop (NanoDrop Technologies). To confirm the identity of the *Fusarium* spp., a portion of the histone *H3* gene was amplified using polymerase chain reaction (PCR) according to the method described by O'Donnell et al. (28,29). The amplicons were purified by ExoSAP IT (USB Corporation) and sent to the University of Hawaii for sequencing. The isolates with identical DNA sequences were grouped together. The sequences (JQ435850 to JQ435858) from the representative isolates in each of the groups were used for BLASTN search against the National Center for Biotechnology Information GenBank database. The primers (6A3AF, 6A3AR, 6CNF, 6ANR, 6G3AF, 6G3AR, 3D15AF, and 3D15AR) developed and described by Suzuki et al. (41) were used to differentiate *F. graminearum* and *F. asiaticum*. Trichothecene types were determined by PCR using the trichothecene-specific multiplex primers and conditions previously described (38,44). The PCR products were separated on a 1% agarose gel and sized with reference to a 100-bp DNA ladder (NEB). The amplicon at 840, 610, and 243 bp corresponds to the NIV, 15ADON, or 3ADON type, respectively (44).

Analysis of variable number tandem repeats. Nine variable number tandem repeat (VNTR) markers (HK913, HK917, HK957, HK965, HK967, HK977, HK1043, HK1059, and HK1073) developed by Suga et al. (39) and one additional VNTR marker HK1003, described by Gale et al. (13) were used to analyze the population structure of the FHB pathogens. Information of the primer pairs used to detect the 10 VNTR markers is provided in Table 1. The forward primer of each primer pair was labeled with IRD700/800 at the 5' end (Eurofins MWG Operon). PCR was performed as described by Suga et al. (39), with some modifications. Each PCR reaction was run in a total volume of 10 µl containing 1× reaction buffer, 200 µM dNTP, 1 µM each primer (forward and reverse), 1 unit of *Taq* polymerase, and genomic DNA at 25 to 30 ng/µl on a PTC-100 thermal cycler (MJ Research). Amplification profile consisted of initial denaturation at 94°C for 3 min; followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. The PCR products were detected on the Li-COR system (Li-COR) according to the method previously described by Puri and Zhong (34). For each primer pair, amplicons with the same size were considered as the same allele and scored on size (bp). A binary system (i.e., 1 = present and 0 = absent) was also used as required for analysis.

Population genetic analysis. Initially, we used Structure 2.3.2 software based on a Bayesian-model-based clustering method (12,33) to assign each multilocus genotype to user-defined genetic *K* clusters. The analysis was performed with *K* = 1 to 5 for five

Fig. 1. Map of Fujian province (lower right), which is located in the southeast region of China (upper). The naturally infected *Fusarium* head blight (FHB) disease nursery is at the city of Jianyang, north of Fujian province. Symptomatic wheat heads were randomly collected from a field (10 by 50 m²) in the nursery for two consecutive years (2008 and 2009).

Table 1. Number of alleles, gene diversity, and fixation index (G_{st}) of 10 variable number tandem repeat (VNTR) markers used to analyze the 144 *Fusarium asiaticum* isolates collected from the disease nursery at Jianyang, China

VNTR ^b	Location ^c	N ^d	Gene diversity ^a			G_{st} ^e
			2008	2009	Overall	
HK1043	Ch1/Ct1.52/41,839	3	0.38 (0.39)	0.44 (0.44)	0.42 (0.42)	0.002
HK957	Ch1/Ct1.91/16,055	7	0.81 (0.84)	0.80 (0.81)	0.81 (0.82)	0.012
HK967	Ch2/Ct1.154/53,868	2	0.00 (0.00)	0.02 (0.02)	0.02 (0.02)	0.005
HK1003	Ch1/Ct4.37/6,986	26	0.90 (0.93)	0.94 (0.95)	0.94 (0.95)	0.017
HK1073	Ch4/Ct1.398/70,812	12	0.75 (0.77)	0.78 (0.79)	0.78 (0.79)	0.022
HK1059	Ch3/Ct1.196/164,228	6	0.74 (0.76)	0.77 (0.78)	0.78 (0.78)	0.024
HK977	Ch3/Ct1.208/47,696	4	0.10 (0.1)	0.29 (0.29)	0.24 (0.24)	0.020
HK917	Ch1/Ct1.82/2,471	3	0.15 (0.15)	0.12 (0.12)	0.13 (0.13)	0.000
HK913	Ch1/Ct1.73/664	6	0.74 (0.76)	0.63 (0.63)	0.70 (0.70)	0.064
HK965	Ch2/Ct1.154/51,671	7	0.64 (0.66)	0.75 (0.75)	0.74 (0.75)	0.038
Mean	...	7.6	0.52 (0.53)	0.55 (0.55)	0.56 (0.56)	0.026

^a Nei's gene diversity (*H*) (26) within populations was calculated using POPGENE 1.32 (49). Values in parenthesis were calculated by ARLEQUIN 3.1 (10).

^b HK1003 was described by Gale et al. (13) and the others were reported by Suga et al. (39).

^c Marker location in the genome was indicated by chromosome (Ch) number/contig (Ct) number/position in contig.

^d Number of alleles.

^e G_{st} fixation indices (26) for individual loci were calculated for the 132 clone corrected isolates using POPGENE 1.32 (49).

iterations with 100,000 Markov Chain Monte Carlo reps after burn-in period of 10,000 (33). The exact number of clusters (K) was determined based on $\ln(P)$ curve for each subpopulation probability and verified with Structure Harvester, a web-based program to visualize Structure output implementing the Evanno method (8). To test whether there was a genetic differentiation between isolates collected from the 2 years, we also grouped isolates into populations according to the year of collection. Isolates collected from 2008 were grouped into one population (FA-08) and those from 2009 were grouped into another population (FA-09).

The genotypic diversity (GD) and index of multilocus linkage disequilibrium ($\bar{r}_d$) were calculated using software MULTILOCUS 1.3 with the algorithm $G = (n/n-1)\{(1 - \sum p_i^2)\}$, where n is the number of individuals sampled and p_i is the frequency of i th genotype (1). The GD has a value of 0 if every individual is of the same genotype or 1 if every individual is different in the population. The index ($\bar{r}_d$) was used to test a hypothesis of random mating within a subpopulation after 1,000 permutations. The index ($\bar{r}_d$) gives a value of zero if there is no association among alleles on unlinked loci, as expected on null hypothesis of random mating. The significant linkage disequilibrium (LD) among VNTR loci and successive P values were generated using a Markov chain length of 10^4 and dememorization of 10^4 , as implemented in ARLEQUIN 3.1.

The genetic differentiation and other population parameters were determined using different statistical tools and techniques. Analysis of molecular variance (AMOVA), population differentiation or fixation index (F_{st}), and number of migrants exchanged between two populations or gene flow (N_m) were calculated using ARLEQUIN 3.11 (10). The allele frequencies, percentage polymorphic loci, gene diversity (26,27), Nei's unbiased genetic distance (D) (27), and fixation index (G_{st}) for individual loci were calculated using POPGENE ver. 1.32 (49). Molecular variance (AMOVA) was performed to test hierarchical partition of genetic variation between two populations and individuals within the whole population (11). The gene flow was calculated by $N_m = (1 - F_{st})/2F_{st}$, where N is the effective population size and m is the migration rate per generation. The statistical significance of pairwise F_{st} was tested by 1,000 permutations.

Aggressiveness evaluation of representative *F. asiaticum* isolates. A greenhouse experiment was conducted to test the aggressiveness of 16 representative isolates of *F. asiaticum*, including 6 15ADON-type isolates, 4 3ADON-type isolates, and 6 NIV-type isolates, on the spring wheat 'Briggs' (susceptible to FHB). Seed were sown in plastic buckets (30 by 20 by 35 cm³) filled with Sunshine pot mix (Sun Gro Horticulture Canada Ltd.) with two rows (8 seeds/row) per bucket. For each isolate, three rows (replicates) of the plants were used for inoculation at the anthesis. Plant growth conditions, inoculum preparation, inoculation, and disease scoring were the same as previously described (34), except that the inoculated plants were kept in a humidity chamber for 72 h instead of overhead misting.

Results

***Fusarium* spp. identification.** In total, 160 *Fusarium* isolates (59 from 2008 and 101 from 2009) were first analyzed using the

Fig. 2. Frequency distribution of chemotypes among *Fusarium asiaticum* isolates collected in **A**, 2008 (59 isolates); **B**, 2009 (100 isolates); and **C**, the 2 years combined (159 isolates). 15ADON = 15-acetyl deoxynivalenol, NIV = nivalenol, and 3ADON = 3-acetyl deoxynivalenol.

species-specific multiplex PCR (41). The result indicated that all isolates except isolate JY09-030 showed the amplicons unique to *F. asiaticum*. Further analysis of histone *H3* gene sequences indicated that JY09-030 (JQ435857) was *F. avenaceum* and the rest of isolates were identified as *F. asiaticum*. Compared with the histone *H3* gene sequence of the *F. asiaticum* strain (*F. graminearum* species complex number 9086), 92% of the *F. asiaticum* isolates analyzed in this study had no or one base difference and only 8% isolates had two to four base differences.

Trichothecene type determination. Trichothecene types were determined for the 159 *F. asiaticum* isolates using the multiplex PCR (25,28). Among them, 126 (79%) of the isolates were of the NIV type while 29 (18%) and 4 (3%) isolates were of 15ADON and 3ADON types, respectively (Fig. 2C). In 2008, 45 (76%) isolates were of the NIV type and 14 (24%) isolates were of the 15ADON type but no 3ADON type was detected (Fig. 2A). In 2009, all three trichothecene types were identified, with 81 (81%) being of the NIV type, 15 (15%) being of the 15ADON type, and 4 (4%) being of the 3ADON type (Fig. 2B).

Population genetics analyses. In all, 144 *F. asiaticum* isolates were analyzed using the 10 VNTR markers, and 76 polymorphic alleles were generated among them. The number of alleles generated by individual VNTR markers varied from 2 to 26 (Table 1). Gene diversity (H) also differed among the 10 VNTR markers, with HK1003 being the highest (0.94), followed by HK957 (0.82), HK1073 (0.78), and HK1059 (0.78). Marker HK967 and HK917 had the lowest H (0.02 and 0.13, respectively; Table 1). Using POPGENE, a fixation index (G_{st}) was generated for each VNTR marker. Marker HK917 had the highest G_{st} value (0.064) followed by marker HK965 (0.038). However, the three markers named HK917 ($G_{st} = 0$), HK1043 ($G_{st} = 0.002$), and HK967 ($G_{st} = 0.005$) had very low fixation indices (Table 1).

Of the 144 isolates, 132 isolates remained after clone removal. All clonal isolates were found in 2008 and clones with the same haplotype belonged to the same trichothecene type. Using STRUCTURE, we assigned the 131 (excluding *F. avenaceum*) clone-corrected isolates into distinct clusters. Assignment of isolates into a specific genetic cluster was based on the average membership value $q = 0.8$ or above. Using this standard, 98 isolates, in total, were assigned into one of the three clusters (numbers 1, 2, and 3) with the highest probability. However, 33 isolates did not fit to any of the clusters and, thus, were not assigned into any populations. Cluster 1 (q mean = 0.941, standard deviation [SD] = 0.036) had a total 45 isolates consisting of 44 NIV-type isolates and 1 15ADON-type isolate ($q = 0.978$), which was named as population NIV-1. Cluster 2 (q mean = 0.908, SD = 0.053) had 18 isolates comprising 16 NIV-type and 2 15ADON-type isolates and named population NIV-2. Because cluster 3 (q mean = 0.937, SD = 0.042) consisted of 18 NIV-type, 15 15ADON-type, and 2 3ADON-type isolates, we further divided them into two subpopulations, one

Table 2. Nei's genetic distance D (above diagonal) and P value from the exact tests for genetic differentiation Φ_{PT} (below diagonal) between four populations of *Fusarium asiaticum* collected from the Fusarium head blight disease nursery in China^a

Populations ^b	NIV-1	NIV-2	NIV-3	DON
NIV-1	...	0.240	0.299	0.419
NIV-2	<0.001	...	0.346	0.399
NIV-3	<0.001	<0.001	...	0.076
DON	<0.001	<0.001	0.139	...

^a D (25) was calculated using POPGENE (49). Φ_{PT} (analogous to F_{st}) was calculated as proportion of variance among and within population relative to the total variance (11) using GENALEX 6.4 (31). $\Phi_{PT} = AP/(WP + AP) = AP/TOT$, where AP = estimated variance among population, WP = estimated variance within in population, and TOT = total variance. The probability of obtaining equal or lower Φ value was determined by 1,000 random permutations.

^b The four populations—NIV-1, NIV-2, NIV-3, and DON—are genetically defined and subdivided according to the member assignment by STRUCTURE and by taking into account the trichothecene types.

named NIV-3, containing all NIV isolates, and the other named DON, consisting of the 3ADON-type and 15ADON-type isolates.

AMOVA among the four populations (NIV-1, NIV-2, NIV-3, and DON) indicated that 17% of genetic variation was due to differences among populations and 83% of variation was from individuals within population. The three populations NIV-1, NIV-2, and DON had a similar level of gene diversity (0.41 to 0.46) whereas NIV-3 had a relatively high gene diversity (0.54) across all loci. Population-wise comparison showed a significant differentiation among them, except between NIV-3 and DON (Table 2). A high gene flow (25.9) and a very low population differentiation (0.019 and $P = 0.139$) was found between NIV-3 and DON.

The genetic distance was low ($D = 0.05$) and genetic identity was high ($I = 0.95$) between the two populations (FA-08 and FA-09) grouped according to the years of collection. The hierarchical AMOVA showed that genetic variation between the populations accounted for only 3.23% while most of the genetic variation (96.77%) was from individuals within population. Genetic differentiation was low ($F_{st} = 0.032$) and gene flow was high ($N_m = 15.13$) between FA-08 and FA-09.

The $\bar{r}_d$ index was low in both populations (i.e., 0.073 for FA-08 and 0.050 for FA-09) but was significantly different from zero at $P < 0.001$. The index of association, another measure of linkage disequilibrium, was 0.575 and 0.424 for FA-08 and FA-09, respectively, and was also significantly different from zero ($P < 0.001$). Analysis of linkage disequilibrium between loci pairs of the 10 VNTR loci showed that almost half (48.8%) of the 45 possible locus-pair combinations were in linkage disequilibrium ($P > 0.05$; Table 3), suggesting that out-crossing was not frequent in the population.

Aggressiveness evaluation. The average disease severity (DS) caused by individual isolates on Briggs ranged from 12.4% (JY08-013) to 96.2% (JY09-45E). Analysis of variance indicated significant differences ($P > 0.0001$) in aggressiveness among the isolates based on the DS caused on Briggs. Isolate JY09-45E (a 3ADON-type isolate) and JY08-043 (a 15ADON-type isolate) induced the highest DS, which was not significantly different from those induced by isolates JY08-009 and JY09-045 (Fig. 3). However, all

NIV-type isolates evaluated were significantly less aggressive compared with the 3ADON and 15ADON isolates (Fig. 3).

Discussion

Our results indicated that *F. asiaticum* is the primary etiological agent 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 for all major cereals (wheat, barley, and rice) in warmer regions of Asian countries, including China, Korea, and Japan (21,35,40). The majority of *Fusarium* isolates collected from FHB-infected wheat and barley samples in eastern and central China along the Yangtze River were identified as *F. asiaticum* in several independent studies (14,19,20,53,55). Qu et al. (35) reported that 79% of the 437 isolates collected from warmer ($>15^{\circ}\text{C}$) regions of China were *F. asiaticum*. Karugia et al. (20) identified 179 *F. asiaticum* isolates among 183 isolates sampled in a small experimental field in Japan. Lee et al. (21) found that all of the 249 isolates collected from rice fields in southern provinces of Korea were *F. asiaticum* whereas isolates from eastern provinces of Korea were either *F. asiaticum* or *F. graminearum* sensu stricto. They hypothesize that *F. asiaticum* has a host preference and specificity to rice, and perithecia production typically favors rice straws under warmer conditions. The Jianyang nursery is located in the warmer region, and the field was planted with rice before wheat was grown for FHB evaluation (J. Huang, *personal communication*). Rice was the major cereal crop in Jianyang and no wheat, barley, or corn were grown around the region. Thus, initial infections might have come from inoculum sources originated from rice straws, as found in Korea (21). However, this hypothesis needs further investigation by comparing isolates originated from the nursery with those from the rice fields surrounding the nursery.

We found that a majority (79%) of the *F. asiaticum* isolates were of the NIV type although isolates of the 15ADON type (18%) and the 3ADON type (3%) were also identified. To our knowledge, this is the first time that all three trichothecene types were found in the same place in southeastern China. Karugia et al. (19) analyzed 208 isolates of *F. asiaticum* collected during 2000 by Gale et al. (14) from four locations in Zhejiang province (approximately 500 km north of Jianyang) 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. (54) analyzed a 1999 collection of 299 isolates from 12 provinces along the middle and lower reaches of Yangtze River and found that 231 (77%) of the isolates were *F.*

Table 3. Significant linkage disequilibrium ($P < 0.05$) between 10 variable numbers of tandem repeat (VNTR) markers in 131 clone-corrected isolates of *Fusarium asiaticum*

VNTR marker pair ^a	P value
HK1043 (1), HK957 (1)	0.004
HK1043 (1), HK967 (2)	0.000
HK1043 (1), HK1073 (4)	0.004
HK1043 (1), HK1059 (3)	0.025
HK1043 (1), HK913 (1)	0.0003
HK957 (1), HK1003 (1)	0.007
HK957 (1), HK1073 (4)	0.009
HK957 (1), HK1059 (3)	0.024
HK957 (1), HK913 (1)	0.003
HK967 (2), HK913 (1)	0.013
HK1003 (1), HK1073 (4)	0.000
HK1003 (1), HK1059 (3)	0.000
HK1003 (1), HK977 (3)	0.037
HK1003 (1), HK913 (1)	0.000
HK1073 (4), HK1059 (3)	0.000
HK1073 (4), HK917 (1)	0.004
HK1073 (4), HK913 (1)	0.004
HK1073 (4), HK965 (2)	0.036
HK1059 (3), HK913 (1)	0.010
HK1059 (3), HK965 (2)	0.032
HK977 (3), HK917 (1)	0.000
HK913 (1), HK965 (2)	0.048

^a Pair-wise estimate of linkage disequilibrium, based on Fisher's exact probability test by Slatkin (37), was calculated for 45 possible marker pairs among 10 VNTR loci using Markov chain length of 10^4 with 10^4 dememorization implemented in ARLEQUIN 3.1 (10). Only significant loci combinations ($P < 0.05$) were shown in the table. Number in parenthesis is the chromosome on which a particular VNTR locus resides.

Fig. 3. Average disease severity (DS) caused by each of the 16 representative isolates of *Fusarium asiaticum* (6 15-acetyl deoxynivalenol [15ADON] isolates, 4 3-acetyl deoxynivalenol [3ADON] isolates, and 6 nivalenol [NIV] isolates) on the susceptible spring wheat 'Briggs' in the greenhouse. Single-floret inoculation was used and infected spikelets in each of the inoculated spikes were counted at 21 days after inoculation. In general, the 3ADON- and 15ADON-type isolates caused significantly higher DS than the NIV isolates ($P < 0.0001$). Vertical bar represents standard error. Isolates followed by the same letters (indicated at the top of bar) were not significantly different at $P < 0.0001$.

asiaticum. Of the 231 *F. asiaticum* isolates, 67% were of the 3ADON type and 23% were the NIV type but only 10% were 15ADON isolates (54). The higher frequency of 15ADON isolates found in the present study may reflect a geographical difference between FHB epidemic regions in China because Jianyang is the furthest place in southeast China where *F. asiaticum* isolates have been sampled and analyzed.

Our population genetics study suggested that the two *F. asiaticum* populations (FA-08 and FA-09) grouped based on the year of collection were genetically similar. This conclusion is supported by the high genetic identity (95%), high gene flow ($N_m = 15.13$), and very low genetic differentiation ($F_{st} = 0.032$) between FA-08 and FA-09. Gale et al. (14) found a high gene flow ($N_m = 7$ to 30) and low F_{st} (0.01 to 0.07) among *F. asiaticum* populations collected from four fields located in Zhejiang province, China. Using the VNTR markers, Karugia et al. (20) analyzed *F. asiaticum* isolates collected in two successive years from a small wheat field (500 m²) in Japan and failed to reveal distinct genetic differentiation between the populations sampled during the 2 years. Zeller et al. (51) 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 (51). These results suggest that a pathogen population from a small, isolated disease nursery may keep stable for some period of time. However, population subdivisions based on trichothecene type, geographic difference, and temperature gradient within and outside China from a larger geographic area have been reported by several studies (16,19,38,44,48,53–55). 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.

Although little genetic differentiation was found between the two subpopulations (FA-08 and FA-09) grouped based on the years of collection, three distinct subpopulations existed (NIV-1, NIV-2, and NIV-3) within the NIV-type isolates, as revealed by the membership assignment using the Structure software and further confirmed by the other genetic population analyses. Interestingly, the non-NIV-type isolates were clustered with one of the NIV-type subpopulations. This result suggests that a significant genetic differentiation occurred in the pathogen population of the disease nursery. Population subdivision has been reported for wheat and barley isolates of *F. asiaticum* collected along the Yangtze River in southern China (53) and for *F. graminearum* isolates sampled from the United States and Canada (15,16,34,45). The genetic differentiation found between the NIV-type populations (NIV-1, NIV-2, and NIV-3) in our study likely suggests lack of enough sexual recombination between the NIV isolates (see below).

Out-crossing of *F. graminearum* was demonstrated in the laboratory by using nitrate non-utilizing mutants (5). Recently, Chen and Zhou (6) reported out-crossing frequency of 5.7 to 20.9% in *F. graminearum* in field conditions in China. However, our LD analysis showed that almost half (48.8%) of the 45 possible locus pairs were in linkage disequilibrium. This result suggests that out-crossing among isolates of the *F. asiaticum* population in the Jianyang nursery might have been very rare. Previously, Gale et al. (14) found 36% restriction fragment length polymorphism loci in linkage disequilibrium among the *F. asiaticum* isolates collected from Zhejiang province. Karugia et al. (20) also showed 10 to 12% of the VNTR loci in linkage disequilibrium condition in Japanese isolates. Thus, out-crossing might not be the major evolutionary force for maintaining high genotype diversity of the population in the Jianyang nursery.

Our greenhouse inoculation experiments indicated that the DON-type isolates (15ADON- and 3ADON-type isolates) were similar in aggressiveness whereas they were more aggressive than the NIV-type isolates on Briggs. This result agrees with the previous studies (15,22), which indicated that DON-type isolates are generally more aggressive on wheat compared with the NIV-type

isolates. The aggressiveness difference between DON and NIV types may be due to different amounts or types of mycotoxins produced by the FHB pathogens (2). DON-type isolates generally accumulated a higher level of trichothecene toxins than the NIV-type isolates (15; Puri and Zhong et al. *unpublished*). Previous studies indicated that both DON and NIV are virulence factors, either of which is required for the *Fusarium* pathogen to cause disease spread in wheat spikes (4,7,22). However, the mechanism of how NIV is involved in pathogen infection and FHB development remains to be investigated.

Among trichothecenes, DON has attracted more attention than NIV (30), which may be due to the fact that DON contaminations were more common in cereal grain samples collected in North America, as is exemplified in the global analysis data of mycotoxin contamination reported by Placinta et al. (32). However, in Asian countries, including China, Japan, Korea, and Vietnam, a high incidence and generally high concentrations of NIV have been reported earlier (42). In a more recent analysis of mycotoxin occurrence and concentrations in food and raw food materials from 12 European countries (36), 16% of the samples were also found to be positive for NIV. Although several studies suggested that NIV has higher cytotoxicity than DON (9,24) in the experiments with animal or human cells and could be linked to a high incidence of cancer of the esophagus and gastric cardia in China (18), there is limited information on the specific toxic effects of long-term NIV exposure in humans or the comparative toxicity of NIV and DON. Considering the fact that NIV-type *Fusarium* pathogens are common in Asian countries, Europe, and the southern United States, and NIV-contaminated grains or food products were frequently detected in the FHB-affected samples, more attention should be paid to NIV and its impacts on cereal crop production as well as animal and human consumptions.

In summary, the FHB pathogen population from the small disease nursery was primarily composed of *F. asiaticum*, with the NIV type being predominant. However, isolates of *F. asiaticum* with 3ADON and 15ADON types were also present. Population subdivision based on year of collection was not significant, although a genetic differentiation was observed within the NIV-type isolates collected in the 2 years. This study provides important information about the population structure of *F. graminearum* complex in the nursery that has been used for FHB resistance evaluation for more than 25 years. The presence of all trichothecene types, which vary in aggressiveness and mycotoxin production, ensures the value of this particular nursery in FHB resistance evaluation and in breeding wheat cultivars with broad-spectrum FHB resistance.

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