Population Analysis of *Fusarium graminearum* from Wheat Fields in Eastern China

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ABSTRACT

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Wheat heads showing symptoms of Fusarium head blight were collected from four commercial fields in Zhejiang Province, China, an area where epidemics occur regularly. A total of 225 isolates were subjected to population-level analyses using restriction fragment length polymorphism (RFLP) as markers. Diagnostic RFLP markers established that all isolates belonged to *Fusarium graminearum* lineage 6. Nine polymorphic probes were hybridized to all isolates, resulting in 65 multilocus RFLP haplotypes (MRH). Probing with the telomeric clone pNla17, which reveals differences among isolates in the hypervariable subtelomeric region, differentiated the 65 MRH further into 144 clones. Mean gene diversity for the four field populations was similar, ranging from $H = 0.306 – 0.364$ over the nine RFLP loci for clone-corrected data. High levels of gene flow were inferred from a low level of population subdivision among all field populations, indicating that they were part of the same population. Pairwise linkage disequilibrium measures did not unequivocally support a random mating population, because one-third of locus pairs were significantly different from the null hypothesis of no-association between alleles. We speculate therefore that sexual recombination may not be frequent and that high levels of genotypic diversity may be maintained by relatively low selection pressure acting on a highly diverse population.

Additional keywords: benzimidazole resistance, *Gibberella zeae*, scab.

Fusarium graminearum Schwabe (teleomorph: *Gibberella zeae* (Schwein.) Petch) is a major causal agent of Fusarium head blight (FHB) or scab of cereals and is a significant fungal disease of wheat and barley in many countries (26). FHB not only causes quantitative yield loss but may also generate problems with grain quality because *F. graminearum* and other FHB-causing *Fusarium* spp. contaminate grain with mycotoxins such as deoxynivalenol (DON). FHB is especially prevalent if moist conditions (resulting from rainfall, high humidity, and dew) and moderate temperatures prevail during flowering (19). Most infections occur during flowering by asexual or sexual spores produced primarily on colonized crop residue (corn stalks and wheat straw, etc.). About 2 weeks after infection, symptoms become visible and the fungus may kill the developing seed around the soft dough stage.

In China, wheat is an important crop, second in importance only to rice, and is grown on approximately 30 million hectares, predominantly as winter wheat. FHB has been a problem in China in some provinces and in some years since 1936, when the first serious outbreak occurred (8). Since 1985, epidemic outbreaks have become more frequent and widespread, though some areas are more prone to epidemics than others. The provinces suffering most from this devastating disease are located along the Yangtze River in eastern and central China (8,36). Although FHB can also be caused by other *Fusarium* spp., *F. graminearum* is by far the most important causal organism in China. In an extensive survey that covered 21 Chinese provinces, 95% of *Fusarium* cultures that were isolated from 2,450 diseased wheat heads were determined to be *F. graminearum*. *F. culmorum* (W. G. Smith) Schwabe was also commonly isolated, but only in northwestern China (36).

The China Wheat Scab Cooperation Group, which was established in the mid-1970s, has provided a crucial impetus to generate more knowledge on the biology of the pathogen and to screen for possible sources of resistance to the pathogen. In regard to pathogen variability, collections of *F. graminearum* isolates from China have been examined for several traits at the phenotypic level, i.e., cultural characteristics, virulence, and fungicide sensitivity (8,36). To date, Chinese isolates of *F. graminearum* have not been subjected to molecular analysis, with the exception of several isolates from culture collections that were examined by O'Donnell et al. (24; K. O'Donnell, *unpublished data*). Seven genetically distinct lineages of *F. graminearum* were defined by these studies. Four isolates from China were determined to belong to the Asian lineage 6, while two isolates belonged to the geographically widespread lineage 7.

The objectives of this study were to develop restriction fragment length polymorphism (RFLP) markers to distinguish among phylogenetic lineages within the *F. graminearum* complex and to determine the lineages that predominate in Zhejiang Province, an epidemic-prone area in China. RFLP markers were also used to identify micro-evolutionary forces that may act on the population of *F. graminearum* in China, especially those related to diversity, gene flow/migration, and recombination. In addition, isolates were analyzed for their reaction to benzimidazole, because resistance to this fungicide class has been reported for *F. graminearum* isolates originating from Zhejiang Province (8).

MATERIALS AND METHODS

Fungal isolates. Diseased wheat heads were collected near harvest time in May 2000 from four farmers' fields near three

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cities in Zhejiang Province, China (Fig. 1). The plant material was collected along transects, with sampling stations within fields being 5 m from each other. At each sampling station, five or six symptomatic wheat heads were selected. The total number of heads collected per field ranged from 31 heads from six sampling stations at Haining II to 42 heads from eight sampling stations at Ninghai. Thirty-five heads were collected from each of the two remaining fields (Haining I and Deqing), with seven sampling stations per field.

Seeds with signs of *F. graminearum* infection were dislodged from diseased heads with forceps. Up to six seeds from individual wheat heads were immersed for 30 s in 70% ethanol, surface sterilized for 3 min in 10% commercial bleach (final concentration of 0.5% sodium hypochlorite), and briefly rinsed in sterile water before placing them on water agar plates (1.5% agar). After 3 to 4 days of incubation at room temperature, fungal colonies showing morphological characteristics of *F. graminearum* were subcultured onto plates containing mung bean agar (40 g of mung beans per liter were boiled for 22 min in water, the liquid was poured through cheesecloth, and 15 g of agar per liter was added to the filtrate before autoclaving). Generally, fungal colonies originating from two seeds per head were subcultured onto individual plates.

The inoculated mung bean agar plates were kept at room temperature for a minimum of 5 days under 12 h light (mix of fluorescent and near UV light) to allow conidiation to occur. A loopful of conidia was streaked onto water agar. The plates were kept at room temperature for at least 5 h to allow conidia to germinate. A single germinated spore was selected under up to ×900 magnification provided by a dissecting microscope (SZX12; Olympus America, Melville, NY). The conidium was placed onto half-strength potato dextrose agar (PDA) (12 g of potato dextrose broth [PDB] per liter [Difco Laboratories, Detroit] and 15 g of agar per liter). After several days, the single-spored culture was further processed for long-term storage and liquid culture. For long-term storage, three or four agar plugs containing mycelium were removed from the plate with a 5-mm-diameter cork borer. The plugs were immersed in 0.75 ml of 50% glycerol in a 2-ml cryogenic vial. All cultures were stored at –80°C. For establishing liquid cultures, mycelium was scraped from the PDA plate and placed into a plate containing half-strength PDB. The broth was made from fresh potatoes because more vigorous growth was observed compared with commercially prepared PDB. Diced white potatoes (125 g/liter) were boiled in water for approximately 10 min until soft. The liquid was then poured through cheesecloth and combined with 10 g of dextrose before autoclaving. The culture in PDB was allowed to grow for a minimum of 5 days until it occupied most of the 9-cm-diameter petri dish. The mycelium was harvested into 15-ml tubes and kept at –80°C until lyophilization and DNA extraction.

Fig. 1. Map of China, including a detailed insert of Zhejiang Province (striped). The insert map of Zhejiang Province shows three major cities in closest proximity to the fields where collections of wheat with head blight symptoms were made. Near Haining two fields were sampled, whereas at the other two locations (Deqing and Ninghai) one field each was sampled.

In vitro benomyl resistance. The reduction of mycelial growth with increasing concentrations of the benzimidazole fungicide benomyl in vitro was determined for two isolates of *F. graminearum*, one lineage 7 isolate from the United States (NRRL 29169), and one Chinese isolate (00-268). Both isolates were plated from long-term storage onto Czapek-Dox agar (CDA) (35 g of Czapek-Dox broth per liter; 15 g of agar per liter). Additional CDA plates were prepared with benomyl concentrations of 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, and 3 ppm of active ingredient. Inoculated plates with no benomyl added served as controls. Duplicate plates for each concentration were inoculated with four mycelial plugs (5-mm diameter) of each of the two aforementioned isolates. The 50% effective dose (ED_{50}) and ED_{90}, which indicates 50 or 90% growth reduction, respectively, in response to various fungicide levels, were established for both isolates. A fungicide concentration that completely inhibited the growth of sensitive isolates of *F. graminearum* on fungicide-amended plates was then selected to test all Chinese isolates for their sensitivity or resistance to benomyl. To test isolates, 2 ml of benomyl-amended CDA was dispensed into each well of 24 multiwell tissue culture plates (Falcon 3047). A different isolate was inoculated into each well directly from long-term storage. Inoculated multiwell plates were kept at room temperature, and growth of isolates was assessed 3 and 5 days after inoculation.

Development of RFLP probes. A genomic library was constructed using *Hin*dIII-digested DNA from *F. graminearum* lineage 7 strain NRRL 29169 ligated into the plasmid vector pUC18 (TaKaRa, Shiga, Japan). Recombinant plasmids were used to transform *Escherichia coli* DH5αMCR (Gibco BRL, Grand Island, NY) using previously established protocols (28,29). Plasmid preparations of recombinant clones were done using lysis-by-boiling (30). Recombinant plasmids were digested with *Hin*dIII, and linearized plasmids and inserts were separated on 0.8% agarose gels. Plasmids were retained in our genomic library if they contained a single insert that was sized between 0.7 and 4.5 kb. Plasmids were labeled pGz1 through pGz165 in consecutive numbers according to size, starting with the smaller insert sizes.

Alkaline blots for screening, prepared in duplicate, contained 5 μg of *Hin*dIII-digested DNA from one or two representatives of the seven described lineages of *F. graminearum* (24), in addition to 12 to 15 field isolates of putative *F. graminearum* isolates each from China and the United States. As size markers, 60 ng each of a 100 bp and a 1-kb ladder (New England Biolabs, Beverly, MA) were included. Blots were probed simultaneously with three ^{32}PdCTP-labeled recombinant plasmids that differed sufficiently in insert size to insure accurate scoring of autoradiograms.

Generation of RFLP data. DNA was extracted from lyophilized mycelium of all cultures according to a protocol developed earlier (28). DNA yield per preparation was generally around 5 to 40 μg. A total of 5 μg of DNA from each isolate was digested with *Hin*dIII overnight at 37°C. Generation of data (i.e., electrophoresis of DNA, transfer of DNA to nylon membranes, hybridization of radioactive probes, washing, and developing and stripping procedures) was performed as described previously (28), except that hybridization and washes were conducted at 65°C for high stringency.

RFLP data analysis. RFLPs generated with each probe were considered different alleles at a single locus and named in numerical fashion. Allelic information from different loci was used to generate a multilocus RFLP haplotype (MRH) for each isolate. In addition to single-copy probes, pNla17, which contains the sequence of the conserved fungal telomere repeat $(TTAGGG)_{17}$ (2) and may reveal differences between isolates in the hypervariable subtelomeric region, was hybridized to DNA for clone determination. Isolates with the same MRH were evaluated for their respective pNla17 patterns. Two or more isolates with the same MRH and pNla17 pattern were considered the same clone;

isolates with the same MRH but different pNla17 pattern were considered independent strains.

All data were analyzed based on clone-corrected data only, i.e., counting each clone only once, using two population genetics software programs: Arlequin version 2.000 (S. Schneider, D. Roessli, and L. Excoffier, Laboratoire de Genetique et Biometrie, Université de Genève, Switzerland) and Genepop version 3.3 (M. Raymond and F. Rousset, Laboratoire de Génétique et Environnement, Université Montpellier, France). Intrapopulation level analyses were conducted for the individual field populations and for the combined data. The mean number of pairwise allelic differences between isolates was calculated in addition to average gene diversity across all loci in the four field populations and the combined data (23,34). Interpopulation level analyses determined genetic differentiation between the different field populations. To test for population structure, analysis of molecular variance (AMOVA) was conducted (10), whereby the total variance (generated by data from all four populations) is partitioned into covariance components, taking into account allelic differences between individuals within populations and allelic differences between individuals from different populations. Gene flow between individual field populations was assessed using Arlequin by determining F_{ST} values (37) (i.e., population pairwise genetic distances) and by determining the effective number of migrants (Nm).

Linkage or gametic disequilibrium in the total sample was assessed using Arlequin and Genepop testing the null-hypothesis of linkage equilibrium. In Arlequin, a test by Slatkin (33) was implemented that was developed from Fisher's exact probability test. Briefly, in a contingency table, this test determines the probability to find a table with the observed haplotype frequencies using a Markov chain (chain length: 100,000; dememorization: 1,000). In Genepop, a similar approach is based on Fisher's exact test, also using a Markov chain. The *P* value in both programs is equal to the proportion of visited tables with a probability smaller or equal to the observed contingency table.

RESULTS

Fungal isolates. From 143 wheat heads collected from four fields, 242 isolates were established. In many instances, two isolates were obtained per wheat head (226 isolates from 113 heads); from 16 heads only one isolate was recovered and 14 heads did not yield any *Fusarium* colonies. Of the 242 isolates, RFLP data were gathered for 225 isolates; fungicide sensitivity was determined for all 242 isolates.

In vitro benzimidazole resistance. The reduction of mycelial growth with increasing concentrations of benomyl for the two isolates was similar and is depicted in Figure 2. The ED_{50} for both isolates was approximately 1 ppm, whereas the ED_{90} was reached at slightly higher levels of benomyl, approximately 1.2 ppm. Neither isolate grew at concentrations of ≥1.5 ppm. For testing the sensitivity or resistance to benzimidazole of all Chinese isolates, we therefore used a concentration of 1.5 ppm of active ingredient. Only 4 isolates of the 242 isolates tested were determined to be naturally resistant to benomyl and grew on benomyl-amended plates up to a tested concentration of 10 ppm of active ingredient. Two isolates were from the population Haining I and the other two isolates were recovered from the population Haining II. The two latter isolates were isolated from the same head and later determined to be the same clone.

Development of RFLP probes. From the genomic library of lineage 7 strain NRRL 29169, 130 recombinant clones with insert sizes ranging from 0.8 to 3.8 kb were used as probes and hybridized to *Hin*dIII-digested DNA of representatives of seven phylogenetically distinct lineages of *F. graminearum*. Of 116 probes that provided scorable data, 71 or 61.2% were monomorphic among all tested isolates. Only 38 probes or 32.8% were polymorphic among lineages. These included 12 probes that also were polymorphic within lineages. In addition to these 12 probes, we identified another seven probes that were polymorphic within a specific lineage but were otherwise monomorphic among lineages.

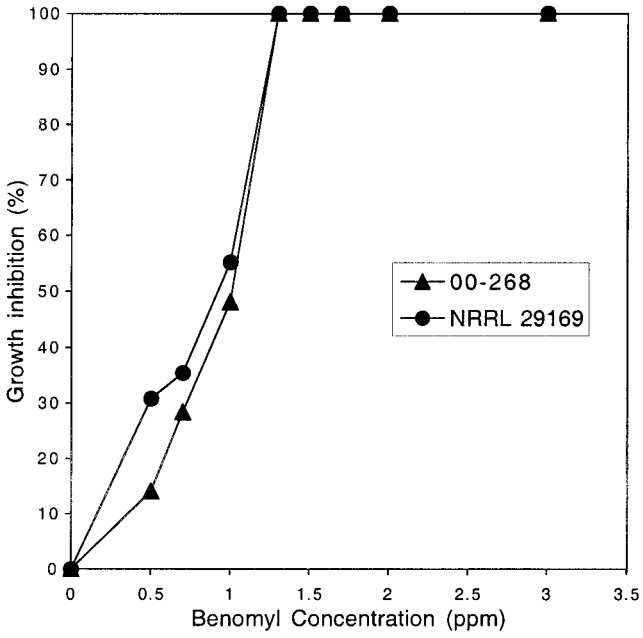

Fig. 2. Growth inhibition in vitro of two strains of *Fusarium graminearum* in the presence of benomyl. Isolate 00-268 is a lineage 6 strain from China, and isolate NRRL 29169 is a lineage 7 strain from the United States.

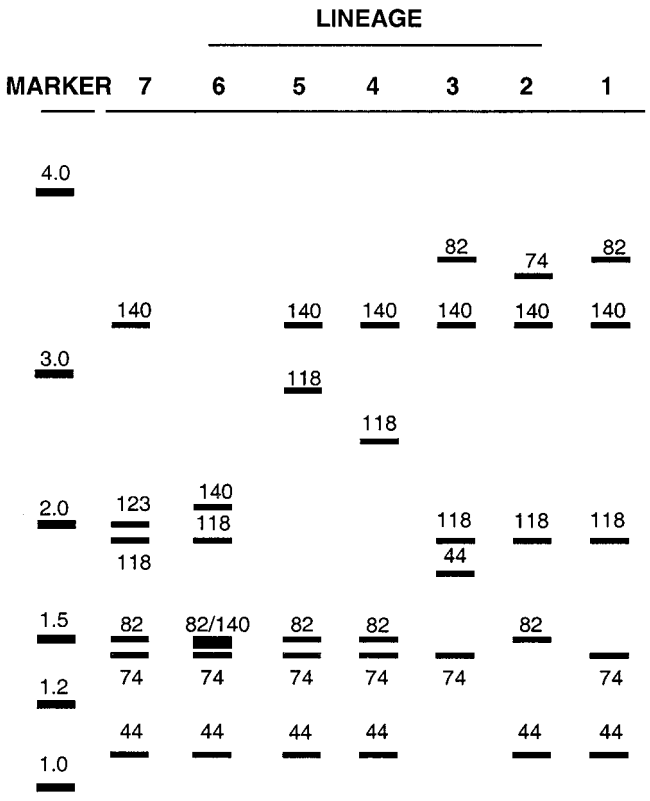

Fig. 3. Diagram of hybridization patterns for seven phylogenetic lineages (1 to 7) of *Fusarium graminearum* probed with six diagnostic restriction fragment length polymorphism (RFLP) probes. Molecular size markers (in kilobases) are given to the left. The numbers above or below the bands denote the probe number (pGz numbers). Whereas five probes revealed length polymorphisms in one or more lineages, pGz123 only hybridized to lineage 7 strains.

Phylogenetic analysis was conducted using allelic information of probes that were polymorphic among but not within lineages. Applying various options available in PHYLIP version 3.5c (J. Felsenstein, Department of Genetics, University of Washington, Seattle) confirmed the close phylogenetic relationship between lineages 1 and 2 (data not shown), but otherwise did not correctly infer the phylogenetic relationship among the seven lineages, which was determined previously by sequence analysis (24).

Out of the 26 probes that were polymorphic among but monomorphic within lineages, we selected six probes that may be useful as diagnostic RFLP probes because collectively their hybridization patterns clearly differentiate between the various lineages (Fig. 3). In addition, nine RFLP probes that were polymorphic among a random selection of Chinese isolates were used to generate RFLP data for field isolates from China.

RFLP data analyses. Probing the *Hin*dIII-digested DNA of 225 field isolates with the six diagnostic probes, in conjunction with comparisons of hybridization patterns of known lineages, classified all isolates as belonging to lineage 6 of *F. graminearum*. Other lineages of *F. graminearum* or species other than *F. graminearum* were not encountered. The nine polymorphic probes were used to hybridize to and collect data from 225 *F. graminearum* isolates. Six of these probes revealed two alleles, two probes revealed three alleles, and one probe detected four alleles. A total of 65 MRHs were differentiated using these nine probes. Using the telomeric probe pNla17 for fingerprinting, the 65 MRHs were further subdivided into 144 clones. Examples of single-locus RFLPs and pNla17 patterns are illustrated in Figure 4.

In general, the same clone was recovered from the same plant or from neighboring plants at a sampling station. Of 73 clones encountered more than once, 65 were found twice and eight were encountered three times. Of the clones found more than once, 66 (or 90.41%) included duplicate isolations from the same wheat head. On the other hand, duplicate isolations from 33 plants resulted in two different MRH, indicating independent sources of infection. Fourteen clones were isolated from neighboring plants, which may be indicative of a limited amount of secondary spread.

Data analysis was conducted for clone-corrected data only, i.e., taking into consideration only one member of the 144 clones. Initial analyses calculated mean number of pairwise differences between isolates and gene diversity (*H*) across nine polymorphic RFLP loci for the four field populations (Table 1). 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 by variation among isolates within populations. In addition, pairwise F_{ST} and effective number of migrants (Nm) also confirmed the high levels of gene flow between all field population pairs (Table 2). Because all four field populations are apparently part of a geographically larger population, data were combined to assess the level of gametic disequilibrium between pairs of loci (Table 3). Both Arlequin and Genepop provided very similar *P* values for different locus pairs. Using Arlequin, 13 of the 36 tested combinations of locus pairs were significant, i.e., rejecting the null hypothesis of equilibrium. In Genepop, 11 combinations were significant.

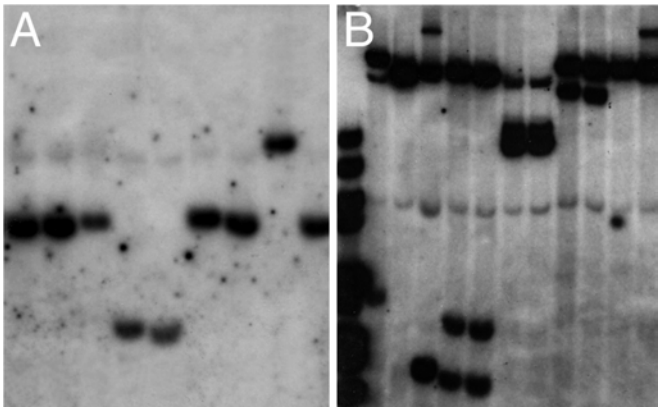

Fig. 4. Restriction fragment length polymorphisms displayed by *Fusarium graminearum* isolates from China. **A,** DNA probed with the single-copy sequence of clone pGz53; and **B,** DNA probed with the telomeric sequence of clone pNla17. Molecular size markers occupy the lane on the left.

DISCUSSION

Current literature presents conflicting results on the level of diversity within *F. graminearum* (14). Though most studies to date have demonstrated high genotypic diversity in collections of *F. graminearum*, with nearly every isolate being of a different molecular genotype, other studies have shown limited diversity. As will become apparent in this discussion, our study attests to both notions of high and low diversity.

Although the RFLP screening encompassed the diversity known for this species, as revealed from the seven phylogenetic lineages, the majority of probes (61.2%) were monomorphic among all isolates. In comparison, similar RFLP screening with 15 isolates belonging to various formae speciales of *F. oxysporum* (28) resulted in merely 22.5% monomorphic sequences. Comparing the RFLP patterns of the two *Fusarium* spp., different genome organizations become apparent. While RFLPs in *F. oxysporum* were mainly caused by duplications or deletions (indels) and by variation attributable to repetitive elements, the genome of *F. graminearum* seems to consist primarily of single-copy sequences, because virtually all 116 probes were visualized as a single band in the DNA of NRRL 29169, the lineage 7 isolate from which the genomic library was derived. As a consequence, most RFLPs were caused by loss or addition of restriction sites, though occasional nonhybridization of specific plasmid inserts also differentiated isolates, especially if they were from different lineages. Transposable elements, representing a significant part of the *F. oxysporum* genome (9), were not identified in *F. graminearum* in this study. In fact, none of the 116 probes from our genomic library was high- or even low-multicopy. Collectively, these data suggest a streamlined, simple, and likely stable *F. graminearum* genome, especially when compared with *F. oxysporum*, whose genome is rather unstable. This instability is caused possibly by the activity

TABLE 1. Mean number of pairwise differences between clones and gene diversity (*H*) across nine polymorphic restriction fragment length polymorphism (RFLP) loci for four *Fusarium graminearum* populations collected from commercial wheat fields in Zhejiang Province, China

Field location[a]	Clones[b]	Pairwise differences and SD[c]	H and SD[d]
Deqing	32	2.871 (1.550)	0.319 (0.192)
Ninghai	43	3.358 (1.756)	0.373 (0.217)
Haining I	30	2.959 (1.592)	0.329 (0.197)
Haining II	39	2.825 (1.522)	0.314 (0.188)
Total[e]	144	3.102 (1.620)	0.345 (0.199)

[a] Field names are based on the city nearest to the specific field sampled (Fig. 1).
[b] The number of clones in a field was established by determining alleles at nine loci for each isolate in addition to its pNla17 pattern. Different clones are characterized by an individual multilocus RFLP haplotype or a different hybridization pattern by use of pNla17, which was used as a fingerprinting probe.
[c] Mean number of pairwise differences (counting the number of different alleles between RFLP genotypes) and standard deviation (SD) were calculated using Arlequin version 2.000 (S. Schneider, D. Roessli, and L. Excoffier, Laboratoire de Genetique et Biometrie, Université de Genève, Switzerland).
[d] Average gene diversity and SD (in parentheses) was determined according to Nei (23) and Tajima (34).
[e] Combined data of all four field populations.

of transposable elements that may result in duplications and karyotypic variability (9).

Investigation of the global diversity of *F. graminearum* has revealed the existence of seven distinct phylogeographic lineages that appear to be reproductively isolated (24). Most isolates from Asia (originating from China, Nepal, and Japan) were categorized into lineage 6, the "Asian clade". Lineage 7 is geographically more widespread and appears to be the predominant or sole lineage found in the Americas and in European countries ("Pan-Northern Hemisphere clade"). Lineage 7 isolates have also been detected from several Asian countries, including China. Two isolates from China had been previously assigned to lineage 7 (K. O'Donnell, *unpublished data*). Both isolates originated from northern China.

One of our objectives was to develop RFLP probes that would reliably assign *F. graminearum* isolates to a lineage, especially lineages 6 and 7. We identified 26 probes that differentiated among lineages and were monomorphic within lineages. Among these 26 probes, we selected six that together were able to distinguish among all lineages. While five probes differentiated one or more lineages through length polymorphisms, one of the probes, pGz123, only hybridized to lineage 7 isolates, resulting in nonhybridization of the other lineages (Fig. 3). By multiplexing three probes at a time, only two hybridizations are necessary for lineage determination of any one isolate. So far, our diagnostic probes have confirmed without fail the identity of approximately 500 field isolates from the United States and Italy as lineage 7 of *F. graminearum* (L. R. Gale and H. C. Kistler, *unpublished data*). All analyzed isolates from Zhejiang Province were confirmed to belong to lineage 6 of *F. graminearum*. Lineage 7 was not detected among the four field populations from eastern China. Because our main interest is currently in lineage 6 and lineage 7, the usefulness of the diagnostic probes in reliably identifying other lineages has to be confirmed with larger sample sizes for the remaining lineages.

The six diagnostic probes may also prove to be useful in verifying species identity. While molecular diagnostic systems have been developed to differentiate between several *Fusarium* spp. commonly isolated from wheat heads (7,31,32), our probes may be useful for authenticating a specific isolate as *F. graminearum*. So far, morphologically similar *Fusarium* spp. either did not hybridize to specific probes or displayed hybridization patterns different from any *F. graminearum* isolates. Specific tests using representatives of closely related *Fusarium* spp. will have to be conducted to confirm these preliminary observations.

In the present study, we analyzed populations of *F. graminearum* from four commercial wheat fields located in eastern China along the Yangtze River. Wheat farmers in Zhejiang Province experience frequent and severe epidemics of FHB and also are confronted with resistance of their resident *F. graminearum* population to benzimidazole fungicides. Carbendazim has been extensively used to control FHB in epidemic areas since the early 1970s. The first resistant isolate was found in 1992 in Zhejiang Province near Haining (8). Since then, some resistant isolates have also been identified from locations north of this region in Jiangsu Province and in the municipality of Shanghai. In Zhejiang Province, results from yearly surveys have shown that the proportion of resistant isolates in the population is increasing. While their frequency in the population was below 3% prior to 1997, a significant increase was seen in 1998 and 1999, when resistant isolates accounted for 18.9 and 25.6%, respectively, of screened isolates (8).

An initial objective of this study was to compare the population genetic structure of fungicide resistant isolates with sensitive isolates from Zhejiang Province. As a first step, we established the ED_{50} and ED_{90} levels for the fungicide. In a previous study, these values were determined to be 0.99 and 7.3 ppm, respectively, for U.S. isolates of *F. graminearum* (16). While our study confirmed the ED_{50} as being approximately 1 ppm for both the lineage 6 and the lineage 7 control isolate, differences were seen with the ED_{90}, because our study determined it to be 1.2 ppm. The discrepancy can be explained by the fact that Jones (16) determined only the growth reduction at 1 and 10 ppm and then extrapolating ED_{90}

TABLE 2. Population pairwise F_{ST}[a] (above diagonal) and effective number of migrants (Nm)[b] (below diagonal) between field populations of *Fusarium graminearum* collected from four wheat fields in Zhejiang Province, China

	Deqing	Ninghai	Haining I	Haining II
Deqing	…	0.048	–0.013	0.066
Ninghai	9.856	…	0.042	0.016
Haining I	Infinite	11.362	…	0.041
Haining II	7.031	30.150	11.594	…

[a] Population pairwise F_{ST} values were calculated using Arlequin version 2.000 (S. Schneider, D. Roessli, and L. Excoffier, Laboratoire de Genetique et Biometrie, Université de Genève, Switzerland).

[b] The effective number of migrants (Nm) was calculated using Arlequin version 2.000, implementing a concept developed by Nei (23).

TABLE 3. *P* values of exact tests evaluating gametic disequilibria between nine pairs of restriction fragment length polymorphism (RFLP) loci based on 144 clones of lineage 6 isolates of *Fusarium graminearum* collected from four wheat fields in Zhejiang Province, China

Locus pair	Arlequin[a]	Genepop[b]
53, 129	0.17658	0.18042
53, 58	0.00019***[c]	0.00008***
53, 81	0.01342*	0.01312*
53, 141	0.00030***	0.00028***
53, 146	0.83903	0.84290
53, 106	0.00957**	0.01050*
53, 148	0.00030***	0.00005***
53, 61	0.87557	0.88669
129, 58	0.28994	0.34760
129, 81	0.16445	0.17018
129, 141	0.03662*	0.05782
129, 146	0.38518	0.56432
129, 106	0.74549	0.74648
129, 148	0.19130	0.19256
129, 61	0.25328	0.24938
58, 81	0.42813	0.43413
58, 141	0.02077*	0.02112*
58, 146	0.75371	0.75303
58, 106	0.02613*	0.02095*
58, 148	0.00000***	0.00000***
58, 61	0.12949	0.12522
81, 141	0.34308	0.34051
81, 146	0.10334	0.18655
81, 106	0.56017	0.76202
81, 148	0.12876	0.24086
81, 61	0.75189	0.75238
141, 146	0.03061*	0.05265
141, 106	0.42031	0.58858
141, 148	0.00536**	0.01653*
141, 61	0.01198*	0.01251*
146, 106	0.46772	0.46773
146, 148	0.70205	0.70644
146, 61	0.60611	0.60170
106, 148	0.00446**	0.01938*
106, 61	0.99990	1.00000
148, 61	0.32087	0.32547

[a] In Arlequin version 2.000 (S. Schneider, D. Roessli, and L. Excoffier, Laboratoire de Genetique et Biometrie, Université de Genève, Switzerland), a test by Slatkin (33) that (Table 3) was developed from Fisher's exact probability test was implemented. The probability of finding a table with the observed haplotype frequencies was assessed using a Markov chain.

[b] In Genepop version 3.3 (M. Raymond and F. Rousset, Laboratoire de Génétique et Environnement, Université Montpellier, France), Fisher's exact probability test was implemented; the probabilities were calculated analogous to Arlequin.

[c] *, **, and *** indicate $P < 0.05$, 0.01, and 0.001.

from the curve, whereas our experiments were more comprehensive and included several concentrations between 1 and 3 ppm. Testing of all field isolates was done at 1.5 ppm, which completely inhibited the growth of benzimidazole sensitive isolates.

We anticipated that our collection of *F. graminearum*, which was compiled in the year 2000, would at least contain 25% fungicide resistant isolates and were surprised by their low frequency in the population. Only 4 of 242 (1.65%) (including two clonemates) were resistant. The discrepancy between the observed and expected numbers may be explained by environmental conditions. While high levels of precipitation characterized the 1998 and 1999 growing seasons and provided conditions that were conducive to infection and disease development, the 2000 growing season was characterized by dry weather and therefore unfavorable disease conditions. The level of fungicide application in 2000 was probably low, therefore eliminating the selective advantage of benzimidazole resistant isolates. A further decrease in the proportion of fungicide resistant isolates in the population may have occurred if resistant isolates are reduced in one or more fitness components compared with wild-type isolates. Due to the limited number of fungicide resistant isolates in the sample, a comparison with sensitive isolates could not be conducted at a population level.

Among 116 screened probes, we identified nine that were polymorphic among lineage 6 isolates of *F. graminearum.* Most RFLP probes that were polymorphic within lineage 6 isolates were monomorphic for lineage 7 isolates and vice versa (data not shown). Therefore, for population analysis of lineage 7 isolates (or any other lineage), a different set of RFLP markers has to be used.

Among 225 isolates, 65 MLH were identified using the nine polymorphic probes. The probes provided allele frequencies and therefore were useful for population genetic analysis, but were not sufficient to distinguish among clones. To differentiate among clones within the same MLH, a fingerprinting probe was needed. The telomeric probe pNla17 contains the sequence of the conserved fungal telomere repeat $(TTAGGG)_{17}$ (2) and may reveal differences in the subtelomeric region. In *Saccharomyces cerevisiae*, for example, the subtelomeric region is hypervariable and consists of an assortment of repeats that may vary in sequence or position at each chromosomal end within a given isolate and from isolate to isolate at the same chromosomal end (27). Hybridization of the telomeric probe to digested DNA may therefore result in a distinct RFLP pattern for genotypically dissimilar isolates, with a maximum number of bands equaling twice the number of chromosomes. The use of the telomeric probe can be valuable for clone determination, especially if other fingerprinting probes (e.g., transposable elements) are not available. In fungi, telomeric probes have been used for diversity assessment in *Calonectria morganii* (25) and *Botrytis cinerea* (18) and for lineage determination in *Ustilago hordei* (1). The relative stability of patterns generated with the telomeric probe was established in the latter two studies by observing an unchanged hybridization pattern for 10 consecutive subcultures and three generations of inbred progeny, respectively. Hybridization of pNla17 to digested DNA of *F. graminearum* resulted in a maximum of eight bands for isolates from both lineage 6 (225 isolates) and lineage 7 (≈500 isolates) in *F. graminearum*. We conclude, therefore, that the genome of *F. graminearum* probably has four chromosomes, in accordance to cytological studies (15).

The telomeric probe pNla17 resolved the 65 MLH into 144 clones. In most cases, duplicate isolations from a single wheat head resulted in the recovery of the same clone, although one-third of duplicate isolations yielded two MLH, indicative of independent infections. Moreover, 14 clones were isolated from neighboring plants. Comparable observations were made for populations of *F. graminearum* from the United States (6,39) and Germany (21), where, occasionally, multiple genotypes from a single head were recovered or the same genotype was observed in neighboring plants. The latter observation may be explained by secondary spread of asexual conidia or a local primary inoculum source, either of asexual progeny or of progeny derived from selfing (6).

Epidemiological studies have shown that airborne ascospores rather than splash-dispersed macroconidia constitute the main source of primary inoculum for initiating FHB infections (11). The distance inoculum, i.e., ascospores, may travel has been estimated to be of the order of up to tens of kilometers or more (12). If one assumes that inoculum is deposited onto a wheat head from an inoculum source that is located meters or kilometers away from that specific wheat head, then it would follow that the same genotype isolated from neighboring plants would originate from secondary infection rather than from a genotypically uniform inoculum source. With 14 clones found on neighboring plants in this study, secondary spread may not be such an exceptional event as has been suggested (11), even though particular environmental conditions may be required.

Population genetic analysis confirmed the four field populations as very similar in their genetic composition. Besides the fact that all isolates from the four fields belonged to lineage 6, gene diversity for the nine RFLP probes within all field populations was also similar, ranging from 0.314 to 0.373 for clone-corrected data, while the average number of pairwise differences between haplotypes ranged from 2.825 to 3.358. Gene flow values were high, indicating that genetic differentiation among the field populations was low. AMOVA determined that only 3.39% of the observed variation was caused by genetic differences among populations. Genetic differentiation did not correspond to geographic distance between fields, because the highest level of gene flow was not between Haining I and Haining II (separated by 5 km), but between Haining I and Deqing. Despite these two fields being separated by approximately 60 km, gene flow analysis, with slightly negative F_{ST} values and an infinite number of migrants (Nm), failed to find any genetic differentiation between them. The population from Ninghai, which is located approximately 200 km from the other three field locations, also did not display higher levels of genetic differentiation in comparison to the remaining three fields. In summary, all four examined field populations belong to a geographically larger population. This level of gene flow strongly suggests that extensive migration of airborne inoculum is sufficient to eliminate any potential localized differentiation.

The population structure of lineage 6 isolates from Zhejiang Province in China is analogous to that of populations of *F. graminearum* from the United States. Two field collections, one from Kansas and one from North Dakota, showed similarly high levels of gene flow, with Nm being 67, even though the two locations were 825 km apart (6,39).

Although this study revealed a homogeneous population within Zhejiang Province, we cannot predict at this point whether high levels of gene flow occur throughout the wheat growing region in China. Zhejiang is located at the southeastern edge of winter wheat production, which is characterized by warm and humid conditions during the latter part of the growing season when FHB infection occurs. The major winter wheat production areas are located farther north in central China (North China Plain), where two-thirds of the winter wheat in China is grown in just four provinces. This region is generally cooler and drier than the Yangtze River area, and epidemics caused by *F. graminearum* are intermittent, though at times severe (8). Spring wheat is predominantly grown in the northeastern provinces and epidemics are frequent and severe in parts of Heilongjiang (8). Due to the variety of climatic conditions and considering the distance between the southern- and northernmost areas of wheat production (approximately 2,500 km), population composition may vary. Therefore, further population studies should include samples covering more production areas. As lineage 7 isolates have been identified from the north of the North China Plain, the deter-

mination of lineage distribution seems to be especially important, as well as the identification of areas where lineage 6 and lineage 7 may coexist, to determine the potential for introgressive hybridization.

A major question to be addressed in this study was whether genotypic diversity in *F. graminearum* is generated by recombination as a consequence of outcrossing, as has been suggested previously by various authors (3,20,21,35). When going through the sexual cycle, *F. graminearum* (or its sexual stage *G. zeae*) is able to self. Its homothallic genome contains both the three MAT1-1 genes and the single MAT1-2 gene, all of which are closely linked (38). Outcrossing in the laboratory is possible, and Bowden and Leslie (4,5) showed that heterozygous perithecia were obtained when complementary auxotrophic mutants were cocultured and selected for recombinant prototrophic growth. Whether outcrossing occurs under field conditions or the potential frequency of such outcrossing is not known; conjecture on high outcrossing rates in nature has been based so far solely on observations of high genotypic diversity. Specific tests to assess recombination in populations of *F. graminearum*, e.g., calculation of gametic disequilibrium, have not been published previously.

Though high genotypic diversity, like that observed for *F. graminearum*, may be an indication of recombination, it is certainly not proof. For example, *F. poae*, another FHB-causing organism, has no known sexual stage and is assumed to only reproduce clonally. Regardless, Kerényi et al. (17) found high diversity of vegetative compatibilities in *F. poae* and 27 isolates from Hungary belonged to 14 vegetative compatibility groups. The authors proposed that the diversity might be the consequence of low selection pressure exerted by its hosts, in addition to the importance of the saprophytic phase in the life cycle, together allowing a multitude of genotypes to persist (17). The authors compared the population structure of *F. poae* to the tremendous amount of genetic variation displayed by nonpathogenic isolates of *F. oxysporum*, an asexual saprophyte. High genotypic diversity also was recently reported for *F. culmorum*, another FHB pathogen with no known sexual stage (21). A population of *F. culmorum* from Russia displayed comparable high levels of genotypic diversity (using RAPDs as molecular markers) as populations of *F. graminearum* from Europe. The authors proposed sexual recombination as the most likely source of the observed diversity, though they also considered other evolutionary forces similar to those stated above for *F. poae*.

As in other studies, we found a high level of genotypic diversity in our field populations. Clones were generally only observed from duplicate isolations from the same wheat head or occasionally from neighboring plants. Because gene flow analysis determined all four field populations as belonging to a geographically larger population, sample data were combined and the extent of gametic disequilibrium of clone-corrected data was measured based on Fisher's exact test. While the majority of the 36 locus pairs did not significantly depart from the null hypothesis of equilibrium, which is consistent with a randomly mating population, a moderately high percentage of locus pairs (36.11 or 30.56% depending on software used) was in gametic disequilibrium. Conversely, most possible allelic combinations were identified among the 65 different MRH. Among 209 potential allele combinations between locus pairs, 184 were found among the various MLH. The missing 25 combinations were predominantly between two low frequency alleles. The interpretation of these results is not clear-cut. Although the most likely explanation for seeing many of the possible combinations is recombination and therefore outcrossing, the moderately high percentage of locus pairs in disequilibrium would argue against frequent outcrossing or would demand another evolutionary force, which would effectively slow the elimination of any existing disequilibria. Evolutionary forces that are known to contribute to gametic disequilibrium, i.e., selection, population admixture, and genetic drift (22), do not seem to be of major importance in the population dynamics of *F. graminearum*. To clarify this issue further, larger sample sizes may be valuable to increase the power of calculations (13). But the ultimate proof and certainly the most definitive test to fully elucidate the role of recombination and outcrossing in populations of *F. graminearum* would be to examine the progeny from naturally occurring perithecia. We plan to use both approaches to further evaluate the importance of outcrossing in natural populations of this homothallic fungal species.

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