Genetic Diversity of *Fusarium* Section *Liseola* (*Gibberella fujikuroi*) in Individual Maize Stalks

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ABSTRACT

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Isolates belonging to *Fusarium* section *Liseola* (teleomorph *Gibberella fujikuroi*), primarily *F. moniliforme, F. proliferatum,* and *F. subglutinans*, are recovered from maize worldwide. Consistent isolation of these fungi from symptomatic and asymptomatic plant tissues suggests that the fungus can systemically colonize maize plants; however, the number of strains that colonize a single plant has not been determined. Using vegetative compatibility groups to differentiate among strains, we have shown that most maize plants are infected by two to three strains belonging to *Fusarium* section *Liseola*. Some of the strains recovered from the stalk usually are recovered from the ear as well. Multiple strains per plant make it more likely that perithecia formation and sexual recombination in this heterothallic fungus can occur under field conditions, because strains of opposite mating type can be found within the same plant. Such multiple infections also make it difficult to attribute particular disease symptoms to a particular strain. The identification of multiple *Fusarium* strains within a maize plant illustrates that when studying this host-pathogen relationship, we are examining a population as well as an individual strain-host plant interaction.

Additional keywords: corn, ear rot, fumonisins, stalk rot.

Fungi belonging to *Fusarium* section *Liseola*, primarily *F. moniliforme* J. Sheld., *F. proliferatum* (Matsushima) Nirenberg, and *F. subglutinans* (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussoun, & Marasas, are distributed widely on maize (*Zea mays* L.) and sorghum (*Sorghum bicolor*) in the United States (25,30). The sexual stage (teleomorph) is *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura. *G. fujikuroi* is a complex species containing at least six genetically distinct mating populations (=biological species) (19,22). In addition to maize and sorghum, members of the *G. fujikuroi* species complex may infect numerous crops worldwide including: asparagus (*Asparagus officinalis*) (11), figs (*Ficus carica*) (2), mango (*Mangifera indica*) (37), nectarines and plums (*Prunus* spp.) (34), pine (*Pinus* spp.) (5), pineapple (*Ananas comosus*) (32), rice (*Oryza sativa*) (35), and sugarcane (*Saccharum officinarum*) (28). Fertile *F. moniliforme* strains belong to either the "A" or the "F" mating populations of *G. fujikuroi* (19). The two mating populations differ in aggressiveness on different hosts (15), ability to synthesize mycotoxins (26,27), sensitivity to some antibiotics (40), and polymerase chain reaction-random amplified polymorphic DNA polymorphisms (10).

In maize, *F. moniliforme* is associated with root, stalk, and ear rots (33), which cause annual losses in Kansas of 4–8% of the crop (14). Infected plant tissues may rot or remain asymptomatic (20). Kernel infection may result from either internal growth in the plant stalks (13), through wounds to the kernel (such as bird, insect, or hail damage), or from growth along the silks through the tip end of the ear (39). Using scanning electron microscopy, Bacon et al (1) showed that *F. moniliforme* is sequestered at the tip cap in kernels. This localization of the fungus has led some workers to suggest that *F. moniliforme* arrives at the tip cap of the kernel from growth occurring on or within the cob initiated from a single infection point (1). *F. moniliforme* can be consistently isolated from all plant tissues (21); therefore, it is likely that the fungus can systemically colonize maize plants (13). However, the number of strains colonizing an individual plant has not been determined.

Determining the number of strains that normally infect a plant is critical in understanding the life cycle and natural history of this pathogen. Colonization of the plant by multiple strains is essential if sexual reproduction is to occur under field conditions, because *G. fujikuroi* isolates are heterothallic and must have a partner of opposite mating type to produce perithecia. If plants are infected systemically, then the same isolates are expected to be recovered from all parts of the plant, whereas if infection is not systemic, then the strains colonizing different portions of the plant are more likely to be different. The number and distribution of strains within the plant also could indicate when and how plants are infected. For example, if soil or seedborne inocula are plentiful and systemic infection occurs, then the same isolates should be recovered from both the stalk and the ear. If infection via growth down the pollen silks is important, then seeds are more likely to be infected with different strains, and these strains should differ from those found in the stalk.

The development of the vegetative compatibility group (VCG) technique (23) for identifying different *F. moniliforme* strains has provided a relatively simple way to distinguish between strains that are morphologically identical. Strains that are vegetatively compatible can form a stable heterokaryon, belong to the same VCG, and are identical at a set of at least 10 *vic* loci. Strains that are vegetatively incompatible are unable to form a stable heterokaryon, belong to different VCGs, and differ at one or more of the *vic* loci. Field strains of *F. moniliforme* belong to many VCGs (4,17,24); thus, strains from the same location that belong to the same VCG are presumed to be clones. In this study, our objectives were to use VCG identification to determine the number of strains from section *Liseola* that colonize an individual maize plant and to determine if isolates from different tissues of the same plant are identical.

MATERIALS AND METHODS

Media. Isolates from *Fusarium* section *Liseola* were recovered from plant tissues by growth on a peptone/pentachloronitrobenzene (PCNB) medium (29). Field-collected isolates were routinely maintained on a modified Czapek-Dox minimal or complete medium (6). Media for the induction and identification of nitrate nonutilizing (*nit*) mutants were those described by Correll et al (6). Carnation leaf agar (CLA) (12) and potato-dextrose agar (PDA; Difco Laboratories, Detroit) were used to culture strains for identification purposes.

Seed preparation and plant culture. For greenhouse studies, maize seed (Inbred LH82; Holden Foundation Seeds, Williamsburg, IA) was disinfested by soaking in sterile distilled water at room temperature for 4 h, followed by a hot water (55 C) dip for 5 min (8). The disinfested seeds were planted in a steamed (2 h) soil/sand mixture in plastic pots. The potted plants were kept in a greenhouse where maize had been grown previously. The plants were watered daily and fertilized once every 2 wk with a 20-20-20 (N-P-K) soluble fertilizer. The plants were harvested at maturity, stripped of all leaves, and the stalk split with a sterile knife. Tissue samples (2-mm^3 blocks) were taken from the crown, the first seven nodes above the crown, the cob, and kernel(s) of each plant and transferred to peptone/PCNB medium. Tissue samples were incubated at 25 C for 5–7 days, and one colony per tissue sample was transferred to minimal medium.

For field experiments, maize seed was disinfested as described above and planted in hills 30 cm apart in a single-row plot 30-m long at the Rocky Ford Experimental Farm, Manhattan, KS. The previous year, this plot was fallow. Plants were thinned to one plant per hill 4 wk after planting. The plants were harvested at maturity, leaves were stripped, and stalks were split with a sterile knife. Tissue samples were taken as described above, and fungal isolates were recovered from the cultured material.

Isolation of *F. moniliforme* from maize ears. Ten maize ears were selected from plants that had not otherwise been assayed. From each ear, five dispersed samples were taken. Each sample was a kernel, its respective glume, and a portion of the cob tissue on which the glume was borne. All of the sample pieces were surface-sterilized by immersion in 1% NaOCl for 30 s and rinsed in sterile distilled water for 20 s. The tissue samples were transferred to peptone/PCNB medium. Tissue samples were incubated, and fungal cultures were recovered as described above.

Ten additional ears were hand-shelled. The kernels from each ear were kept separate, and, from each ear, 15 single-seed subsamples were selected. The seeds were surface-sterilized, and fungus was recovered as described above.

Generation and characterization of *nit* mutants. The basic protocols used follow those of Correll et al (6), except that mutants were generated on media containing 2% $KClO_3$ instead of the 1.5% $KClO_3$. *nit* mutants were maintained on complete medium or were frozen as spore suspensions in 15% glycerol at −80 C until pairing studies were complete (41). *nit* mutants were assigned to *nit1, nit3,* and NitM phenotypic classes based on differential growth on media with KNO_3, KNO_2, $(NH_4)_2SO_4$, and hypoxanthine as sole nitrogen sources (6). Mutants in different classes that are in the same VCG can complement and form a prototrophic heterokaryon that can grow on minimal medium. At least one NitM or *nit3* mutant and several *nit1* mutants were derived for each isolate. Genetic nomenclature follows that proposed by Yoder et al (41) for filamentous fungal plant pathogens.

Assignment of strains to a VCG. Wherever possible, VCG assignments were based on complementation reactions between NitM and *nit1* mutants. Otherwise, complementation reactions between *nit3* and *nit1* were used. Pairs of strains that formed vigorous growth on minimal medium were said to be vegetatively compatible and to belong to the same VCG (23). All other pairs were vegetatively incompatible, and the strains belonged to different VCGs. Complementation tests were conducted on 24-well hybridoma plates as described by Klittich and Leslie (18). Isolates from field plants were paired only with other isolates from the same plant (or ear). Isolates from the greenhouse plants were paired with all the isolates recovered from the greenhouse plants.

RESULTS

All fungal isolates were recovered from apparently healthy, asymptomatic plant tissue. Recovered fungal isolates were identified to the *Liseola* section by morphology and growth on PDA and/or CLA. No tests were made to determine the mating populations to which the different strains belonged, but based on previous work (3), most are expected to be in the A mating population of *G. fujikuroi*.

Greenhouse plants. Isolates belonging to *Fusarium* section *Liseola* were collected from all plant tissues. Up to three strains (an average of 2.3 strains per plant) were recovered from each plant, but all the recovered strains belonged to one of five VCGs (Table 1). The distribution of the strains within each plant varied, with the strain designated "a" found in eight of the 10 plants and the strain designated "b" found in seven of the 10 plants. None of the other strains were found in more than three of the 10 plants. In six of the 10 plants (plants 3–6, 9, and 10), the isolates from the cob and the kernel tissues were identical. In all cases, the strains recovered from the seed and the cob also were recovered from the stalk. These results are consistent with a hypothesis of systemic infection. However, the source of inoculum and point of infection could not be determined, and the possibility that the a and b inocula simply overwhelmed all other strains cannot be excluded.

Field plants. Eleven plants grown under field conditions were examined in a manner similar to that used for the plants grown in the greenhouse (Table 2). From each plant, two to five strains were recovered, with an average of three strains per plant. Unlike the greenhouse plants, none of the field plants was infected with only a single *Fusarium* strain, and seven of the 11 plants examined were infected by three or more strains. In 10 of the 11 plants, the strains recovered from the stalk also were recovered at least once from the ear. Ten of the 11 stalks were infected by more

TABLE 1. Vegetative compatibility group (VCG) diversity of *Fusarium* isolates recovered from greenhouse-grown maize[y]

Plant	Crown	Nodes							Cob	Seed	No. of strains
		1	2	3	4	5	6	7			
1	a	a	a	a	a	b	a	b	...[z]	a	2
2	b	...	b	c	...	c	b	d	c	b	3
3	b	b	b	c	b	b	b	d	b	b	3
4	a	a	a	a	a	b	b	b	b	b	2
5	a	a	a	a	a	a	a	a	a	a	1
6	a	a	...	a	...	a	b	b	a	a	2
7	b	a	b	a	b	b	a	b	b	a	3
8	a	e	a	a	b	a	e	a	...	a	3
9	c	a	a	a	a	a	a	a	a	a	2
10	a	a	a	d	d	d	...	...	d	d	2

[y] a–e are designations for the five recovered VCGs.
[z] No data available. *Fusarium* isolates were recovered, but nitrate nonutilizing mutants could not be generated.

than one strain, but only seven of the 11 ears were infected by more than one strain. The significance of these differences, if any, is not clear.

Ears. Two samples of ears from field-grown plants also were examined. In the first sample (Table 3), five sets of isolates were taken from each of 10 ears. Each set was composed of an isolate from a kernel, its glume, and cob tissue supporting the glume and kernel. In a separate sampling of 10 hand-shelled ears (Table 4), fungal isolates were recovered from 15 randomly selected kernels. The individual kernels were used to estimate the number of strains within an ear, and the sets of isolates were used to determine if cob, glume, and seed material were all infected with the same strain of the fungus.

Of the 50 sample sets (Table 3) containing *Fusarium* isolates from the cob, glume, and kernel, only 44 could be evaluated. Two isolates from the ear identified as ear 9 were heterokaryon self-incompatible (7) but were able to form heterokaryons with other strains from that ear that belonged to the same VCG. The number of strains per ear ranged from one to five with an average of 2.2 strains per ear. Only two of the 10 ears had more than two isolates in a single ear. Of the 44 sets examined, 30 of 44 (68%) had the same isolate in the glume, cob, and kernel. The cob and glume were the same in 34 of 44 (77%) cases, the cob and kernel were the same in 33 of 44 (75%) cases, and the glume and kernel were the same in 35 of 44 (80%) cases. In only two of 44 cases (5%) were three strain types recovered from a single set, and both of these were from the ear identified as ear 2. If all three isolates from a set did not belong to the same VCG, then there was no clear pattern as to which members of the set would be alike and which would be different.

The number of strains identified in the 146 kernels from the 10 shelled ears ranged from one to four per ear (Table 4). Four of the 10 ears contained strains from only a single VCG. Thus, the relatively low numbers observed in the smaller samples from the preceding ears appear to be an accurate reflection of the number of strains present within an ear and not merely an artifact of the sampling procedure.

DISCUSSION

In this study, we have shown that asymptomatic maize plants are ubiquitously infected with *Fusarium* strains from section *Liseola*. This finding by itself is not new, but it does strengthen the hypothesis that these fungi are endophytes within the maize plant. That these fungi could be recovered from plants grown from disinfested seed in steamed soil under greenhouse conditions suggests that inoculum is airborne and infection efficiency is high. Understanding the basis of this endophytic association is necessary before we can control losses due to stalk and ear rots and limit the amount of fumonisin mycotoxins produced in sound grain.

One of our primary objectives was to determine if maize plants were infected by multiple *Fusarium* strains and the approximate number of strains per plant. In this study, we found that all of the field plants, 12 of 20 field ears, and nine of 10 greenhouse-grown plants were infected by two or more strains of the fungus. These numbers probably represent a lower limit on the number of fungal strains per plant, because only one fungal isolate was taken from each plant tissue sample. It is possible that increasing the number of tissue pieces sampled would have increased the number of distinct strains recovered. Based on the number of

TABLE 2. Vegetative compatibility group (VCG) diversity of *Fusarium* isolates recovered from field-grown maize[y]

		Nodes							Ear						
									Base		Middle		Tip		No. of
Plant	Crown	1	2	3	4	5	6	7	Cob	Seed	Cob	Seed	Cob	Seed	strains
1	1	1	1	1	1	2	3	3	2	3	4	4	5	5	5
2	6	6	7	7	8	6	6	6	6	6	6	6	6	6	3
3	9	9	9	9	10	9	9	9	11	11	11	11	11	11	3
4	12	13	13	12	12	12	12	12	12	13	13	13	13	13	2
5	14	15	15	14	14	15	14	15	16	16	16	16	16	15	3
6	17	17	17	17	17	18	18	18	17	17	17	17	17	17	2
7	19	19	19	19	19	19	19	...[z]	20	20	20	19	20	20	2
8	21	22	22	22	22	22	22	23	22	22	22	24	22	24	4
9	25	25	26	26	26	25	27	26	28	28	26	26	26	26	4
10	29	29	...	30	30	29	29	29	30	30	30	30	30	30	2
11	31	31	32	32	32	31	31	31	33	32	32	31	32	31	3

[y] 1–33 are identification numbers for VCGs of strains recovered from a single plant. Pairings were not made between strains recovered from different plants, so the total number of unique VCGs in the field may be less than 33.
[z] No data available.

TABLE 3. Vegetative compatibility group (VCG) diversity of *Fusarium* isolates recovered from cobs (C), glumes (G), and kernels (K)

	Set[w]															
	A			B			C			D			E			No. of
Ear	C	G	K	C	G	K	C	G	K	C	G	K	C	G	K	strains
1	34[x]	34	34	34	34	34	34	34	34	34	34	34	34	34	34	1
2	35	35	36	35	35	35	35	36	36	35	36	35	36	35	35	2
3	37	37	37	38	38	38	38	39	40	37	40	39	41	40	41	5
4	42	42	42	42	43	43	43	43	43	42	42	42	...[y]	...	...	2
5	44	44	44	44	44	44	45	45	45	45	45	45	...	...	...	2
6	46	46	46	46	46	46	46	46	46	46	46	46	...	...	...	1
7	47	47	47	48	48	48	47	47	48	48	48	49	...	...	...	3
8	50	50	51	50	50	50	51	51	51	50	51	50	...	...	...	2
9	52	52	52	52	52	52	52	52	52	52[z]	52[z]	53	...	...	...	2
10	54	55	55	55	55	55	55	55	55	55	55	55	54	55	55	2

[w] Each set contains one kernel and its respective glume and cob tissue.
[x] 34–55 are identification numbers for VCGs of strains recovered from a single plant. Pairings were not made between strains recovered from different plants, so the total number of unique VCGs in the field may be less than 22. Sets underlined contain strains from more than one VCG.
[y] No data available.
[z] Heterokaryon self-incompatible strains (3). These strains did form heterokaryons with other strains in the same VCG.

distinct strains we observed in the seed from the shelled ears (Table 4), however, we think it is unlikely that there are usually more than five or six fungal strains per plant. The norm for maize appears to be infection by two or three *Fusarium* section *Liseola* strains per plant.

Most plants appear to have multiple infections; therefore, many plants are likely to contain two strains of opposite mating type that can cross to produce perithecia and complete the life cycle (22), and the possibility of sexual recombination under field conditions is significant. If most plants were singly infected, then sexual reproduction could not occur on the plants, because all isolates of *G. fujikuroi* are heterothallic outcrossers. Ubiquitous multiple infections make the attribution of disease symptoms to a particular strain much more difficult, because many diagnosticians will usually recover only a single strain from a diseased plant. If the recovered strain is not responsible for the observed symptoms, then misinterpretation of the resulting data may occur. Similarly, data collected from experiments in which a single strain was inoculated into the plant may be confounded by the presence of "background" infections from other strains. Our results are consistent with the misidentification problems reported by Jardine and Leslie (15) who found that lesions in the greenhouse-grown sorghum plants in their studies usually contained strains other than the one that was inoculated.

The distribution of the different strains within the maize plant also is interesting. The source of the fungi in these plants was not the seed, instead the fungus was transmitted through other sources, e.g. soil, pots, water, plant debris, insect vectors, airborne inoculum, etc., because the planted seeds were disinfested with a hot-water treatment prior to planting. In most cases, at least one isolate could be recovered from several portions of the same plant. This finding suggests either a systemic infection, probably early in host plant growth, or multiple infections by the same strain. Based on preliminary studies (16), in which maize seeds were infested with known strains of *F. moniliforme* that were then recovered at a high rate from the mature plants, we favor the systemic-infection hypothesis.

In general, fungal isolates from the kernels, their respective glumes, and their cob tissues all belonged to the same VCG. In only two of 44 cases were all three isolates from a set in different VCGs. These results support the hypothesis of Bacon et al (1) that *Fusarium* isolates could arrive at the kernel after growth in the cob. These results also suggest that if infection of the ears occurred through the silks, then most portions of the ear must have received the same strain as inoculum. In the case of wounds, it is not clear if strains sporulating in the wounds were introduced at or after the time the wound was made, or if they were, instead, a convenient location for a local strain to sporulate. Based on our data, we suggest that whatever infection method is used by the fungus to infect the ear, it must be such that infection within an ear is relatively uniform and that only a few fungal strains are usually present per ear.

TABLE 4. Vegetative compatibility groups (VCGs) of *Fusarium* isolates recovered from 15 kernels of single ears of maize

Ear	No. of isolates	No. of VCGs[z]
1	15	2
2	15	1
3	15	1
4	15	1
5	15	4
6	15	3
7	14	4
8	13	2
9	14	1
10	15	2

[z]Number of VCGs from a single ear. VCGs from a given ear are not the same as any other VCGs from that ear. Pairings were not made between strains recovered from different ears, so the total number of unique VCGs in the field may be less than 21.

F. moniliforme can reduce or eliminate infection of maize kernels by other fungi (31,36,38,39). Zummo and Scott (42) have shown that *F. moniliforme* infection inhibits kernel infection by *Aspergillus flavus* in inoculated maize ears and leads to reduced aflatoxin contamination of the kernels. Most maize plants are infected by multiple strains of *F. moniliforme*; therefore, it may be possible to use selected (and perhaps modified) strains of *F. moniliforme* to reduce infection of maize by *Aspergillus*. Similarly, if infection is systemic, it may be possible to introduce strains that do not produce mycotoxins, such as fumonisins (9,26) and to reduce the overall level of fumonisin contamination in maize grain. It also may be possible to develop synergistic strain sets that are more competitive than any single strain alone.

The identification of multiple *Fusarium* strains within a maize plant indicates that both population and individual strain-host plant interactions are important in the evaluation of this host-pathogen interaction. The ubiquity of the infection serves as a reminder that fungal penetration and colonization of the host plant are necessary, but not sufficient conditions, for disease induction and that our understanding of the highly evolved interactions between this host and its endophyte/pathogen is rudimentary at best.

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