Population Structure and the Relationship Between Pathogenic and Nonpathogenic Strains of *Fusarium oxysporum*

T. R. Gordon and D. Okamoto

Assistant professor and staff research associate, Department of Plant Pathology, University of California, Berkeley 94720.
This work was supported, in part, by grant 89-37151-4487, from the USDA-CRGO. We acknowledge helpful discussions with T. D. Bruns concerning the use of parsimony analysis.
Accepted for publication 14 August 1991 (submitted for electronic processing).

ABSTRACT

Gordon, T. R., and Okamoto, D. 1992. Population structure and the relationship between pathogenic and nonpathogenic strains of *Fusarium oxysporum*. Phytopathology 82:73-77.

One hundred isolates of *Fusarium oxysporum* were collected from soil with a history of Fusarium wilt of muskmelon in a 35 m^2 area within an agricultural field in the San Joaquin Valley of California. Twenty-nine strains from this collection, each representing a different vegetative compatibility group, were found to be nonpathogenic to muskmelon in greenhouse seedling tests. Each of these 29 strains was examined for polymorphisms in mitochondrial (mt) DNA by probing restriction digests of total DNA with cloned fragments from the mt genome of *F. oxysporum* f. sp. *melonis*. Changes in length (insertions or deletions) and restriction sites, relative to the reference strain of *F. o. melonis*, were inferred from the number and size of mtDNA restriction fragments that hybridized to *Pst*I fragments from the reference strain. Thirty-seven changes were scored as present or absent in each strain. There were six unique character combinations (haplotypes) among the 29 nonpathogenic strains, all of which differed from *F. o. melonis*. One mtDNA haplotype was associated with 14 different vegetative compatibility groups. Parsimony analysis revealed a distant relationship between *F. o. melonis* and the mtDNA haplotypes associated with nonpathogenic strains. However, two of the nonpathogenic strains were more distantly related to other nonpathogens than they were to *F. o. melonis*.

Fusarium oxysporum Schlechtend.:Fr. is an asexual, soilborne fungus found in agricultural soils throughout the world. *F. oxysporum* is made up of numerous formae speciales distinguished by their ability to cause a vascular wilt disease on a limited taxonomic range of host plants (3). This species also includes nonpathogenic strains capable of persisting through asymptomatic colonization of plant roots and saprophytic growth on nonliving organic matter (13,15,30,39).

Diseases caused by *F. oxysporum* are most effectively controlled through the use of disease-resistant cultivars. Often, however, this form of control is compromised by the appearance of a new pathogenic race, capable of causing disease on the otherwise resistant host. At present, little is known about the origin of new races, or the extent to which the appearance of a previously known race in a new location represents an independent origin of that race or simply dispersal, assisted by commerce and agricultural practices, from a single site of origin. The present distribution of some formae speciales would be consistent with the latter explanation (8,20,32). However, there also are indications that multiple origins may have occurred, such as the association of a given virulence phenotype with more than one vegetative compatibility group (VCG) (9,20), and significant diversity in mitochondrial (mt) DNA within a race (19).

Further insight into these questions requires a better understanding of how diversity in *F. oxysporum* is partitioned, both geographically and with respect to virulence. Given the large number of pathotypes in this species and their extensive distribution, a comprehensive inventory of genetic diversity cannot easily be assembled. To date, workers interested in pathogenic strains of *F. oxysporum* have rendered this task more manageable by restricting their attention to one or several formae speciales (4,8,18,21,32), an essentially taxonomic or phylogenetic approach to the problem.

A complementary and equally valuable approach is to subdivide the species spatially, that is, to make populations the principal object of study rather than individual sub-specific taxa. In this type of study all strains of *F. oxysporum* within a population would be examined, including those for which virulence cannot be demonstrated. This should provide a more comprehensive picture of diversity within a population and may also shed light on whether or not pathogenic strains are introduced or indigenous at any given location. For example, if virulence to a particular host arose only once and a single strain was thereafter widely distributed, we might expect this pathogen to be genetically distinct from co-occurring nonpathogenic strains at most locations where it now is found. On the other hand, if a pathogen has been recently derived, or selected, from a local population of *F. oxysporum* we would expect it to show a close relationship to nonpathogenic strains at that location.

Recently, we initiated a study of a population of *F. oxysporum* within a single agricultural field in California. In this field, all isolates of *F. oxysporum* f. sp. *melonis* W. C. Snyder & H. N. Hans., cause of Fusarium wilt of muskmelon, were represented by a single VCG (20) and a single mtDNA haplotype (19). In contrast, nonpathogenic strains at this location were included in 29 different VCGs (14). Based on these results, we suggested that the nonpathogenic strains were derived from a population indigenous to the soil before its cultivation, whereas the pathogen recently was introduced (14). However, pathogenicity may not be an appropriate basis for subdividing populations of *F. oxysporum*. Although we have tested at least one representative of each of 29 VCGs of *F. oxysporum* for pathogenicity on muskmelon, tomato, cotton, sugar beet, and alfalfa and found all strains to be avirulent (14, and *unpublished data*), it remains possible that other host plants would reveal pathogenic potential in some of these strains. Thus, an alternative means of assessing the affinity of *F. o. melonis* for co-occurring strains clearly is needed. For this reason, we have characterized strains in a local population of *F. oxysporum* by identifying length and restriction site changes in mtDNA and used these data to assess interstrain relationships. A preliminary report has been published (12).

MATERIALS AND METHODS

Details of the collection procedure were fully described elsewhere (14) and are briefly summarized here. Soil samples were collected at 20 randomly selected locations from within a 35 m^2 area of an agricultural field in Fresno County, California, on the west side of the San Joaquin Valley. A total of 100 isolates

of *F. oxysporum* (five from each soil sample) were obtained from soil dilution plates using Komada's selective medium (23). Single spore subcultures were identified as *F. oxysporum* on carnation leaf agar (31) based on the presence of short monophialides bearing microconidia in false heads and characteristically shaped macroconidia (7,31). One representative of each of the 29 VCGs identified in the collection of 100 isolates (14) was included in the present study.

Total DNA was extracted from each isolate using the miniprep extraction procedure described by Lee et al (25), with modifications described by Jacobson and Gordon (19). One further modification was the substitution of 100% ethanol for isopropanol in the first DNA precipitation step. DNA samples were stored at −20 C.

Restriction digests of total DNA from all strains were done using either *Hae*III or *Pst*I according to the manufacturer's recommendations (Bethesda Research Laboratories, Bethesda, MD). Double digests (*Pst*I and *Hae*III) of total DNA from strains representative of each unique restriction digest pattern were done to determine the location of *Pst*I sites within *Hae*III fragments. *Eco*RI, alone and in double digests with either *Hae*III or *Pst*I, also was used as needed to facilitate mapping the location of changes relative to a reference strain of *F. o. melonis* (19).

Restriction fragments were separated electrophoretically, usually in either 1% or 0.7% agarose; for double digests in which it was necessary to resolve fragment sizes <0.5 kb, 1% agarose + 2% Nusieve (FMC Bioproducts, Rockland, ME) was used. After electrophoresis, restriction fragments were transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, NH) using a pressure blotting device (Stratagene, La Jolla, CA) and cross-linked to the membranes by exposure to UV light using a Stratalinker 1800 (Stratagene) in the auto crosslink mode.

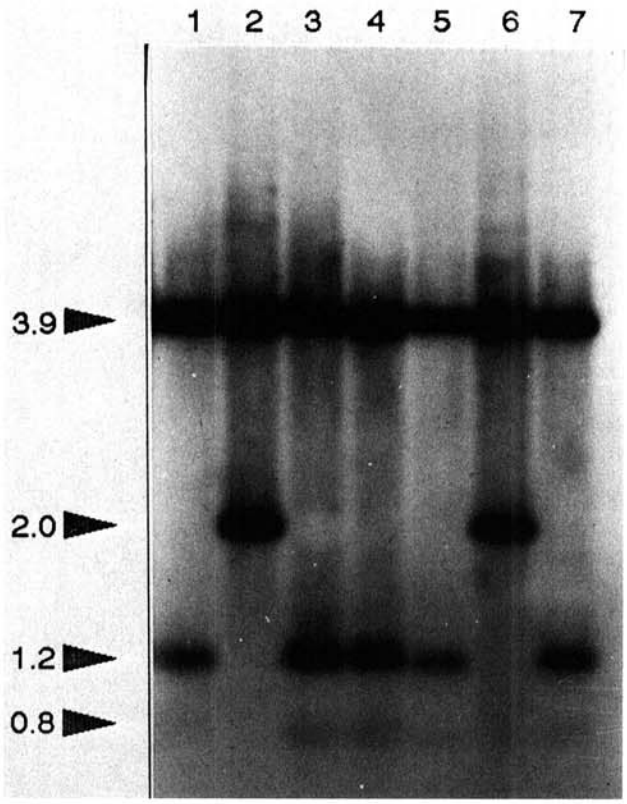

Fig. 1. A Southern blot of total DNA digested with *Hae*III and probed with a 2.0-kb fragment (pFOM-6) cloned from the mitochondrial genome of *Fusarium oxysporum* f. sp. *melonis*. This probe hybridized with two fragments in *F. o. melonis* (lane 6) and in the nonpathogenic strain shown in lane 2. All of the other nonpathogenic strains shown (lanes 1, 3, 4, 5 and 7) are missing the 2.0-kb fragment but have gained 1.2- and 0.8-kb fragments. This was interpreted as a gain of a *Hae*III site within the original 2.0-kb fragment. This change corresponds to character 35.

Restriction fragments bound to nylon membranes were probed sequentially with each of nine recombinant plasmids; each plasmid contained a *Pst*I fragment previously cloned from the mt genome of *F. o. melonis* (19). Recombinant plasmids were labeled with [α-^{32}P]dCTP using a random primer extension kit (Amersham, Arlington Heights, IL). Southern hybridizations, post-hybridization washing of membranes, autoradiography, and stripping of radioactive probes from membranes were performed as previously described (19).

Differences in the number and size of mtDNA restriction fragments that hybridized with each probe were used to identify restriction site changes (Fig. 1) and changes in length (Fig. 2) relative to a reference strain of *F. o. melonis* (19). Each change was treated as a single character and scored as either present (1) or absent (0) in each strain. These data were analyzed using PAUP Release 3.0L (D. L. Swofford, Illinois Natural History Museum) on a Macintosh SE-30 to produce most parsimonious trees (37) and to estimate the extent to which the data supported branches on these trees using bootstrap (10).

RESULTS

Eight of the nine clones used as probes revealed some polymorphism among strains of *F. oxysporum*. Only pFOM-8, the smallest clone (1.0 kb), failed to show any unique polymorphisms (i.e., polymorphisms not also revealed by a contiguous clone). Length and/or restriction site changes that could explain the observed polymorphisms were deduced from a direct comparison of fragments in the nonpathogenic strains to homologous fragments in the reference strain of *F. o. melonis* (Figs. 1 and 2). Homologous fragments of the same mobility were assumed to occupy the same position.

Changes in mtDNA shown in Table 1 represent the minimum number required to explain the observed differences. For example, wherever an insertion mapped to the same location as a restriction site gain, we assumed the enzyme recognition sequence was internal to the inserted fragment and counted this as only one

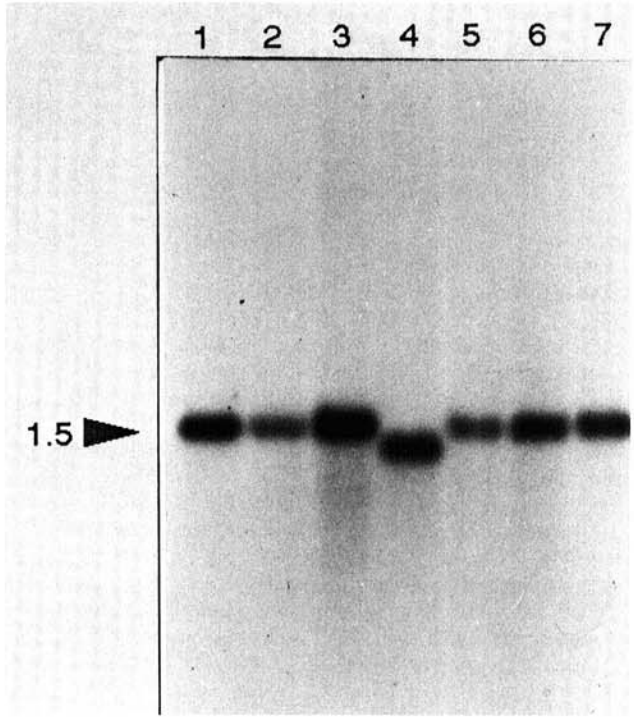

Fig. 2. A Southern blot of total DNA digested with *Pst*I and probed with a 1.5-kb fragment cloned from the mitochondrial genome of *Fusarium oxysporum* f. sp. *melonis* (pFOM-7). This probe hybridized with a 1.5-kb fragment in *F. o. melonis* (lane 6) and all of the nonpathogenic strains shown, with one exception (lane 4), which has a 0.1-kb deletion relative to the reference strain. This change corresponds to character 37.

change (i.e., the insertion). Also, we identified length changes based on the smallest fragment with altered mobility. Whereas we regarded this as one change, it could have been the result of several smaller insertions (or deletions). Consequently, it is likely we have underestimated the actual number of differences among strains.

The 37 changes shown in Table 1 were treated as characters and scored as present or absent in each of the 29 strains. Six unique character combinations (haplotypes) were identified among the nonpathogenic strains of *F. oxysporum* and given a single letter designation (A–F); *F. o. melonis* represented a seventh mtDNA haplotype (G) (Table 1). mtDNA haplotypes representative of nonpathogenic strains were unevenly distributed among the 29 VCGs. Fourteen VCGs shared one mtDNA haplotype, and a second mtDNA haplotype was common to eight VCGs. Two mtDNA haplotypes were represented by single VCGs. Assuming all isolates within a VCG shared a common mtDNA haplotype, then two haplotypes accounted for 92% of the isolates sampled from this population. Although only five isolates were collected from each sampling site within this field, we recovered two or more different mtDNA haplotypes from six of these sites.

TABLE 1. Characters and character states in seven mitochondrial DNA haplotypes of *Fusarium oxysporum*

Character number	Clone[a]	Change[b]	Haplotype[c]						
			A	B	C	D	E	F	G
1	pFOM-1	0[d]	0	0	0	0	0	1	1
2	pFOM-1	+*Hae*III site	1	1	0	0	0	0	0
3	pFOM-1	+*Pst*I site	1	1	0	1	0	0	0
4	pFOM-1	+*Hae*III site	0	0	1	0	1	0	0
5	pFOM-2a	0	0	0	0	0	0	0	1
6	pFOM-2a	+0.20 kb	1	1	1	1	1	1	0
7	pFOM-2a	+1.50 kb	0	0	1	0	1	0	0
8	pFOM-2a	+*Pst*I site	1	1	1	1	1	1	0
9	pFOM-2a	+3.70 kb	1	1	0	1	0	1	0
10	pFOM-2a	−3.25 kb	1	1	0	1	0	1	0
11	pFOM-2a	+0.70 kb	1	1	0	1	0	1	0
12	pFOM-2b	0	0	0	0	0	0	0	1
13	pFOM-2b	−0.40 kb	1	0	0	0	0	0	0
14	pFOM-2b	−1.00 kb	0	0	1	0	0	0	0
15	pFOM-2b	+1.10 kb	0	0	1	0	0	0	0
16	pFOM-2b	+*Pst*I site	1	1	0	1	0	1	0
17	pFOM-3	0	0	0	1	0	0	0	1
18	pFOM-3	+0.15 kb	1	1	0	1	0	1	0
19	pFOM-3	+0.25 kb	1	0	0	0	0	0	0
20	pFOM-3	−10.4 kb	0	0	0	0	1	0	0
21	pFOM-3	−7.60 kb	0	1	0	1	0	1	0
22	pFOM-3	+7.00 kb	0	1	0	1	1	1	0
23	pFOM-3	+0.10 kb	0	1	0	1	0	0	0
24	pFOM-3	−*Hae*III site	0	0	0	0	1	1	0
25	pFOM-3	+*Hae*III site	0	0	0	0	1	1	0
26	pFOM-4	0	0	0	1	0	1	0	1
27	pFOM-4	−3.40 kb	1	1	0	1	0	1	0
28	pFOM-4	+4.80 kb	1	1	0	1	0	1	0
29	pFOM-5	0	0	0	0	0	0	0	1
30	pFOM-5	+*Hae*III site	0	0	1	0	0	0	0
31	pFOM-5	+*Hae*III site	0	0	1	0	0	0	0
32	pFOM-5	−*Pst*I site	1	0	0	0	0	0	0
33	pFOM-5	−0.35 kb	1	0	0	0	0	0	0
34	pFOM-6	0	0	0	1	0	1	0	1
35	pFOM-6	+*Hae*III site	1	1	0	1	0	1	0
36	pFOM-7	0	0	0	1	0	0	0	1
37	pFOM-7	−0.10 kb	1	0	0	0	0	0	0

[a] The recombinant plasmids that revealed unique polymorphisms when used as probes of *Hae*III and *Pst*I digests of total DNA. Each plasmid contained an insert that represented a *Pst*I fragment cloned from the mitochondrial genome of *Fusarium oxysporum* f. sp. *melonis*.

[b] Each change represents either a gain or loss of a restriction site (e.g., +*Hae*III site or −*Hae*III site), or an insertion or deletion (e.g., +0.1 kb or −0.1 kb). These changes are relative to a reference strain of *F. o. melonis*.

[c] Six unique combinations of characters were identified among 29 vegetative compatibility groups of *F. oxysporum*, these are represented as mitochondrial DNA haplotypes A–F. Haplotype G represents the reference strain of *F. o. melonis*. Character states are either present (1) or absent (0).

[d] Fragment unchanged.

The relationships among mtDNA haplotypes were evaluated using Wagner parsimony analysis (37). This analysis produced four most parsimonious unrooted trees, each with a total of 45 steps or character changes. A bootstrap 50% majority-rule consensus tree is shown in Figure 3. According to bootstrap, the major branch in this tree was supported at the 99% level indicating that 99 of the 100 data sets obtained by resampling the original data produced a tree that included this branch. The branch in question separated seven mtDNA haplotypes into two major clusters, one of which was composed only of nonpathogenic strains. Again assuming common mtDNA haplotypes within a VCG, this one cluster included 95% of the nonpathogenic isolates in the collection. The remaining 5% were represented by the two mtDNA haplotypes that clustered loosely with *F. o. melonis*.

F. o. melonis was separated from the closest mtDNA haplotype (C) by 12 changes. The differences that distinguished *F. o. melonis* from mtDNA haplotype C were revealed by four of the nine clones that were used as probes. The greatest distance between mtDNA haplotypes, 29 steps, separated *F. o. melonis* (or mtDNA haplotype C) from mtDNA haplotype A. A pairwise comparison of either *F. o. melonis* or mtDNA haplotype C with mtDNA haplotype A (Table 1) revealed 25 differences rather than 29. This discrepancy reflects character state reversals inherent in the topology of the most parsimonious trees.

DISCUSSION

Many methods now are available to assess variability in fungi. Direct sequencing of conserved regions such as nuclear and mt rRNA genes has proven to be valuable in studies of intra- and interfamily level relationships (5). However, these conserved regions may reveal insufficient variability to be useful in resolving relationships within a species (5,11). At the population level, restriction fragment length polymorphisms in nuclear DNA have been employed to characterize variation between individual strains (17,27,28). Variability in mtDNA also has been utilized to study intra-specific variation in fungi (19,35,38) but has not been widely used to study fungal populations. Variation in mtDNA, however, commonly is exploited for studies of population biology in animal species (2).

A physical map of the mt genome facilitates analysis of variability in fungi by making it possible to map the location of each change. Length changes can thereby be distinguished from

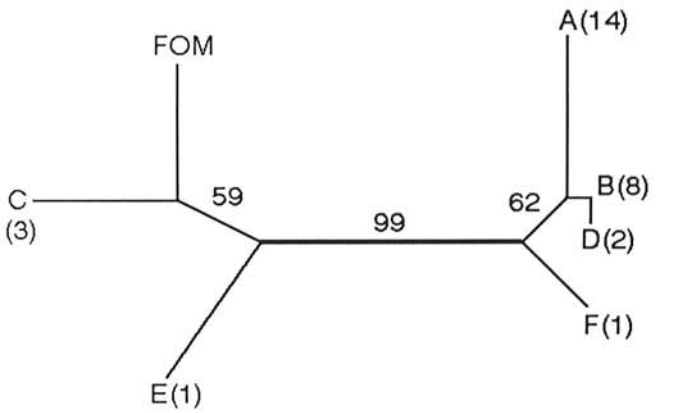

Fig. 3. An unrooted most parsimonious tree of relationships among seven mitochondrial DNA haplotypes representing nonpathogenic strains of *Fusarium oxysporum* (A–F) and *F. oxysporum* f. sp. *melonis* (FOM). The numbers in parentheses following each letter refer to the number of vegetative compatibility groups associated with that haplotype. The numbers shown on each of the three major branches indicate the level of support for these branches, according to bootstrap. The length of each branch is proportional to the number of changes between the nodes (or node and terminus) joined by that branch.

site changes, insuring that individual length changes are not counted more than once (5,38). Unfortunately, this type of analysis can be relatively time-consuming, and, because length changes are common, alignment of maps from different taxa may be somewhat ambiguous. We found mtDNA to be useful in *F. oxysporum* because the clones available for use as probes represented approximately 95% of the mt genome of this species, which made data acquisition relatively rapid; mtDNA revealed variation among strains within a population; and the structural differences between mtDNA haplotypes were not so great as to preclude mapping the location of individual changes.

All the diversity in mtDNA was among the nonpathogenic strains of *F. oxysporum* with no diversity in *F. o. melonis* at this location (19,20). Furthermore, based on mtDNA, *F. o. melonis* is very different from the nonpathogenic strains being separated by a minimum of 12 character changes. However, even greater differences exist among nonpathogenic strains. Also, a previous study of *F. o. melonis* showed that as many as 17 changes in mtDNA separated pathogenic strains in this forma specialis (19). Consequently, it is not immediately obvious that *F. o. melonis* lacks affinity for other strains in this population.

Any judgment concerning whether or not *F. o. melonis* is an integral part of this population clearly depends on what we consider to be the limits of diversity for a local population of this fungus. If we choose to regard the diversity in mtDNA haplotypes that we have observed among nonpathogenic strains to be consistent with a well established natural population of *F. oxysporum*, then *F. o. melonis* should be considered a part of that population. An alternative interpretation of our findings is a more complex origin of the population of *F. oxysporum*. That is, what we refer to as a population might be a composite of mtDNA haplotypes indigenous to the soil before its cultivation with an admixture of haplotypes that were introduced through agricultural practices. The indigenous population is most likely to be represented by the large cluster composed only of nonpathogens (Fig. 3). These mtDNA haplotypes are either associated with more than one VCG or are closely related to multiple VCG haplotypes. It is improbable that such a closely related group of strains would be assembled through chance introductions. On the other hand, mtDNA haplotype E and *F. o. melonis* are very distant from the putatively indigenous population ($\geq$18 steps) and both are represented by only one VCG; they also are well separated from each other (14 steps). Based on these criteria, we regard these two strains as candidates for recent introductions. This interpretation is consistent with the relatively recent discovery of Fusarium wilt of muskmelon in California (16) and the close relationship between strains of *F. o. melonis* found in California and those found in the eastern United States (18,19), where the disease has been known to occur for more than fifty years (24).

The proper disposition of mtDNA haplotype C is less obvious. To preserve our hypothesized distinction between the introduced and indigenous strains, we have to assume that haplotype C was introduced as three different VCGs. This would be most easily explained if haplotype C originated from a population indigenous to a nearby field, from which it was introduced on more than one occasion. Such uncertainties underscore the need for more extensive sampling of populations of *F. oxysporum*. We do not yet know the extent to which populations even in adjacent fields might differ from the one we have studied.

The strong correlation between mtDNA haplotype and VCG that has been reported for pathogenic strains of *F. oxysporum* (19,22) was not apparent among the nonpathogens included in our study (Fig. 3). This discrepancy would be consistent with strong selection for a pathogenic clone leading to the observed association between VCG and mtDNA haplotype, and a considerably weaker effect of selection on root-colonizing strains with a broad host range.

Greater diversity of VCGs than mtDNA haplotypes, such as we have reported, might be expected in a sexually reproducing population, where recombination through meiosis would generate a large number of unique vegetative compatibility genotypes (1,33). For this reason, the diversity we observed might be considered polyphyletic, that is, the result of sexual reproduction at some time in the past. Of course, it also is possible that the population we sampled was initiated by a limited number of VCG-mtDNA clonal types, and the present greater diversity of VCGs reflects a faster rate of evolution at the loci that influence this trait, relative to the rate of change in the mt genome.

Other studies of *F. oxysporum* also have identified considerable diversity, especially in terms of VCGs (9,32), but over much broader geographic areas than we sampled in the present study. Thus, we do not know if the diversity we have identified on a local scale represents an exceptional situation for this fungus. Diversity in mtDNA within natural populations of the *Armillaria mellea* complex has been reported with most of the variation between rather than within biological species (34,35). High levels of diversity also have been reported for the foliar pathogens *Septoria tritici* (27) and *Rynchosporium secalis* (26).

Clearly, it is important to develop a better concept of what constitutes a fungal population. This information is needed to develop a better understanding of the dynamics of dispersal of fungal pathogens and the origin of novel pathotypes. Recent studies on population structure in foliar fungal pathogens (6,17,27) have provided insight into the influence of sexual reproduction on pathogen diversity and disease development that may contribute to more effective deployment of resistant cultivars. Much less is known about the structure of fungal populations in soil.

Among soilborne fungi, *F. oxysporum* represents an attractive model for the study of fungal populations. First, it is composed of nonpathogenic as well as pathogenic strains. Because nonpathogens are broadly adapted to colonization of plant roots and organic matter (13,15,30) and, presumably, are under less severe selection pressure than pathogens, they should provide a broader view of population structure than pathogenic phenotypes alone would provide. Also, natural populations of this fungus are found in native grasslands that have not been subjected to cultivation (29, 36, and *unpublished data*). Studies of *F. oxysporum* in these native situations could greatly enhance our understanding of what constitutes the spatial limits of a fungal population and may also reveal the extent to which these populations contribute to the diversity observed in agricultural settings.

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