

Highly Diverse Endophytic and Soil *Fusarium oxysporum* Populations Associated with Field-Grown Tomato Plants

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The diversity and genetic differentiation of populations of *Fusarium oxysporum* associated with tomato fields, both endophytes obtained from tomato plants and isolates obtained from soil surrounding the sampled plants, were investigated. A total of 609 isolates of *F. oxysporum* were obtained, 295 isolates from a total of 32 asymptomatic tomato plants in two fields and 314 isolates from eight soil cores sampled from the area surrounding the plants. Included in this total were 112 isolates from the stems of all 32 plants, a niche that has not been previously included in *F. oxysporum* population genetics studies. Isolates were characterized using the DNA sequence of the translation elongation factor 1 α gene. A diverse population of 26 sequence types was found, although two sequence types represented nearly two-thirds of the isolates studied. The sequence types were placed in different phylogenetic clades within *F. oxysporum*, and endophytic isolates were not monophyletic. Multiple sequence types were found in all plants, with an average of 4.2 per plant. The population compositions differed between the two fields but not between soil samples within each field. A certain degree of differentiation was observed between populations associated with different tomato cultivars, suggesting that the host genotype may affect the composition of plant-associated *F. oxysporum* populations. No clear patterns of genetic differentiation were observed between endophyte populations and soil populations, suggesting a lack of specialization of endophytic isolates.

Fusarium oxysporum is a well-known phytopathogenic fungus that affects hundreds of crops worldwide. Most studies of the fungus focus on the ability of *F. oxysporum* to cause vascular wilt on a particular host. However, environmental surveys of a wide range of habitats frequently find *F. oxysporum* in the absence of plant disease (1). These *F. oxysporum* populations, often referred to as nonpathogenic, although lack of pathogenicity is usually not confirmed, are a cosmopolitan component of soil communities. *F. oxysporum* can also infect and colonize plants asymptotically as an endophyte. This endophytic lifestyle has not been well researched, despite reports of endophytic colonization by *F. oxysporum* on nearly 100 plant species (2).

The interaction of endophytic *F. oxysporum* and plants may give insight into how pathogen recognition and plant resistance operate. In susceptible plants, pathogenic *F. oxysporum* penetrates the root, colonizes the root cortex, and then spreads through the xylem to the rest of the plant, causing occlusion of the xylem and ultimately wilting and death (3, 4). In host cultivars that are resistant to the pathogen, the spread of the fungus is blocked, and only limited colonization of host tissue occurs (5, 6, 7). Nonpathogenic *F. oxysporum* inoculated onto plants can colonize the root cortex similarly to pathogens, but host responses, such as thickening of cell walls and protuberances into intercellular spaces, seem to limit the growth of endophytes, similar to defenses limiting the growth of pathogens in resistant plants (3, 4, 8). Nonpathogenic *F. oxysporum* can also have a differential ability to colonize and multiply in the root cortex of different plant species. For example, research done by Leoni et al. (9) showed how *F. oxysporum* f. sp. *cepae* (which causes *Fusarium* basal rot of onions) can reach high population levels in the roots of plants, such as black bean or Sudan grass, in the absence of disease. Although the above observations suggest that endophytic *F. oxysporum* is not systemic, *F. oxysporum* endophytes can be recovered from naturally colonized plant stems (10). However, most population genetics studies have

focused on nonpathogenic *F. oxysporum* isolated from roots (11, 12, 13, 14, 15).

Despite the cosmopolitan nature of *F. oxysporum* and its ecological diversity, the structure and diversity of soil and endophytic *F. oxysporum* populations are not well understood. A recent typing scheme proposed by O'Donnell et al. (16) used DNA sequences of the ribosomal DNA intergenic spacer region (IGS) and the translation elongation factor 1 α (TEF) gene to define sequence types (STs) and found 256 sequence types among 850 isolates, mainly plant pathogens. Subsequent studies using the same typing scheme found 26 new sequence types among isolates obtained from soil in Sardinia (17) and 46 new sequence types from non-cultivated soils across Australia (18). These novel sequence types suggest that focusing only on plant-pathogenic *F. oxysporum* may underestimate the total species diversity. Other studies indicate that *F. oxysporum* populations from soil are geographically heterogeneous and greatly influenced by resident plant populations (19, 20, 21), suggesting that some plants may exert a selective effect on soil populations of *F. oxysporum* (22). Studies comparing pathogenic and nonpathogenic *F. oxysporum* populations associated with the same host have found much higher levels of diversity

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in the nonpathogenic populations than in the pathogens (11, 14, 23, 24).

An additional unaddressed topic is the diversity of *F. oxysporum* within an individual plant. Endophytic *F. oxysporum* is commonly isolated from plants infected with pathogenic *F. oxysporum* (for examples, see references 23 and 25), suggesting that colonization of plants by multiple genotypes of *F. oxysporum* may be common. Also, different genotypes may interact with their host plants in different ways; for example, studies focusing on developing nonpathogenic *F. oxysporum* fungi as biocontrol agents have found particular isolates to be better biocontrol agents than others (26, 27, 28). A few studies have reported overrepresentation of a few genotypes within endophytic populations (12, 22), suggesting a possible selective effect by the host on soil populations.

This study investigated the population structure of *F. oxysporum* associated with asymptomatic tomato plants grown under field conditions. We hypothesized that endophyte populations would be genetically differentiated from soil populations. Additionally, if only specific genotypes of *F. oxysporum* could colonize plants endophytically, we expected that endophyte populations would be less diverse than soil populations. The objective of the study was to compare the compositions and diversities of *F. oxysporum* populations to look for evidence of specialization of *F. oxysporum* to different environmental niches, such as soil or plants. A further goal was to assess the diversity of *F. oxysporum* fungi found within individual plants and associated with different plant parts, namely, roots, crowns, and stems.

MATERIALS AND METHODS

Sampling and fungal isolation. *F. oxysporum* populations were sampled from two tomato fields located in the Russell E. Larson Research Center, The Pennsylvania State University, Pennsylvania Furnace, PA. These are experimental fields used for agricultural research and are located approximately 1.5 km from each other; they were chosen for sampling of *F. oxysporum* populations because they had no known history of *Fusarium* wilt of tomato. Within each field, four plots were randomly chosen for sampling of tomato plants and soil. Each plot consisted of four plants in a row with 1-foot spacing. Within a plot, six 4-inch soil cores were collected, two cores from between each pair of plants, and bulked together to make a composite soil sample per plot, for a total of 16 plant samples and 4 soil samples per field. Plants in field 1 were all of the processing tomato cultivar Heinz 9907. In field 2, each set of four plants consisted of a different cultivar, the processing tomato cultivars Gem 611 and Heinz 3402 and the fresh market cultivars FL 47 and Mountain Fresh. These cultivars are all resistant to *F. oxysporum* f. sp. *lycopersici* races 1 and 2. No fungicides had been applied to the plants. Plants in field 1 were sampled at the green fruit stage, and plants in field 2 were sampled after the fruits were mature.

Sampling from plants was done by carefully digging up the plants, washing off the soil under tap water, and surface disinfecting entire plants with a solution of 2% commercial bleach (6.15% NaOCl) for 1.5 min. Small (approximately 5-mm-long) pieces of the root and stem were placed on Nash-Snyder *Fusarium*-selective growth medium for fungal isolation (29). Soil samples were air dried for at least 10 days and sieved to remove large particles. Five grams of soil was suspended in 50 ml sterile water by mixing for 20 min, and then 10-fold serial dilutions of 10^{-1} to 10^{-4} were plated on Nash-Snyder medium (24). A maximum of 10 isolates were collected per plant: 4 from the roots and stem, respectively, and 2 from the crown, for a maximum total of 40 isolates for each set of four plants (one plot), and a maximum of 40 isolates were collected per composite soil sample (one plot), selected randomly from dilution plates. *F. oxysporum* isolates were identified based on colony morphology, and monoconidial cultures were grown on potato dextrose agar. Isolates were

stored long-term at 4°C in 1.5-ml microcentrifuge tubes in sterile sand mixed with 250 μ l of potato dextrose broth.

DNA extraction and sequencing. DNA was extracted following the protocol of Cenik (30). Briefly, mycelium was grown in potato dextrose broth in 1.5-ml microcentrifuge tubes for at least 3 days at room temperature, centrifuged to remove the medium, and ground in an SDS-based extraction buffer. Cellular debris was precipitated with sodium acetate, and finally, DNA was precipitated with isopropanol. The TEF gene, a region commonly used for *Fusarium* identification, was amplified using the primers EF1 and EF2 under conditions described previously (16). Amplification was checked on agarose gels stained with ethidium bromide, and the PCR products were cleaned with Exo-Sap (Affymetrix, Santa Clara, CA) before sequencing. Sequencing was done using the primers EF1 and EF2 at the Pennsylvania State University Genomics Core Facility (University Park, PA).

Data analysis. Sequences were compared to reference sequences in GenBank and the *Fusarium*-ID database (31) to confirm the isolates' identities as *F. oxysporum* and to identify if the sequences were present in the *Fusarium*-ID database or if they represented new sequence types. Isolates that were not *F. oxysporum* (5% of the sequenced samples) were excluded from further analysis. Sequence types were defined and randomly numbered for each allele, including gaps as informative characters. Population size was estimated using the nonparametric estimator Chao 2, calculated in EstimateS 8.2.0 (R. K. Colwell, 2006 [http://www.purl.oclc.org/estimates]). Population genetic diversity was measured with Shannon's diversity index ($^S H_A$) based on the frequency of sequence types, using the program GENALEX 6 (32). Diversities were compared between subpopulations based on Shannon's mutual-information index ($^S H_{UA}$), and significance was assessed using the log-likelihood test statistic, G (33). Genetic differentiation among subpopulations was measured using two analyses: the nearest-neighbor statistic (S_{nn}) (34), which is based on the nucleotide sequence and determines how often isolates with the most similar DNA sequences (the nearest neighbors) are found in the same population, and the haplotype statistic (H_{ST}), which is based on allele frequency, specifically the following equation: $H_{ST} = 1 - (\text{average haplotype diversity in the subpopulations} / \text{average haplotype diversity in the total population})$ (35). Analyses were performed using DnaSP 5.10 (36), and statistical significance was assessed with 1,000 permutations using Monte Carlo simulations (35). Gaps were considered a fifth character for H_{ST} analysis but not for S_{nn} . Sequences from different locations (i.e., plants or soil from each of the eight plots) were compared using principal-coordinate analysis to look for clustering of sequences from particular environments. The principal-coordinate analysis was performed using UniFrac (37), with sequences weighted for abundance and nonnormalized. A phylogenetic network of the sequence types was estimated with statistical parsimony analysis and performed using TCS v. 1.21 (38). Maximum-parsimony analysis was inferred from the DNA sequences using the program PAUP* v.4.0b10 (D. L. Swofford, 2002; Sinauer Associates, Sunderland, MA). Parsimony analysis was performed using the heuristic-search option with the MulTrees option on, 1,000 random addition sequences, the tree-bisection-reconnection branch-swapping algorithm, and gaps not considered characters. Support for each branch was assessed with 1,000 bootstrap replicates. Included in the phylogenetic analysis were sequences representative of the known diversity in *F. oxysporum* based on previous phylogenetic analyses (16, 39, 40) downloaded from GenBank or the *Fusarium*-ID database (31).

Nucleotide sequence accession numbers. The sequences identified in this work have been deposited in GenBank under accession numbers KJ920404 to KJ920429.

RESULTS

Fungal isolation. *F. oxysporum* was successfully isolated from all eight soil samples and all 32 plants from both fields, with a total of 609 isolates collected and analyzed: 314 isolates from soil and 295 from plants. A total of 16 populations were analyzed, 8 from each

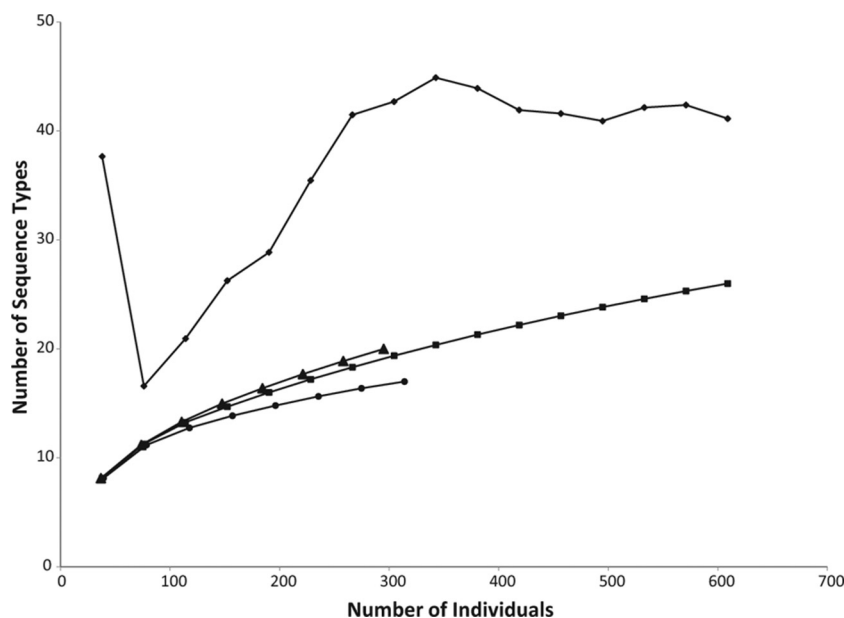


FIG 1 Species accumulation curves showing the expected number of sequence types when sampling a given number of individuals. The curves show the observed numbers of sequence types for samples from plants and soil combined (squares), plants only (triangles), and soil only (circles). The Chao 2 nonparametric richness estimator, calculated in EstimateS 8.2.0, is also shown for samples from plants and soil combined (diamonds).

field, with four plots per field. From each plot, we established one population from plants and one from the corresponding soil. For this research, we used a very broad definition of population as a group of individuals of the same species occupying a particular space or niche (in our case, a plant or a soil) at a particular time (41). Isolates from plants were considered to be endophytes, because the plants were asymptomatic and surface disinfested. A maximum of 10 isolates were collected for each plant, and the majority of the plants yielded 9 or 10 isolates. The plant that yielded the fewest isolates (six) was heavily colonized by *Geotrichum* spp. Soil samples contained approximately 1,000 CFU of *F. oxysporum* per gram soil, based on estimations from soil dilution plating.

Within each plant, *F. oxysporum* was isolated from the roots, crown, and stem, although some plants yielded only one isolate per plant part. *F. oxysporum* was isolated from the top third of the stem (approximately 60 cm above the soil) for 19 of the 32 plants, suggesting that colonization of the entire stem is not uncommon. Whether *F. oxysporum* could also be found in the leaves and fruit was not assessed.

F. oxysporum was the most common *Fusarium* sp. observed. Other *Fusarium* species observed but excluded from further study (5% of the sequenced samples) were identified, using the Fusarium-ID database (31), as *Fusarium commune*, *Fusarium proliferatum*, and members of the *Fusarium solani* species complex, the *Gibberella fujikuroi* species complex, and the *Fusarium incarnatum-Fusarium equiseti* species complex. *F. commune* was frequently isolated, but this was due to difficulties in distinguishing it morphologically from *F. oxysporum* and not to its relative abundance in the samples.

Sequence types and population diversity. A fragment of approximately 630 bp of the TEF gene was amplified for all isolates. Fifty-seven sites were polymorphic, with 45 single-nucleotide polymorphisms and 12 insertions/deletions (indels). Including

indels as informative characters, 26 different STs were observed (GenBank accession numbers KJ920404 to KJ920429). The species accumulation curves of populations from plants, soil, and plants and soil combined show that sampling was approaching but did not reach saturation (Fig. 1). The total population richness was estimated to be 41 STs based on the Chao 2 estimator, with a 95% confidence interval of 29 to 88 STs. The Chao 2 richness estimate for soil was 21 STs, compared to 17 observed STs, and it was 40 STs in plants, compared to 20 observed STs. Twenty-one of the 26 STs were found in field 1, and 18 of the 26 STs were found in field 2. Two STs, 5 and 13, were especially common and composed 35% and 30% of all isolates, respectively. These two STs were equally common in soil and in plant tissues and were found in both fields, although ST 5 was twice as common in field 2 as in field 1 and ST 13 was three times more common in field 1 than in field 2. The most abundant STs were found in both locations and in both soil and endophyte populations, but some of the rare STs, including 10 that were observed only once, were unique to field 1 (8 STs), field 2 (5 STs), plant tissues (8 STs only in roots or crowns and 1 ST only in stems), or soil (6 STs). Of the average of 9.2 isolates per plant, an average of 4.2 STs were found per plant, ranging from 2 to 7. One to 4 STs were found per stem and per root system, with an average of 2.2 STs per stem (out of an average of 3.5 isolates per stem) and 2.6 STs per root system (out of an average of 3.8 isolates per root system).

The diversities of populations from plants and from soil were compared using Shannon's diversity index (Table 1). Based on this index, endophytic populations were significantly more diverse than soil populations for field 2 and for both fields combined. The diversities were not significantly different between plant and soil populations for individual plots, except for plot 6 in field 2, in which plant populations were significantly more diverse than soil populations.

Sequences were compared to the Fusarium-ID database to de-

TABLE 1 Pairwise comparisons of Shannon's diversity indices between populations from soil and plants

Sampling location	sH_A		${}^sH_{UA}$	G	P value ^a
	Plant population	Soil population			
All locations	2.729	2.672	0.081	68.437	<0.001
Field 1	2.607	2.440	0.077	33.354	0.031
Field 2	2.529	2.364	0.147	60.822	<0.001
Plot 1 (Heinz 9907)	2.318	2.478	0.150	16.044	0.098
Plot 2 (Heinz 9907)	1.653	2.084	0.117	13.001	0.224
Plot 3 (Heinz 9907)	2.709	1.703	0.225	24.041	0.020
Plot 4 (Heinz 9907)	2.436	2.512	0.185	19.702	0.073
Plot 5 (FL 47)	2.542	2.190	0.180	18.484	0.047
Plot 6 (Gem 611)	2.710	1.619	0.351	37.426	<0.001
Plot 7 (Heinz 3402)	1.386	2.052	0.158	16.405	0.022
Plot 8 (Mountain Fresh)	2.277	2.748	0.218	21.746	0.026

^a Values are *P* values of the log-likelihood test of differences between soil and endophyte populations, with statistically significant values in boldface (Bonferroni-adjusted $P < 0.0045$ [0.05/11]).

termine if the sequence types had been found in previous samplings. Only 12 of the 26 sequence types had a 100% match to sequences in the database. The two most common sequence types, ST 5 and ST 13, have been previously found associated with many different plant species. Most of the 14 novel sequence types were rare, with only one or two isolates of each, although two of the novel sequence types, ST 4 and ST 8, were found 44 and 32 times, respectively.

Genetic differentiation among populations. The genetic differentiation between various subpopulations was calculated based on both haplotypes (H_{ST}) and the nucleotide sequence (S_{nn}) (Table 2). Gaps in the sequence alignment were considered in calculations based on haplotypes, but the results were similar when gaps were excluded (results not shown). Populations were compared between the two fields to determine if they were differentiated based on physical separation. Total populations were significantly different between the two fields, which were approximately 1.5 km apart ($P < 0.001$ for both S_{nn} and H_{ST}), as were the soil and endophyte populations in each field ($P < 0.001$ for both S_{nn} and H_{ST}). Populations from plots in field 1, all planted with the same tomato cultivar, were compared to determine if populations are also differentiated among locations several meters apart. Based on S_{nn} , endophyte populations for plot 1 were significantly different than for plots 2 and 3, but not in any other pairwise comparison. Soil populations were not significantly differentiated among the plots based on S_{nn} or H_{ST} . Physical separation, therefore, does not appear to be a significant cause of population differentiation on a small scale (meters) but is significant on a larger scale (kilometers).

Populations were compared among plots in field 2, planted with different tomato cultivars, to determine if the host genotype affects *F. oxysporum* populations. Different sequence types predominated in each cultivar, most notably ST 5, composing 72% of the endophyte population from Heinz 3402 compared to 47% in Gem 611, 27% in FL 47, and 21% in Mountain Fresh (Fig. 2). These differences were reflected in the genetic differentiation analyses using H_{ST} , which is based on allele frequency. Endophyte populations from Heinz 3402 were significantly different from

populations in Gem 611 and Mountain Fresh (Table 2). However, based on S_{nn} , which incorporates the genetic distance between alleles, Heinz 3402 was significantly differentiated only from Mountain Fresh. Differences in allele frequency between Heinz 3402 and Gem 611, therefore, may be based on closely related clones. Soil populations were also compared to determine if differences between cultivars are biased by the physical separation among plots. In pairwise comparisons, no soil populations were significantly differentiated based on either S_{nn} or H_{ST} , suggesting that the genetic differentiation observed among endophyte populations was due to the host genotype rather than different *F. oxysporum* populations in different locations in the field.

Populations were also compared between endophyte and soil populations to determine if populations were differentiated based on the environmental niche. The total soil population was differentiated from the total endophyte population for both fields combined and for field 2 based on S_{nn} ($P < 0.001$), but they were not differentiated in field 1. The differentiation observed between the total soil population and the total endophyte population could be biased by the combination of populations from different locations and different tomato cultivars, so soil and endophyte populations were compared for each plot. Endophyte and soil populations were significantly differentiated for plot 5, planted with Gem 611 ($P < 0.001$ for both S_{nn} and H_{ST}), but not for any of the other plots.

A principal-coordinate analysis was performed on the sequence types to look for clustering of isolations from different substrates. The first coordinate explained 56.79% of the variation, the second coordinate explained 22.40% of the variation, and the third coordinate explained 12.49% of the variation. In the plot of the first and second coordinates (Fig. 3), the samples were clustered based on whether they were from field 1 or field 2, but there was no clear pattern of clustering based on plot or substrate, for either soil or plants, although samples from soil tended to be less dispersed in the plot than samples from plants.

Phylogenetic analysis. A statistical parsimony network of the sequence types was inferred, and the sequence types grouped into two networks (Fig. 4). Three loops were present in the larger network, indicating homoplastic relationships or possibly recombination between isolates. Sequence types mainly from soil were frequently closely related to sequence types mainly from plants, although one branch of the tree was composed entirely of endophytic isolates (STs 17, 14, and 15).

A phylogenetic analysis of the tomato-associated sequence types compared to other *F. oxysporum* isolates showed that the isolates were highly diverse and belonged to many different clades within *F. oxysporum* (Fig. 5). While some STs were closely related, no clear pattern was observed based on whether isolates were from plant tissues or soil, and isolates from soil and plants often shared the same sequence type. The two most common STs, 5 and 13, were not closely related. Most STs, including ST 5 and ST 13, were not closely related to isolates known to be pathogenic to tomato, *F. oxysporum* f. sp. *lycopersici* races 1, 2, and 3 and *F. oxysporum* f. sp. *radicis-lycopersici*.

DISCUSSION

The main objectives of this study were to investigate the genetic diversity of *F. oxysporum* populations associated with soil and asymptomatic tomato plants and to look for evidence of specialization to particular environmental niches among nonpathogenic

TABLE 2 Genetic differentiation among *F. oxysporum* subpopulations from different fields, plots, and substrates

Sampling location and comparison ^a	S _{nn}	P	H _{ST}	P
All soil isolates (314) vs. all plants isolates (295)	0.52244^b	<0.001	0.00292	0.039
Field 1 (311) vs. field 2 (298)	0.59486	<0.001	0.05196	<0.001
Soil in field 1 (158) vs. soil in field 2 (156)	0.63467	<0.001	0.07442	<0.001
Plants in field 1 (153) vs. plants in field 2 (142)	0.55456	<0.001	0.03237	<0.001
Field 1				
Soil (158) vs. plants (153)	0.51963	0.011	-0.00064	0.5140
Soil (158) vs. roots (61)	0.61855	0.016	0.00134	0.1930
Soil (158) vs. stems (61)	0.60957	0.1050	-0.00169	0.6130
Roots (61) vs. stems (61)	0.52225	0.0690	0.00364	0.2000
Plot 1, soil (39) vs. plants (38)	0.53453	0.0820	0.00000	0.3740
Plot 2, soil (40) vs. plants (40)	0.48901	0.5250	0.00354	0.2630
Plot 3, soil (39) vs. plants (38)	0.52804	0.0930	0.03640	0.005
Plot 4, soil (40) vs. plants (37)	0.54687	0.037	0.00360	0.2490
By plot (plants only)	0.29965	<0.001	0.04364	0.001
Plot 1 (38) vs. plot 2 (40)	0.61930	<0.001	0.08801	<0.001
Plot 1 (38) vs. plot 3 (38)	0.59271	0.001	0.01693	0.044
Plot 1 (38) vs. plot 4 (37)	0.57874	0.004	0.03091	0.014
Plot 2 (40) vs. plot 3 (38)	0.51912	0.1230	0.03503	0.008
Plot 2 (40) vs. plot 4 (37)	0.49443	0.4640	0.01174	0.1020
Plot 3 (38) vs. plot 4 (37)	0.48607	0.6080	-0.00411	0.6420
By plot (soil only)	0.25322	0.2640	0.02106	0.029
Plot 1 (39) vs. plot 2 (40)	0.51622	0.1570	0.01159	0.1170
Plot 1 (39) vs. plot 3 (39)	0.53279	0.0860	0.05149	0.003
Plot 1 (39) vs. plot 4 (40)	0.47066	0.8000	-0.00825	0.9010
Plot 2 (40) vs. plot 3 (39)	0.48180	0.6470	0.00149	0.2910
Plot 2 (40) vs. plot 4 (40)	0.51827	0.1790	0.00086	0.3270
Plot 3 (39) vs. plot 4 (40)	0.52057	0.1310	0.03139	0.020
Field 2				
Soil (156) vs. plants (142)	0.52643	<0.001	0.01069	0.007
Plot 5 (Gem 611), soil (40) vs. plants (37)	0.62040	<0.001	0.08284	<0.001
Plot 6 (FL 47), soil (38) vs. plants (36)	0.50618	0.2780	-0.00176	0.4900
Plot 7 (Heinz 3402), soil (39) vs. plants (36)	0.48576	0.5740	0.01350	0.0920
Plot 8 (Mountain Fresh), soil (39) vs. plants (33)	0.51499	0.2470	0.01975	0.044
By cultivar (plants only)	0.28595	0.006	0.06590	<0.001
Gem 611 (37) vs. FL 47 (36)	0.50582	0.3120	0.00977	0.1190
Gem 611 (37) vs. Heinz 3402 (36)	0.57121	0.003	0.07524	<0.001
Gem 611 (37) vs. Mountain Fresh (32)	0.47132	0.7830	0.00284	0.3060
FL 47 (36) vs. Heinz 3402 (36)	0.51714	0.1860	0.01999	0.048
FL 47 (36) vs. Mountain Fresh (32)	0.52684	0.1540	0.03992	0.008
Heinz 3402 (36) vs. Mountain Fresh (32)	0.61245	<0.001	0.13219	<0.001
By plot (soil only)	0.26894	0.025	0.01992	0.020
Plot 5 (Gem 611) (40) vs. plot 6 (FL 47) (38)	0.56327	0.008	0.02852	0.020
Plot 5 (Gem 611) (40) vs. plot 7 (Heinz 3402) (39)	0.50211	0.3490	0.00425	0.2150
Plot 5 (Gem 611) (40) vs. plot 8 (Mountain Fresh) (39)	0.55374	0.022	0.04594	0.002
Plot 6 (FL 47) (38) vs. plot 7 (Heinz 3402) (39)	0.49057	0.4670	-0.00354	0.5510
Plot 6 (FL 47) (38) vs. plot 8 (Mountain Fresh) (39)	0.51342	0.2180	-0.00240	0.5500
Plot 7 (Heinz 3402) (39) vs. plot 8 (Mountain Fresh) (39)	0.51798	0.1570	0.00912	0.1120

^a The number of samples from each sampling location is given in parentheses.

^b Statistically significant values are in boldface (Bonferroni-adjusted $P < 0.001$ [0.05/45]).

F. oxysporum. Over 600 isolates were collected from plants and soil samples from two fields, and endophytic *F. oxysporum* was successfully isolated from all plants sampled.

Populations of *F. oxysporum* living saprophytically in soil or nonpathogenically in asymptomatic plant roots have been previously reported to be highly diverse. Most studies of *F. oxysporum* populations used vegetative compatibility groups (VCGs) as a genetic diversity estimator and usually found nonpathogenic popu-

lations to comprise large numbers of VCGs even within one field, with the majority of isolates belonging to single-isolate VCGs (13, 15, 23, 24, 42). Studies using various molecular markers have reported 25 mitochondrial DNA (mtDNA) haplotypes among 197 isolates from soil and asymptomatic melon roots (11), 23 ribosomal IGS-restriction fragment length polymorphism (RFLP) types among 129 isolates from soil and asymptomatic carnation roots (24), and 46 TEF sequence types among 214 *F. oxysporum*

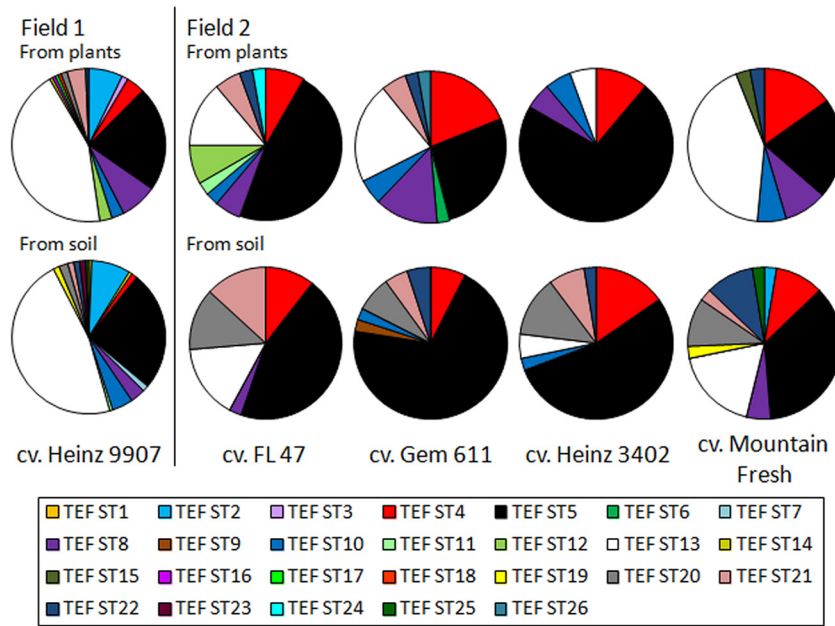


FIG 2 Comparison of sequence type frequencies among populations. The frequency of each sequence type is shown for all plots in field 1 (153 isolates from plants and 158 isolates from soil) and for each cultivar in field 2: FL 47 (36 isolates from plants and 38 isolates from soil), Gem 611 (37 isolates from plants and 40 isolates from soil), Heinz 3402 (36 isolates from plants and 39 isolates from soil), and Mountain Fresh (33 isolates from plants and 39 isolates from soil). Sequence type frequencies were calculated using GENALEX 6 (32).

isolates from soils across Australia (18). Twenty-six TEF sequence types were found in the two tomato fields, suggesting a level of diversity comparable to those in previous studies, given the differences in sampling strategies and markers used.

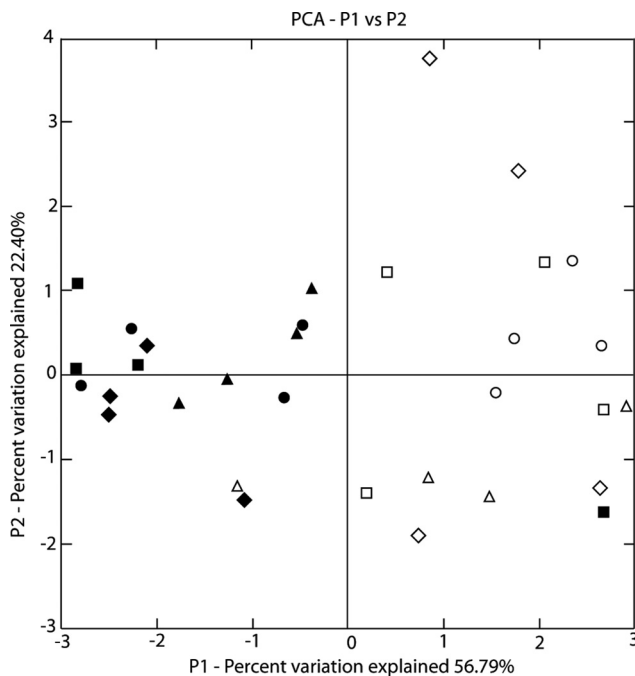


FIG 3 Principal-coordinate analysis (PCA) of sequences from different plots (P) and substrates. The symbols represent samples from different substrates (circles, soil; squares, root; diamonds, crown; triangles, stem). The black symbols represent samples from field 1, and the white symbols represent samples from field 2. Sequences were weighted for abundance, and the analysis was not normalized.

F. oxysporum populations in the two tomato fields for both soil and plant samples were significantly different ($P < 0.001$ for both S_{nn} and H_{ST}) (Table 2), although the two fields were only approximately 1.5 km apart. The populations in the two fields were not entirely isolated, as half of the 26 total STs were found in both fields, but the distribution of sequence types was heterogeneous between the two fields. The populations of *F. oxysporum* in each field might differ due to factors such as management practices, previous planting history, or soil type (21).

F. oxysporum populations were also surprisingly diverse within each plant, with an average of 4.2 STs found per plant, including roots and stems. *F. oxysporum* was isolated from the stems of all plants, a niche that has not been studied for *F. oxysporum*, as previous population genetics studies of nonpathogenic *F. oxysporum* have focused on the roots or crown of plants (11, 12, 13, 15, 23). These isolations from tomato show that endophytic *F. oxysporum* also commonly colonizes plant stems. Within an individual stem, the presence of multiple STs was common, with an average of 2.2 STs per plant. The ability of multiple inoculated *F. oxysporum* genotypes to infect the same plant has been previously reported, such as for two pathogenic isolates in muskmelon (43), two endophytic isolates in carnation (15), and a pathogen and an endophyte in tomato (44) and in carnation (28). In addition, isolation of nonpathogenic *F. oxysporum* from plants also infected with pathogenic *F. oxysporum* is common (for example, see references 25 and 45). Endophytic *F. oxysporum* fungi, therefore, do not necessarily exclude other *F. oxysporum* isolates from colonizing the same plant, and wild plants might commonly harbor a population of *F. oxysporum* endophytes rather than just a single individual.

One unexpected result of this study was that endophytic populations were equally or sometimes more genetically diverse than populations from the surrounding soil (Table 1 and Fig. 1). Our

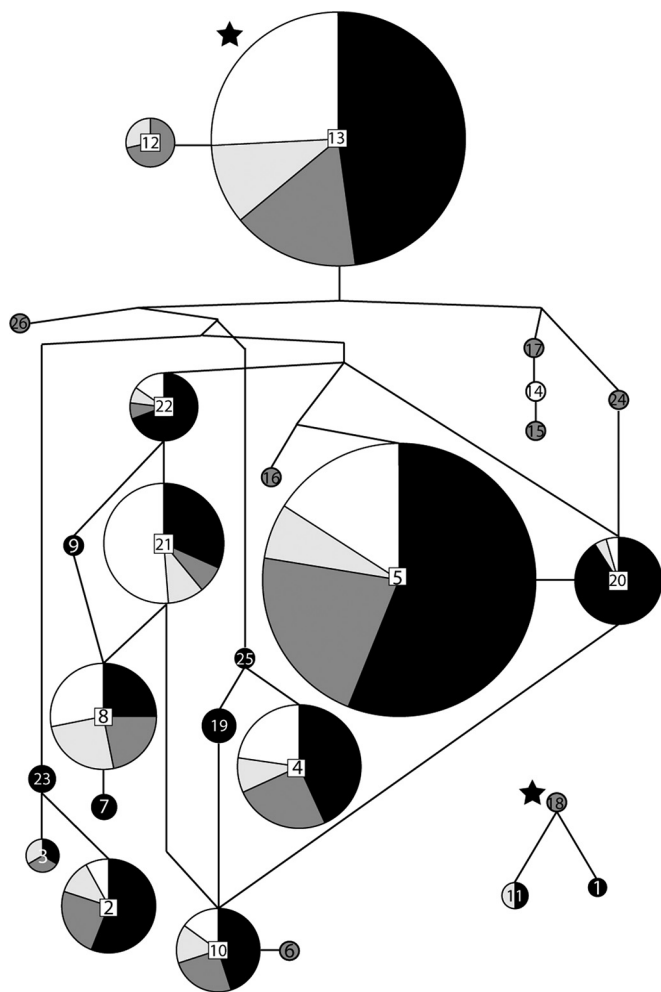


FIG 4 Statistical parsimony network of the translation elongation factor 1 α sequences types, constructed in TCS v. 1.21 (38). Sequence types are represented by circles, with the number given to each sequence type in the center of the circle, and the size of the circle is proportional to the number of isolates with that sequence type. The shading represents the percentage of isolates in each sequence type from different substrates (black, soil; dark gray, roots; light gray, crowns; white, stems). The inferred ancestral sequence types are indicated with stars. Branch lengths are not to scale.

initial hypothesis was that endophytic populations would be less diverse than soil populations if the interaction between the plant and the fungal endophyte is somewhat specific, leading to selection by the plant acting as a filter on the resident fungal soil population. Instead, Shannon's diversity index indicated that overall, populations isolated from plants were significantly more diverse than populations from soil ($P < 0.001$). In individual plots, the levels of diversity were not significantly differentiated, except for plot 6, in which levels of diversity were much higher for the endophyte population ($P < 0.001$). In the principal-coordinate analysis (Fig. 3), isolates from the crown and root are the most widely spread out, while isolates from the soil tend to cluster together. These results might be an artifact of the sampling scheme, as a greater volume of plant material than of soil was collected, but calculations of the population sizes based on the Chao 2 nonparametric richness estimator suggest that the populations were only slightly undersampled. One possible explanation is that STs found

only in the plants could have colonized the plants prior to transplantation, either by seed transmission or during the seedling stage before planting. This hypothesis is supported by the higher degree of genetic differentiation among plots for endophyte populations than for soil populations (Table 2), suggesting that soil populations are relatively uniform across the field and not the source of the unique endophytic genotypes. Given that some *F. oxysporum* isolates obtained from soil have been found to suppress disease caused by pathogenic *F. oxysporum* (46) while other endophytic *F. oxysporum* isolates may be pathogenic to plant species besides the endophyte host (10, 47), the introduction of new genotypes of endophytic *F. oxysporum* conceivably could increase or decrease the incidence of *Fusarium* wilt and other diseases and may have important implications for agriculture.

No definitive evidence was found to support the hypothesis that certain genotypes of *F. oxysporum* are specialized to colonize tomato plants as endophytes. If only specific genotypes can form an endophytic association with tomato, we hypothesized that populations within the plants would be genetically differentiated from populations in the soil. This was true across both fields combined and for field 2 (based on S_{nn} analysis; $P < 0.001$) (Table 2). However, closer analysis did not show consistent differentiation between populations from different parts of the plants (roots and stems) and the soil or between soil and plant populations for each plot within the fields (Table 2). Moreover, diversity was generally not statistically different for populations from plants and populations from soil (Table 1). If only specialized genotypes could colonize the plant, diversity within the plant should be lower than diversity in the soil, as is seen for pathogenic isolates of *F. oxysporum* compared to soil populations (14).

One result that may suggest selection of specialized genotypes within the population is the predominance of two STs, 5 and 13, in all samples. The majority of isolates belonged to ST 5 and ST 13, including isolates from both fields and from both plants and soil. ST 5 and ST 13 may be better adapted to colonizing tomato plants, or perhaps plants in general, than other isolates, leading these STs to prevail in endophytic populations, as was suggested for a population of *F. oxysporum* isolated from asymptomatic celery roots in which most isolates belonged to one of two VCGs (12). The high prevalence of these sequence types in the soil could be due to a selective effect of the plant roots on soil populations, or larger populations in the plant may lead to larger populations in the surrounding soil. Alternatively, the frequent isolation of ST 5 and ST 13 may reflect their abundance in the local *F. oxysporum* soil population and not a selective effect by tomato. Soil was not sampled from fields not cultivated with tomato, so it cannot be determined if STs 5 and 13 are equally common in other local fields in the absence of tomato plants.

Based on haplotype network results, isolates were not necessarily more closely related to other isolates from the same niche than to isolates from other substrates, whether soil or the roots, crown, or stem of the plants (Fig. 4). Likewise, in a phylogenetic analysis of the isolates from tomato compared to *F. oxysporum* isolated from other hosts, genotypes associated with asymptomatic tomato, either from plants or from soil, were not necessarily closely related (Fig. 5). Some sequence types differed by only one single-nucleotide polymorphism and therefore clustered together in the phylogeny, while other sequence types were quite different. These results agree with the findings of a large phylogenetic analysis of *F. oxysporum* (16), in which the included putative nonpathogens

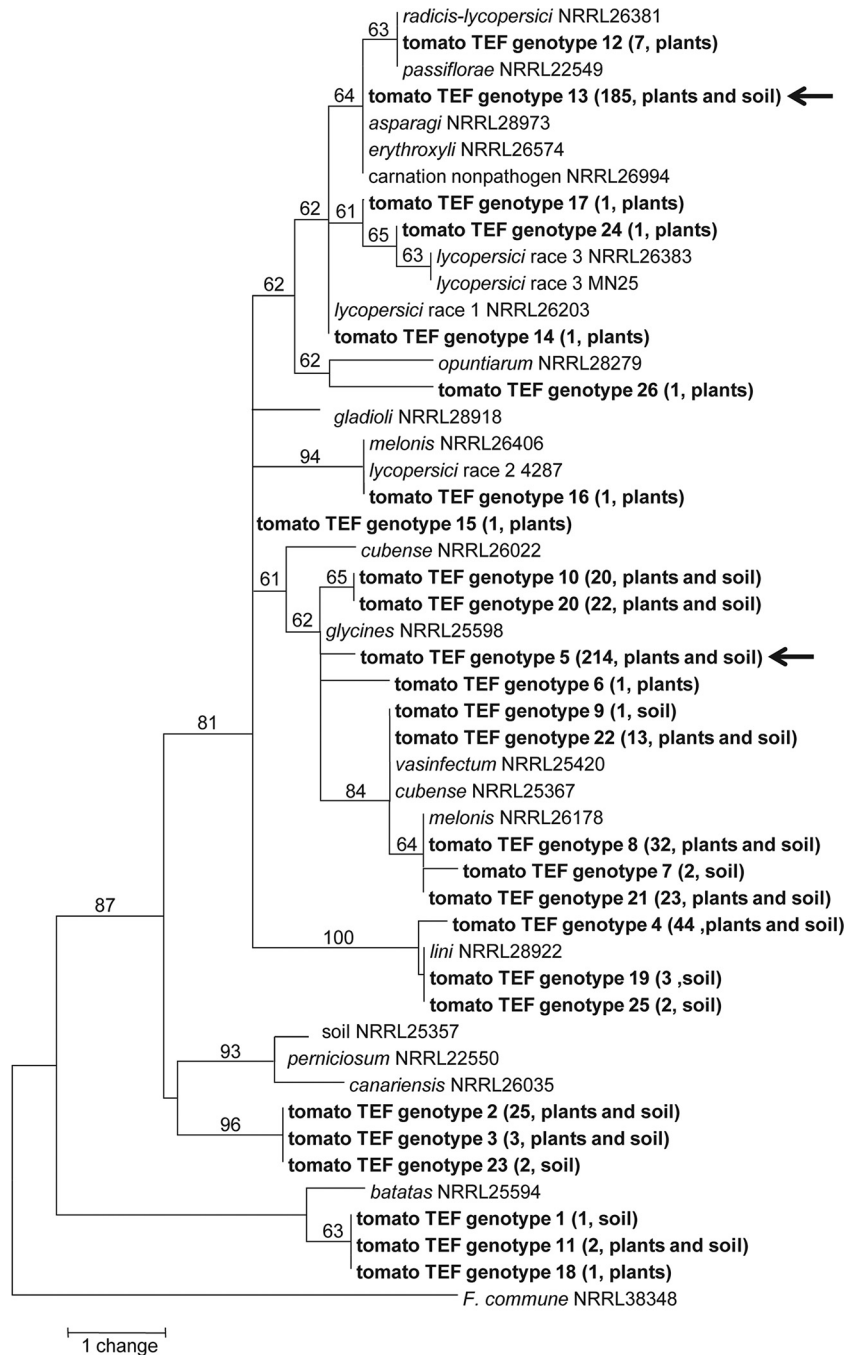


FIG 5 One of two most parsimonious trees based on the translation elongation factor 1 α gene using maximum-parsimony analysis (81 steps; consistency index = 0.926; retention index = 0.968; rescaled consistency index = 0.897). The tree is rooted with *F. commune* NRRL 38348, and bootstrap support of >50% based on 1,000 replicates is indicated for each branch. Sequence types generated in this study from tomato fields are in boldface, with the number of isolates per sequence type and the original substrates (plants or soil) given in parentheses. The arrows indicate the two most abundant sequences types, ST 5 and ST 13.

from several host plants did not belong to any specific lineage within the complex.

The potential effect of the host genotype on the population composition was studied using the samples from field 2, where four different tomato cultivars were sampled. Populations of endophytic isolates were significantly different among cultivars based on H_{ST} ($P < 0.0001$) (Table 2). Isolates from Heinz 3402 in particular were differentiated from those of Gem 611 and Moun-

tain Fresh based on H_{ST} . No clear patterns were observed based on whether the cultivars were bred for fresh market (FL 47 and Mountain Fresh) or for processing (Gem 611 and Heinz 3402). The differentiation of endophyte populations among cultivars suggests that particular host genotypes interact differently with *F. oxysporum* genotypes, leading to different *F. oxysporum* populations within each cultivar. This interpretation is tenuous, however, as differentiation among endophyte populations was also

observed among the plots in field 1, which contained only one cultivar, Heinz 9907, and an uneven distribution of STs in the field could influence the results. However, Gem 611 and Heinz 3402 were planted next to each other and harbored distinct populations, and no significant differentiation was seen among soil populations in field 2, which suggests that differences in endophyte populations are not due to uneven spatial distribution of STs. While the patterns observed are suggestive of the host genotype influencing endophyte populations, firm conclusions cannot be drawn from these results.

Overall, the large amount of diversity observed among these populations highlights the importance of nonpathogenic lifestyles to the biology of *F. oxysporum*. Both soil and endophyte populations were composed of many sequence types, even associated with just one plant. Some results were suggestive of specialization of endophytic populations, but generally, they support the hypothesis that most, if not all, *F. oxysporum* isolates are capable of living as endophytes. These endophytic and soil populations may represent a source of unstudied diversity within *F. oxysporum*. *F. oxysporum* is well known for its variety of agriculturally important characteristics, such as pathogenicity on many plant hosts; production of secondary metabolites, including mycotoxins; and biocontrol activity, and better exploration of endophytic and soil environments may discover even more economically or scientifically relevant traits.

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