Population Genetic Analysis Corroborates Dispersal of *Fusarium oxysporum* f. sp. *radicis-lycopersici* from Florida to Europe

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ABSTRACT

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Fusarium oxysporum isolates from tomato plants displaying crown and root rot symptoms were collected in central and southern Florida and analyzed using vegetative compatibility grouping (VCG) and nuclear restriction fragment length polymorphism (RFLP) data. VCG 0094 of *F. oxysporum* f. sp. *radicis-lycopersici*, previously known only from northwestern Europe, was predominant among 387 isolates assessed. In addition, two newly described VCGs (0098 and 0099) were detected at low frequencies. Floridian VCG 0094 isolates displayed a continuum of compatibilities, which is in contrast to the three distinct subgroups previously

identified among European VCG 0094 isolates. RFLP haplotypes were constructed using one repetitive and three low-copy probes. Population subdivision of VCG 0094 from various Floridian counties and from northwestern Europe (Belgium, the Netherlands, and the United Kingdom) was evaluated by analysis of molecular variance. A "natural" population structure was revealed, differentiating populations from the east and west coasts of Florida. In addition, isolates from Europe were statistically indistinguishable from the Palm Beach County, FL, population. Furthermore, gene diversity among Palm Beach County VCG 0094 isolates was more than five times greater than among European isolates. Results from both VCG and RFLP analyses strongly support the inference that the European VCG 0094 constitutes a founder population that resulted from intercontinental migration of a few isolates from Palm Beach County, FL.

Fusarium oxysporum Schlechtend:Fr. f. sp. *radicis-lycopersici* W.R. Jarvis & Shoemaker is a fungal plant pathogen that causes crown and root rot disease on tomato (14). The disease was first reported in 1969 from Japan (26). In the 1970s, it was present in widely separated geographic locations including Japan (42), Ontario (15), Ohio (9), New Hampshire (30), California (23), and Florida (38). Since then, the pathogen appears to have spread farther, so that it is now common in many tomato-growing areas of the United States, Canada, Europe, Israel, and Japan (19,26).

Even though *F. oxysporum* f. sp. *radicis-lycopersici* is a relatively newly recognized pathogen, considerable genetic diversity has been identified within this forma specialis. Six multi-member vegetative compatibility groupings (VCGs) were identified among 218 isolates obtained from eight countries (19). Two of these VCGs (0090 and 0091) were widely distributed, occurring in North America, Europe, and Israel. VCG 0094 was found only in northwestern Europe (Belgium, France, the Netherlands, and the United Kingdom,) and was dominant in Belgium, the Netherlands, and the United Kingdom (17,19). The remaining three VCGs (0092, 0093, and 0096) were found only in Israel (19).

F. oxysporum f. sp. *radicis-lycopersici* is the first forma specialis of *F. oxysporum* in which subgroupings of VCGs have been reported. Two VCGs (0090 and 0091) could be divided into two subgroups (19), while detailed analysis revealed three subgroups among VCG 0094 isolates from northwestern Europe (17). The assignment of isolates to specific subgroups within a VCG was based on their capacity to form strong heterokaryons with isolates of one subgroup, whereas their interactions with isolates of other subgroups were weak and slow.

In Florida, *Fusarium* crown and root rot of tomato was first reported as early as 1975 near the town of Jupiter in Palm Beach County, FL (38). Even though the disease is widespread in all tomato-growing counties of central and southern Florida, local isolates of *F. oxysporum* f. sp. *radicis-lycopersici* have so far not been examined for VCG.

The objectives of the current study were to determine the genetic diversity among Floridian isolates of the tomato crown and root rot pathogen, initially based on VCG as the criterion for population subdivision. We also developed nuclear restriction fragment length polymorphism (RFLP) markers to test whether the aforementioned diversity or population diversification within this forma specialis (and within VCGs) may also be reflected on a geographic level. To estimate population genetic parameters within single VCGs, we used large sample sizes covering the major tomato-growing counties of central and southern Florida.

This study also is part of a project to develop molecular markers to easily distinguish between *F. oxysporum* f. sp. *radicis-lycopersici* and the tomato wilt pathogen, *F. oxysporum* f. sp. *lycopersici*. Both pathogens occur in most tomato-growing regions, can be present in the same field (U. L. Rosewich, unpublished data), and can cause overlapping symptoms on tomato plants (18). Before diagnostic methodologies can be developed for these pathogens, however, we need to determine the genetic make-up and diversity of the causal organisms in question. A preliminary report of this work has been published (34).

MATERIALS AND METHODS

Fungal collections. *F. oxysporum* samples from tomato plants displaying crown rot symptoms were collected from nine counties located in central and southern Florida (Fig. 1), and fungal cultures were established as follows. After removing the epidermis from crown segments, the vascular tissue was surface-sterilized in

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5% sodium hypochlorite for at least 1 min, followed by rinsing in sterile deionized water. Small pieces of tissue were then plated onto three different media: acidified potato dextrose agar (PDA) (24 g of potato dextrose broth [PDB] [Difco Laboratories, Detroit], 15 g of agar, and 12 drops of 50% lactic acid per liter), $1/4$ -strength PDA (6 g of PDB and 15 g of agar per liter), and water agar (15 g of agar per liter). After 2 to 7 days of incubation at room temperature, resulting colonies were visually and microscopically examined for morphological characteristics typical of *F. oxysporum*. Mycelium was subcultured onto PDA (24 g of PDB and 15 g of agar per liter) containing 100 parts per million of streptomycin and, eventually, a single-spored isolate was retained for each plant. Isolates were labeled according to their Floridian county of origin, using county identifiers followed by serial numbers. Microconidia of each isolate were maintained for long-term storage at -80°C in 50% glycerol.

This study also included 22 isolates of VCG 0094 from north-western Europe (Belgium, the Netherlands, and the United Kingdom) that have been part of earlier studies (17,19).

Isolation of nitrate nonutilizing (*nit*) mutants and complementation tests. *nit* mutants were generated and used in complementation tests as previously described (4,19). Twelve pairs of complementary *nit* mutant testers, representing the six previously established VCGs and subgroups of *F. oxysporum* f. sp. *radicis-lycopersici* (17), were used to determine the compatibility of the new isolates with these VCGs.

Development of RFLP probes. Genomic libraries were constructed using either *EcoRV*- or *HindIII*-digested DNA of two Floridian isolates of *F. oxysporum* f. sp. *radicis-lycopersici* (CL-5 and CL-19) cloned into Bluescript KS or pUC118 using standard technology (37) with modifications (35). Recombinant plasmids were used to transform *Escherichia coli* DH5 α MCR (Life Technologies, Inc., Gaithersburg, MD). Plasmid preparations of resulting clones were obtained by the lysis-by-boiling method (37). Plasmid-bearing *E. coli* was kept in glycerol at -80°C for long-term storage.

We used 15 *F. oxysporum* isolates in the screening to identify polymorphic RFLP probes. Fourteen of these isolates originated from tomato and included representatives of *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici*, in addition to a nonpathogenic *F. oxysporum* from tomato. Also, one isolate

from banana (*F. oxysporum* f. sp. *cubense* strain S1) was included in the screening. Together, the 15 isolates encompassed the three major clades of the *F. oxysporum* complex (31). The DNA of each isolate was digested separately with *HindIII* and *EcoRV*, and Southern blots were prepared as described below. All probe-enzyme combinations were examined for RFLPs and for the number of sites to which these probes hybridized.

Sequencing of certain DNA probes was done at the DNA Sequencing Core Laboratory at the University of Florida, Gainesville. Sequences were analyzed by the sequence similarity search tool BLAST 2.0 (Gapped BLAST) (1) provided by the National Center for Biotechnology Information.

Electrophoretic karyotyping. Fungal germlings from 5×10^8 conidia were generated as previously reported (2). Germlings were protoplasted by suspending in 20 ml of sulfate-citrate solution (1.2 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 50 mM sodium citrate $\cdot 2\text{H}_2\text{O}$, pH 5.8) in the presence of 0.1 g of Glucanex (batch no. 14102011; Novo Nordisk Ferment AG, Basel, Switzerland) and then gentle rocking for 2 to 3 h until most cells were protoplasts. The protoplasts were filtered through cheesecloth into two 15-ml tubes and centrifuged at $900 \times g$ for 20 min. The protoplasts, which were a brownish layer on the top of the sulfate-citrate solution, were carefully removed using a large bore pipette and transferred into a 50-ml tube. Twenty milliliters of a solution consisting of 0.8 M sorbitol, 100 mM NaCl, and 10 mM Tris, pH 7.4, was added to the protoplasts and the tubes were centrifuged at $800 \times g$ for 10 min. The supernatant was removed with a sterile pipette and the protoplast pellet resuspended very gently in 0.5 ml of a solution consisting of 0.8 M sorbitol and 50 mM EDTA, pH 8.0. Protoplast concentration was determined with a hemacytometer and adjusted to 10^9 per ml. The preparation of agarose-embedded protoplasts for pulsed-field gel electrophoresis followed preestablished protocols (2,32).

Chromosome separation was performed by contour-clamped homogeneous electric field electrophoresis (3) as previously reported (2). Small chromosomes (<3 million base pairs [Mb]) were separated using 0.8% FastLane agarose gels (FMC BioProducts, Rockland, ME). Electrophoresis was at 4°C at 80 V with a ramped switch time of 40 to 800 s for 96 h. Larger chromosomes (>3 Mb) were separated on 0.6% agarose gels at 40 V with a ramped switch time of 1.4 to 5.8×10^3 s for 240 h. Southern hybridizations of chromosomal DNA were done in the same manner as for restriction enzyme-digested total DNA (discussed below).

Generation of RFLP data. Microconidia from long-term storage were streaked onto PDA plates and grown for several days at room temperature. Mycelium was scraped off and transferred to petri plates (15 cm diameter) containing approximately 70 ml of PDB. After 7 days of growth in still culture at room temperature, the resulting mycelium was harvested by filtration through cheesecloth, rinsed with sterile deionized water, blotted dry, and placed into 15-ml polypropylene tubes. The mycelium was kept frozen at -80°C and lyophilized for at least 48 h before DNA extraction.

Up to 0.2 g of freeze-dried mycelium was pulverized and 7 ml of DNA extraction buffer (22) was added. The tubes were vortexed for 1 min and kept in a 65°C waterbath for 60 min. A standard chloroform extraction followed. To the aqueous phase, 200 μl of NaCl-cetyltrimethylammonium bromide (CTAB) (10% CTAB and 0.7 M NaCl) and 2 ml of CTAB precipitation buffer (1% CTAB; 50 mM Tris, pH 8; and 10 mM EDTA, pH 8) were added. Tubes were inverted 10 to 20 times and incubated at room temperature for 20 min before centrifugation at $14,000 \times g$ at 21°C in a refrigerated Sorvall RC-5B centrifuge (Sorvall Instruments, Kendro Laboratory Products, Newtown, CT). The DNA pellet was resuspended in 7 ml of NaCl-Tris-EDTA (TE) buffer (1 M NaCl; 10 mM Tris, pH 8; and 1 mM EDTA, pH 8), followed by a second chloroform extraction. The DNA in the aqueous phase was precipitated by adding 7 ml of isopropanol and then pelleted at $14,000 \times g$ for 20 min at 21°C . The DNA was resuspended in 400 μl of TE (10 mM Tris and 1 mM EDTA, pH 8.0), transferred to a microfuge tube, and precipitated by

Fig. 1. Map of central and southern Florida depicting counties from which tomato plants showing typical crown rot symptoms were sampled. The counties also represent the main tomato-growing areas in central and southern Florida (CL = Collier, DA = Dade, DE = De Soto, HE = Hendry, LE = Lee, MN = Manatee, MA = Martin, PB = Palm Beach, and SL = St. Lucie).

adding 0.5 volume of 7.5 M ammonium acetate and two volumes of 95% ethanol. Tubes were gently inverted 15 to 20 times and kept at room temperature for 20 min before centrifugation at $11,000 \times g$ in a microfuge. The DNA pellet was resuspended in 50 to 100 μ l of TE and allowed to dissolve at 4°C for several days before DNA concentration was determined using a TKO 100 fluorometer (Hoefel Scientific Instruments, San Francisco).

A total of 5 μ g of DNA of each isolate was digested with *EcoRV* at 37°C overnight. Generation of RFLP data (i.e., restriction fragment separation by electrophoresis, transfer of DNA to nylon membranes, hybridization, washing, developing, and stripping procedures) was performed as described previously (35), except that hybridization and washes were carried out at 65°C for high stringency.

RFLP data analysis. Restriction fragments revealed by each probe were considered distinct loci and their presence and absence were scored as “1” and “0,” respectively. This information was used to generate RFLP haplotypes.

All RFLP haplotypes were analyzed using the Arlequin population genetics data analysis package, version 1.1 (Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland). Depending on the analysis, genetic data were arranged into “populations” and analyzed according to (i) VCG affiliation alone or (ii) VCG affiliation and geographic source. Analyses used both clone-corrected (whereby each haplotype was counted only once) and uncorrected data.

Intrapopulation level analyses determined molecular diversity indices. The mean number of pairwise differences between RFLP haplotypes and its standard deviation was calculated according to Tajima (40). Average nucleotide diversity, including its standard deviation within each group, was calculated according to Nei (29) and Tajima (39).

Interpopulation level analyses tested for the presence of population structure using analysis of molecular variance (AMOVA) (8). AMOVA produces estimates of variance components and *F* statistic analogues (Φ statistics), reflecting the correlation of diversity at different levels of hierarchical subdivision. AMOVA was performed for various subsets of total data gathered. We also used AMOVA to determine extant populations by sequentially consolidating the genetically most-similar geographic groups, which were determined by pairwise Φ_{ST} (analogous to F_{ST}) comparisons (8). This was done until “among-population diversity” (and Φ_{ST}) was at maximum.

RESULTS

VCG. In total, 387 *F. oxysporum* isolates from symptomatic tomato plants from central and southern Florida were evaluated for VCG.

Out of 12 pairs of testers representing the six established VCGs and subgroups of *F. oxysporum* f. sp. *radicis-lycopersici* (17,19), only those of VCG 0094 were compatible with isolates from Florida.

Initially, a sample of 73 isolates from Collier County was used for vegetative compatibility tests. *nit* mutants were generated from each isolate and paired with tester strains. Based on strong complementation with VCG 0094 testers 01150-6 and 01152-31, either alone or together (19), 28 isolates were assigned to this VCG. Sixteen isolates, whose *nit* mutants reacted weakly or slowly with these testers, were further examined for compatibility with two additional testers of VCG 0094. The new testers (D-69/7 and D-69/19) have recently been chosen to represent a subgroup II of *F. oxysporum* f. sp. *radicis-lycopersici* (17). In addition, *nit* mutants of the 16 isolates were paired among themselves in various interisolate combinations to determine vegetative compatibility. For some isolates, strong complementation with VCG 0094 II testers confirmed them as belonging to VCG 0094, whereas some other isolates showed weak or uncertain reactions. On the other hand, strong complementation was evident in some combinations of the 16 isolates, suggesting the existence of a new, fourth subgroup (designated

VCG 0094 IV) in addition to the previously found three (17). Based on their ability to form strong heterokaryons with many mutants, two complementary *nit* mutants were chosen as testers of VCG 0094 IV, CL-2/2 (parental isolate CL-2) and CL-41/16 (parental isolate CL-41).

Complementary *nit* mutants from each of the 29 isolates from the initial Collier County sample not assigned to VCG 0094 were paired among themselves in various interisolate combinations to determine vegetative compatibility. Ten isolates were compatible with one another, thus constituting a new VCG. Two complementary *nit* mutants, CL-7/6 (parental isolate CL-7) and CL-75/4 (parental isolate CL-75), were chosen as testers of this new group, which was designated VCG 0098 according to the VCG numbering system of *F. oxysporum* (16,20,33). The new tester pairs of VCGs 0094 IV and 0098 were added to the set of testers used to determine vegetative compatibility of isolates of *F. oxysporum* f. sp. *radicis-lycopersici* from Florida.

Out of 69 isolates from Manatee County, 11 were assigned to VCG 0094 and none was compatible with VCG 0098. Of the remaining isolates, 38 were compatible with one another, forming a distinct VCG. Tester isolates of this distinct group (DA-1/7 from parent DA-1 and MN-27/9 from parent MN-27) were vegetatively compatible with two isolates (MM-2 from Arkansas and DC-2 from Florida) of the tomato wilt pathogen (*F. oxysporum* f. sp. *lycopersici*) VCG 0033 (25). Consequently, the isolates of the group were assigned to *F. oxysporum* f. sp. *lycopersici* VCG 0033, despite their apparent association with crown rot symptoms.

Based on complementation with the different testers, about 180 additional isolates from Florida were assigned to VCGs 0094, 0098, or 0033. RFLP haplotype similarity (discussed below) among 13 of the remaining, nonassigned isolates suggested a common clonal origin. Interisolate complementation subsequently confirmed them as belonging to a new distinct group, designated VCG 0099. Table 1 summarizes the frequencies of the different VCGs in nine Floridian counties. Pathogenicity and incitement of crown rot symptoms have been confirmed for all three VCGs for a subset of isolates used in this study (12,19).

Some interisolate pairings between 87 nonassigned isolates revealed several minor groups, each comprising two to four vegetatively compatible isolates. More testing (including pathogenicity testing) will be required before VCG code numbers can be given to these apparently new groups (16,20).

Isolates of VCG 0094 varied considerably in their tendency to form complementary heterokaryons with one another, as well as

TABLE 1. Frequency of vegetative compatibility groupings (VCGs) in *Fusarium oxysporum* from tomato displaying crown rot symptoms from central and southern Florida^a

County	Number of isolates ^b	VCG			
		FORL 0094 ^c	FORL 0098 ^c	FORL 0099 ^c	FOL 0033 ^d
Collier	84 (104)	0.753	0.185	0.062	0
Lee	12 (20)	0.417	0.333	0.250	0
Dade	7 (8)	0.714	0	0	0.286
De Soto	4 (5)	0.500	0.250	0.250	0
Hendry	7 (9)	1.000	0	0	0
Palm Beach	102 (115)	0.902	0.078	0.020	0
Martin	33 (46)	0.727	0.061	0.061	0.152
St. Lucie	5 (11)	0.800	0	0	0.200
Manatee	49 (69)	0.234	0	0	0.766
Total	300 (387)	0.703 (211)	0.100 (30)	0.043 (13)	0.153 (46)

^a A complete list of all isolates used in this study is available on request from the corresponding author.

^b Number of isolates that could be assigned to a VCG out of the total (in parentheses).

^c Identified VCGs of *F. oxysporum* f. sp. *radicis-lycopersici*.

^d *F. oxysporum* f. sp. *lycopersici*; vegetative compatibility among isolates within the Floridian collection was demonstrated; representative isolates were vegetatively compatible with isolates DC-2 and MM-2 of VCG 0033 (25).

with the testers representing three subgroups of this VCG (subgroups I and II from Europe and subgroup IV from Florida). Many isolates formed strong heterokaryons with the testers of only one subgroup, while their interaction with the other testers was weak, slow, uncertain, or negative. On the other hand, a number of isolates formed moderate or strong heterokaryons with the testers of two or three subgroups and even with those of a fourth one (subgroup III from Europe, which only recently became available and could not be widely used in the current study). The selection of subgroup IV testers and their use were essential for assigning VCG 0094 to many Floridian isolates that were weakly compatible with the European testers. However, some isolates still remained that showed weak, uncertain, or negative interaction with subgroup IV testers as well. Grouping of such isolates was eventually inferred from their positive interactions with other, nontester strains of VCG 0094. Weak, slow, and uncertain interactions have been described in detail for intersubgroup combinations in other VCGs of *F. oxysporum* f. sp. *radicis-lycopersici* (19). The quantitative variation in the growth of complementary heterokaryons of VCG 0094 spanned a wide range including strong, moderate, weak, and no growth. The same range of variation was observed whether isolates were paired with each other or with the testers, but the frequency of strong complementation was higher in pairings with the testers, as expected. Interestingly, isolates that formed strong heterokaryons with all the testers did not necessarily do the same with other isolates of VCG 0094.

Varying interaction was likewise observed among the 30 isolates of VCG 0098. Nevertheless, 27 of them could be assigned to this VCG by strong or moderate interaction with its testers; the remaining three isolates exhibited weak, uncertain, or no interaction with the testers, and their assignment to VCG 0098 was inferred from strong interaction with other isolates from this VCG.

VCG 0099 was considerably more variable. Its low frequency enabled us to compare the interactions among all 13 isolates, using two to five *nit* mutants per isolate and scoring the complementation as strong, moderate, weak, or absent. Only one isolate, LE-20, displayed strong or moderate complementation with all other isolates, while CL-52 was compatible with 10 of them. Each of the remaining isolates was compatible with only six to nine isolates. Overall, 33% (26 out of 78) isolate combinations indicated weak, uncertain, or no compatibility.

Development of RFLP probes. During the screening, 80 clones with single inserts were probed to *EcoRV*- and *HindIII*-digested DNA of 15 *F. oxysporum* isolates. Of the 80 probes tested, 18 (22.5%) defined monomorphic loci for all isolates. Additional results of the screening are summarized in Table 2.

TABLE 2. Restriction fragment length polymorphism (RFLP) patterns of various *Fusarium oxysporum* strains^a

RFLP pattern ^b	FOC S1 ^c	FO HE-5 ^d	FOL 0030 ^e	FOL 0033 ^f	FORL 0099 ^g
No hybridization	13	11	3	5	1
Same banding pattern	30	34	36	43	59
Different banding pattern	37	35	41	32	20

^a Strains probed to 80 single-insert clones from two genomic libraries of two random isolates of *F. oxysporum* f. sp. *radicis-lycopersici* vegetative compatibility grouping (VCG) 0094. The *F. oxysporum* strains were arranged from left to right according to increased phylogenetic relationship to *F. oxysporum* f. sp. *radicis-lycopersici* (31).

^b The RFLP patterns of the various strains of *F. oxysporum* were compared with patterns of eight isolates of *F. oxysporum* f. sp. *radicis-lycopersici* VCGs 0094 and 0098.

^c *F. oxysporum* f. sp. *cubense* VCG 0126 isolate S1 (22).

^d Nonpathogenic *F. oxysporum* HE-5 isolated from tomato crown tissue, Hendry County, FL.

^e *F. oxysporum* f. sp. *lycopersici* VCG 0030 isolate 5397 (25), Bradenton, FL.

^f Three isolates of *F. oxysporum* f. sp. *lycopersici* VCG 0033 from Florida and Georgia.

^g *F. oxysporum* f. sp. *radicis-lycopersici* CL-58 VCG 0099.

Twenty probes differentiated between the phylogenetically indistinguishable VCGs 0094 and 0098 on one hand and the phylogenetically closely related VCG 0099 on the other (31). These probes were evaluated further for their usefulness in RFLP analysis. Five of these twenty polymorphic probes were repetitive (10 or more bands). We chose one of them (pRL 82.8) (Fig. 2A) to be included in the analysis as a DNA fingerprinting probe. An unfiltered BLASTX search of pRL 82.8 determined a moderate sequence homology to the reverse transcriptases from *Magnaporthe grisea* MGR583 (41) with an E value of 8e-11 and from *Colletotrichum gloeosporioides* CgT1 (13) with an expect (E) value of 8e-09. In addition to the repetitive probe pRL 82.8, we identified three low-copy probes that were also suitable for population genetics analysis (pRLs 32 [Fig. 2B], 59, and 85), as they identified easily scorable RFLPs within all three Floridian VCGs of *F. oxysporum* f. sp. *radicis-lycopersici*. pRL 32 represents an *EcoRV* fragment, while the other three probes (pRLs 59, 82.8, and 85) were *HindIII* fragments.

On close inspection of the 20 probes that differentiated between VCGs 0094 and 0099, we noticed that hybridization of DNA digested with the same restriction enzyme as was used for DNA cloning often resulted in hybridization to more than one band. Exclud-

Fig. 2. Examples of restriction fragment length polymorphisms for A, a DNA fingerprinting probe (pRL82.8) and B, a low-copy probe (pRL 32) among *Fusarium oxysporum* f. sp. *radicis-lycopersici* isolates collected from one tomato field in Palm Beach County, FL. Isolates in lanes 6 and 18 belong to vegetative compatibility grouping (VCG) 0098; isolates in lanes 3, 5, and 8 are nonassigned isolates. The remaining isolates belong to VCG 0094.

ing the five clearly repetitive probes, the 15 polymorphic probes hybridized to 3.07 loci per probe ($46/15 = 3.067$) (minimum number of bands for the same enzyme-digested DNA of six isolates of VCG 0094). In comparison, all other (monomorphic) probes taken together hybridized, on average, to only 1.13 bands (69 bands per 61 probes).

Electrophoretic karyotyping and chromosomal distribution of polymorphic probes. Agarose-embedded protoplasts were prepared for 49 isolates of *F. oxysporum* f. sp. *radicis-lycopersici* from VCGs 0094, 0098, and 0099. A selection of isolates from all three VCGs contained seven chromosome-size DNA bands ranging in size from 2.5 to 6.5 Mb. In this size range, no polymorphism could be detected among the chromosomes of the isolates examined (data not shown). In addition, 47 of the 49 isolates examined had three “minichromosomes,” small chromosomes predominantly between 0.85 to 1.5 Mb, while the remaining two isolates had four minichromosomes. Thirty-seven of the forty-nine isolates (76%), including representatives of all three VCGs, had an identical minichromosome pattern (Fig. 3A).

Fig. 3. A, Minichromosomes of isolates from three vegetative compatibility groupings (VCGs) of *Fusarium oxysporum* f. sp. *radicis-lycopersici*. The most common chromosome pattern is demonstrated for the example of VCG 0099 and the last examples of VCGs 0094 and 0098. **B,** Chromosomal distribution of the low-copy probe pRL 32.

The four polymorphic probes used in this study were hybridized to Southern blots of chromosome-size DNAs of selected isolates of *F. oxysporum* f. sp. *radicis-lycopersici* from VCGs 0094, 0098, and 0099 to determine their chromosomal distribution. Clones pRL 32 and pRL 85 hybridized exclusively to the three minichromosomes (between 0.85 to 1.5 Mb) (Fig. 3B). pRL 59 hybridized to two chromosomes: 6 (3.5 Mb) and 9 (5.7 Mb). pRL 82.8 showed strongest hybridization to minichromosomes, but also hybridized weakly to chromosomes 5 (3.0 Mb) and 6 (3.5 Mb).

RFLP data analysis. The four single-insert polymorphic probes (pRLs 32, 59, 82.8, and 85) were used to generate RFLP haplotypes for the *EcoRV*-digested DNA of all isolates including isolates that could not be grouped into a VCG. Restriction fragments revealed by each probe were considered distinct RFLP loci and were scored as present (“1”) or absent (“0”). Each unique combination of 1s and 0s at 121 loci constituted a different RFLP haplotype.

The presence of population subdivision was tested using AMOVA. For initial analysis, all isolates classified into VCG 0094 were arranged according to geographic source (individual Floridian county or Europe). A moderate level of population subdivision between the entities was found ($\Phi_{ST} = 0.139$) that was highly significant ($P < 0.001$). To elucidate the “natural” population structure, we then sequentially consolidated the two geographic populations that displayed the lowest pairwise Φ_{ST} values until we reached maximum population differentiation. By doing so, we increased an initial Φ_{ST} value from 0.134 (between “county populations”) to 0.171 (between “natural populations”). The pairwise Φ_{ST} values between the “natural” populations of *F. oxysporum* f. sp. *radicis-lycopersici* VCG 0094 are presented in Table 3.

Because of the apparent dominance of VCG 0094 in Florida and in northwestern Europe, we examined the relationship between the Floridian and European populations and their origins. Three simple hypotheses could explain the presence of VCG 0094 on different continents. First is that VCG 0094 originated in Florida or in some as-yet-unknown ancestral population and then was transported to Europe. The second is that VCG 0094 originated in Europe or in an ancestral population and then was transported to Florida. Finally, it is possible that VCG 0094 arose in an ancestral population and then was transported independently to Florida and Europe. To distinguish the three hypotheses, intra- and interpopulation comparisons were made. To distinguish newer populations, measures of intrapopulation diversity (mean number of pairwise differences among RFLP haplotypes and gene diversity) were used. The basic assumption is that newly established populations exhibit lower levels of molecular diversity due to a founder effect and the limited time to accumulate mutations. To determine the origin of the populations, interpopulation comparisons (by AMOVA) were used; haplotypes within newly established populations might be expected to be similar to haplotypes from which the new population arose. On the other hand, populations arising from independent draws from a relatively large, unsampled ancestral population likely would exhibit a greater degree of population subdivision.

Molecular diversity indices (mean number of pairwise differences and average gene diversity) were compared for the Floridian and European populations of VCG 0094 (Table 4). The European

TABLE 3. Pairwise Φ_{ST} values (above diagonal) and their P value (below diagonal) between natural populations of *Fusarium oxysporum* f. sp. *radicis-lycopersici* VCG 0094 from Florida and Europe

	West coast ^a	East coast ^b + Europe ^c	St. Lucie County
West coast ($n = 89$)		0.155	0.279
East coast + Europe ($n = 127$)	<0.001		0.385
St. Lucie County ($n = 4$)	0.003	<0.001	

^a West coast counties: Collier, Dade, De Soto, Hendry, Lee, and Manatee.
^b East coast counties: Martin and Palm Beach.
^c Europe: Belgium, the Netherlands, and the United Kingdom.

population exhibited five times less diversity than the Floridian population using either diversity assessment. When each haplotype found in Florida was sampled only once (clone-corrected data), diversity levels for the European population were approximately seven times less than the Floridian population. To evaluate the relationship between the European and Floridian populations further, pairwise Φ_{ST} values between populations of VCG 0094 from Europe and each Floridian county were calculated (Table 5). Population subdivision was moderately high for all comparisons except Europe versus Palm Beach County; in the latter comparison, the Φ_{ST} value was not significantly different from zero.

DISCUSSION

We have characterized isolates of *F. oxysporum* f. sp. *radicis-lycopersici* from the central and southern tomato-growing regions of Florida by vegetative compatibility and nuclear RFLPs. The 254 isolates of *F. oxysporum* f. sp. *radicis-lycopersici* that could be classified grouped into three VCGs. None of them corresponded to the widespread VCGs 0090 and 0091, previously reported to be present in North America (19). More than 80% (211 out of 254) of these isolates belonged to VCG 0094, the remainder grouped into the smaller, new VCGs 0098 and 0099.

VCG 0094 was first described for isolates from Belgium (19) and was later found to be present in France, the Netherlands, and the United Kingdom (17). In these northwestern European countries, VCG 0094 was dominant, encompassing 70% of the isolates examined (17). The European isolates of VCG 0094 have been further divided into three subgroups; isolates within each subgroup readily formed complementary heterokaryons with one another, whereas heterokaryosis was considerably less frequent and complementation weaker or absent between isolates of different subgroups. Although weak heterokaryotic growth can sometimes be mutant dependent, its consistent occurrence in interisolate pairings between otherwise strongly complementing mutants suggested a reduced level of compatibility between isolates of different subgroups.

Unlike the distinct organization of European VCG 0094 into three subgroups, the interpretation of vegetative compatibilities of Floridian VCG 0094 isolates was more complicated, as they displayed a large variation in interisolate compatibility. For example, isolates CL-68 and CL-90 were not compatible with each other, but both were strongly compatible with isolate PB-23 and, hence, not separable into different subgroups. The relationships between VCG 0094 isolates in Florida can be described as a continuum or array of varying degrees of compatibility across the population. Dis-

tantly related isolates were considered members of the same VCG, essentially by their positive interaction with the testers and other strongly complementing strains that served as bridges within VCG 0094, either alone or together. Furthermore, two weakly compatible isolates from France, which could not be subgrouped in a previous study (17), were eventually confirmed as members of VCG 0094 by strongly interacting with the testers of subgroup IV from Florida.

In addition to VCG, we characterized isolates of *F. oxysporum* f. sp. *radicis-lycopersici* from Florida and Europe using nuclear RFLPs. Whereas most genetic diversity studies of *F. oxysporum* have focused on polymorphisms that occur among strains of different VCGs and formae speciales (10,22,24,27), fewer studies have been successful in elucidating some population genetic processes within individual VCGs of *F. oxysporum* (11,28). Extensive screening of two genomic libraries generated from two isolates of *F. oxysporum* f. sp. *radicis-lycopersici* led to the identification of 20 polymorphic sequences that differentiated between isolates of the three phylogenetically closely related Floridian VCGs. Among these 20 polymorphic probes, 5 were repetitive and 15 were low copy. Further examination of three low-copy sequences revealed that each of them hybridized to at least two chromosomal-sized DNA bands including minichromosomes. In contrast, monomorphic probes were, with few exceptions, single copy. A reasonable explanation for these observations is that the polymorphisms accumulated due to duplication, a previously described phenomenon in *F. oxysporum* (21). Therefore, we deemed it appropriate to score their presence and absence at specific RFLP loci rather than to simply assign alleles to the different RFLP patterns.

DNA sequence analysis of the repetitive pRL 82.8 clone that was useful for generating DNA fingerprints revealed a moderate sequence homology with genes coding for reverse transcriptases from *M. grisea* MGR583 (37) and *C. gloeosporioides* CgT1 (10). pRL82.8, therefore, may be part of a non-long-terminal-repeat class 1 retroelement (5).

The current set of one repetitive and three low-copy RFLP probes has enabled us to elucidate the population genetics of *F. oxysporum* f. sp. *radicis-lycopersici* from Florida and Europe. Initially, we were interested in determining whether we could detect subdivision between geographic populations of the prevalent VCG 0094 (arranged according to Floridian county or Europe). This analysis was possible due to the large amount of polymorphisms identified among Floridian VCG 0094 isolates. In fact, we were able to differentiate 142 different haplotypes among 198 isolates.

By sequentially pooling the geographic populations with the lowest Φ_{ST} values, we increased genetic differentiation between groups.

TABLE 4. Mean number of pairwise differences and average nucleotide diversity of restriction fragment length polymorphism (RFLP) haplotypes for isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* from Florida and Europe

VCG ^a	Locality	Sample size	Polymorphic loci ^b	Mean number of pairwise differences and SD ^c	Nucleotide diversity and SD ^d
0094	Europe	22	9	0.983 (0.692)	0.008 (0.006)
	Total Florida	198	92	5.476 (2.646)	0.045 (0.024)
	Total Florida clone-corrected	142	92	6.890 (3.261)	0.057 (0.029)
	Palm Beach County	86	73	5.141 (2.516)	0.042 (0.023)
	Collier County	60	60	5.100 (2.508)	0.042 (0.023)
	Martin County	19	16	3.275 (1.763)	0.027 (0.016)
	Manatee County	11	16	3.236 (1.806)	0.027 (0.017)
	Hendry County	7	7	8.381 (4.421)	0.069 (0.042)
	Dade County	5	8	3.400 (2.085)	0.028 (0.020)
	Lee County	5	15	7.200 (4.067)	0.060 (0.039)
	St. Lucie County	4	9	4.500 (2.793)	0.037 (0.028)
	DeSoto County	1	0	Not applicable	Not applicable
	Total Florida	22	21	3.632 (1.913)	0.030 (0.017)
	Total Florida clone-corrected	15	21	4.686 (2.431)	0.039 (0.023)
0099	Total Florida	13	45	10.989 (5.318)	0.091 (0.049)

^a Vegetative compatibility grouping.

^b Among a total of 121 RFLP loci.

^c Mean number of pairwise differences and standard deviation (SD) (in parentheses) according to Tajima (40).

^d Average nucleotide diversity and SD (in parentheses) according to Nei (29) and Tajima (39).

Among VCG 0094 isolates, we found a natural division for isolates from the west and east coasts of Florida. The only county population that could not be consolidated was St. Lucie County, whose isolates displayed very distinct haplotypes. As this study considered only four isolates from this Floridian county, it is still premature to speculate whether St. Lucie County VCG 0094 isolates indeed represent a distinct population.

The division between east and west coast populations raises some interesting issues. First, the moderate level of population subdivision suggests that there is no current immigration into Florida and into its individual counties from a general source, e.g., by infected seed (26) that might be expected to homogenize these populations. Second, the data also indicate that migration within Florida (i.e., between counties) may be common, but probably spatially limited. *F. oxysporum* f. sp. *radicis-lycopersici* forms conidia on the surface of diseased tomato stems, and it has been demonstrated that these conidia can become airborne (36). Although it is unknown how far they can travel, the data may indicate that airborne conidia contribute to county-to-county dispersal.

The RFLP data also has provided an indication of the origin of VCG 0094. Floridian isolates of VCG 0094 displayed much higher gene diversity than isolates collected from three countries in Europe (Belgium, the Netherlands, and the United Kingdom). Sample sizes for European and Floridian VCG 0094 populations differed ($n = 22$ and 198, respectively), but Table 4 illustrates that differences in diversity indices cannot be attributed to differences in sample size. Diversity indices for individual county populations of VCG 0094 were at least three times higher than for the European isolates, even though we had a smaller sample size for six Floridian counties than for Europe. In addition, populations of VCGs 0098 and 0099, which were also represented by a low sample size ($n = 22$ and 14, respectively), displayed 4 to 11 times higher diversity indices, as measured by mean number of pairwise differences and average nucleotide diversity. Furthermore, analysis of population subdivision data (pairwise Φ_{ST}) between Floridian populations arranged according to county and isolates from Europe indicated that the European population was quite similar, with a very low Φ_{ST} value, to isolates from Palm Beach County. Along with the smaller molecular diversity values for the European population, we postulate that a founder population from Palm Beach County established the northwestern European population. Coincidentally, Palm Beach County was also the first location in Florida where crown rot was reported (38).

Detailed analysis of vegetative compatibilities also indicated that VCG 0094 in Florida and Europe differed from each other in their internal structure and that this difference can be a clue to the micro-evolutionary relatedness between these geographic populations. A secondary population (European isolates) derived from only a few founder strains is expected to be composed of a small number of relatively homogeneous clones (in this case, subgroups) as compatible with one another as their parental strains had been. On the other hand, the range of compatibilities shown to exist in the original (Floridian) population apparently reflects differences accumulated in the course of VCG 0094 evolution in Florida.

The data presented and discussed here are inconsistent with the hypotheses that VCG 0094 was introduced to Florida from Europe or that the Floridian and European populations may have been independently established from an unknown ancestral population. It is somewhat more difficult to rule out the possibility that the Floridian VCG 0094 population was established from a progenitor population prior to migration to Europe. Though we cannot provide a definite proof that VCG 0094 originated in Florida, it nevertheless seems the most likely hypothesis. The population structure of Floridian VCG 0094 is advanced, with its genetic diversity, apparent geographic subdivision, and continuum array of vegetative compatibilities. Therefore, VCG 0094 from Florida does not display signs of notable human interference, which would be expected from a plant pathogen population established by founder events.

Besides VCG 0094, we identified two new VCGs, 0098 and 0099. About 10% of Floridian isolates grouped into VCG 0098. RFLP data were unable to show genetic differences between Floridian isolates of VCGs 0094 and 0098, indicating that these two VCGs are very closely related ($\Phi_{ST} = 0.008$ for clone-corrected data). Since isolates within VCG 0098 show less genetic variation than VCG 0094 and fewer isolates of VCG 0098 were found, we speculate that VCG 0098 may have recently evolved from VCG 0094.

The existence of VCG 0099 was initially suggested by the strains' similarity based entirely on RFLP data; to our knowledge, this is the first instance in which a VCG had been predicted and actually discovered based on RFLP analysis. Only 13 of the 300 isolates that could be assigned to a VCG in this study belonged to VCG 0099. These 13 isolates were acquired from five different counties and, based on RFLP analysis, their genetic diversity was twice as high as that of the more prevalent VCG 0094. This diversity suggests that a recent origin of VCG 0099 is unlikely. One speculative explanation for this observation is that VCG 0099 was previously more abundantly represented in Florida, but due to competition from isolates of VCG 0094, its frequency declined. Detailed competition studies are needed to further address this possibility.

Many of the isolates examined for vegetative compatibility did not group with the established VCGs (87 out of 387 isolates). We constructed RFLP haplotypes for 66 of such isolates, and 18 of them had haplotypes similar to those of isolates from VCGs 0094 and 0098. Phylogenetic clustering of unclassified isolates with multiple-member VCGs has been found to be common among isolates from *F. oxysporum* f. sp. *lycopersici* (6,7). Single-gene mutations in putative incompatibility genes have been suggested to explain these data and may also hold true for our observations.

Four of these isolates (from Collier County) were compatible with each other, suggesting the emergence of yet another VCG within this forma specialis. In the future, we will conduct pathogenicity tests toward tomato of all unclassified isolates to determine whether all are nonpathogens or whether tomato crown rot pathogenicity can be identified in several other genetic backgrounds of *F. oxysporum*.

Finally, many isolates obtained as crown rot samples were eventually identified as *F. oxysporum* f. sp. *lycopersici* VCG 0033, the tomato wilt pathogen, based on VCG, RFLP data, and pathogenicity tests. In fact, strains of both *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici* were isolated as crown rot pathogens in two of the sampled fields. This may reflect the fact that under certain conditions the symptoms of these two *Fusarium* diseases on tomato may overlap, making it more difficult to differentiate these diseases and their respective pathogens.

This study has measured the extent of genetic variation within *F. oxysporum* f. sp. *radicis-lycopersici* in Florida. Currently, we are conducting a similar study of the genetic variation within Floridian populations of *F. oxysporum* f. sp. *lycopersici*. The final objective of that study is to develop diagnostic markers that can quickly and reliably differentiate between these two tomato pathogens in Florida.

TABLE 5. Pairwise comparison of Φ_{ST} and their P values of restriction fragment length polymorphism (RFLP) haplotypes of European isolates of *Fusarium oxysporum* f. sp. *radicis-lycopersici* vegetative compatibility grouping (VCG) 0094 to isolates from Florida from VCG 0094 originating from eight counties

County	Pairwise Φ_{ST}	P
Collier	0.1832	<0.001
Lee	0.5050	<0.001
Dade	0.4840	<0.001
Hendry	0.4052	<0.001
Palm Beach	0.0049	0.211
Martin	0.0903	<0.001
St. Lucie	0.7026	<0.001
Manatee	0.3995	<0.001

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