Two Genetically Distinct Populations of *Fusarium oxysporum* f. sp. *lycopersici* Race 3 in the United States

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ABSTRACT

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Isolates of *Fusarium oxysporum* f. sp. *lycopersici* from the United States, Australia, and Mexico were examined for vegetative compatibility, pathogenicity on a set of differential tomato cultivars, mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs), and isozyme polymorphisms. The collection of 113 isolates included representative isolates of races 1, 2, and 3, and the three previously described vegetative compatibility groups (VCGs). A new and previously undescribed VCG, VCG 0033, was identified that contained race 3 isolates of *F. oxysporum* f. sp. *lycopersici* from locations in Arkansas, North Carolina, and Florida. Previously described race 3 isolates belonged to VCG 0030. Recently recovered race 3 isolates from Mexico also belonged to VCG 0030. VCGs 0030 and 0033, which both contain race 3 isolates, represent genetically distinct populations based on vegetative compatibility, mtDNA RFLPs, and isozyme differences. These data support the hypothesis that isolates of *F. oxysporum* pathogenic on tomato may represent two genetically distinct evolutionary lineages. No differences in pathogenicity or virulence (aggressiveness) were detected among race 3 isolates from each of the two VCGs in greenhouse inoculation tests.

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely cultivated food crops. In 1985, tomatoes were grown on over 2.5 million ha (over 6.3 million acres) and production worldwide exceeded 60 million metric tons (21). Production in North and Central America alone accounted for 10.8 million metric tons (1985 figures) (21). In southeastern Arkansas, approximately 405 ha (1,000 acres) of commercial, fresh-market tomatoes are grown annually. Approximately 600,000 20-lb boxes of fresh-market tomatoes are sold annually in Arkansas, with an estimated crop value of approximately $10 million (P. E. Cooper, *personal communication*).

Fusarium wilt of tomato, caused by the vascular wilt pathogen *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans. is a destructive disease of tomato worldwide (39). Three races of *F. oxysporum* f. sp. *lycopersici* have been reported (1,7,13,19) that can be distinguished by pathogenicity to tomato cultivars that contain a single dominant resistance gene (31). Races 1 and 2 have been reported in most tomato-growing regions of the world. In the mid-1950s, Fusarium wilt caused by race 1 was severe in Arkansas and threatened to eliminate commercial tomato production in the state (17). Most commercial tomato cultivars now being grown for commercial markets are resistant to races 1 and 2 of *F. oxysporum* f. sp. *lycopersici*. Race 3 of *F. oxysporum* f. sp. *lycopersici* has been reported in Australia (19), Florida (38), and California (13), and more recently in northwestern Florida and southwestern Georgia (7) and Mexico (37). Currently, few commercially acceptable cultivars with race 3 resistance are available (21).

Neutral genetic and molecular markers have been useful in characterizing diversity in fungi (6,28). Vegetative compatibility and mitochondrial DNA (mtDNA) and nuclear DNA restriction fragment length polymorphism (RFLP) analyses have helped elucidate the evolutionary relationship among races within several formae speciales *of F. oxysporum* (2,16,23,25,29). In a number of formae speciales, vegetative compatibility has been shown to be correlated with race. However, Elias and Schneider (14) reported that vegetative compatibility was not closely correlated with race in *F. oxysporum* f. sp. *lycopersici*. Some vegetative compatibility groups (VCGs) contained multiple races, and races 1 and 2 of *F. oxysporum* f. sp. *lycopersici* were found within multiple VCGs. For example, isolates that belonged to races 1, 2, and 3, with a widespread geographical distribution, belonged to a single VCG (VCG 0030), whereas races 1 and 2 were found in all three of the predominant VCGs. Only six race 3 isolates of *F. oxysporum* f. sp. *lycopersici* have previously been examined for vegetative compatibility (15); five isolates belonged to a single VCG (VCG 0030) and one isolate was not vegetatively compatible with any other isolates of *F. oxysporum* f. sp. *lycopersici* examined.

Elias and Schneider (15) used isozymes to examine 111 isolates of *F. oxysporum* f. sp. *lycopersici* for genetic similarity. Genetic similarity of isolates appeared to be greater within than that between VCGs of *F. oxysporum* f. sp. *lycopersici*. However, nine isozyme electrophoretic phenotypes were identified that contained isolates representing different VCGs, diverse geographic origins, different races, and even different formae speciales.

RFLPs also have been shown to be useful in examining intraspecific variation in *F. oxysporum* (2,16,20,23,25,29). Elias et al. (16) used RFLP analysis of total genomic DNA to examine the relationship between race 1 and race 2 isolates of *F. oxysporum* f. sp. *lycopersici* representing several VCGs. In that study, they determined that isolates within a VCG were more genetically similar to one another than to isolates in different VCGs. They also concluded that some race 1 and 2 isolates may have arisen independently from one another. They concluded that different races may have evolved from within several of the VCGs found within this forma specialis. Such microevolutionary events were proposed by several models of race evolution for *F. oxysporum* (8,24).

The distribution of and genetic similarity among race 3 isolates of *F. oxysporum* f. sp. *lycopersici* in the United States have not been addressed. Our objectives were to document the distribution of race 3 of *F. oxysporum* f. sp. *lycopersici* in Arkansas and compare race 3 isolates from Arkansas with race 3 isolates of *F. oxysporum* f. sp. *lycopersici* from other geographic locations, using vegetative compatibility, mtDNA RFLPs, and isozyme variation (30).

MATERIALS AND METHODS

Isolates. Isolates of *F. oxysporum* were recovered from symptomatic tomatoes in Arkansas between 1992 and 1994. Stem sections were surface disinfested with a 10% household bleach solution (0.5% so-

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dium hypochlorite) for 1 min and plated on water agar. Cultures of *F. oxysporum* were purified from individual microconidia recovered with a micromanipulator. Germinated conidia were transferred to potato dextrose agar (PDA). All isolates were stored on sterile filter paper at 4°C (10). Isolates representing previously described races and VCGs of *F. oxysporum* f. sp. *lycopersici* were received from other researchers and included as references in our study.

A total of 113 isolates of *F. oxysporum* f. sp. *lycopersici* were studied (Table 1). Seventy-five isolates were recovered from symptomatic tomato plants in five commercial fields in southeastern Arkansas between 1992 and 1994. In 1992, 68 isolates were recovered from cv. Mt. Delight (resistant to races 1 and 2) from two commercial tomato fields and from cv. Bradley (resistant to race 1) in one commercial field in Bradley County, AR. In 1993, a single isolate was recovered from cv. Mt. Spring (resistant to races 1 and 2) in Bradley County and, in 1994, six isolates were recovered from cv. Mt. Spring (resistant to races 1 and 2) from two commercial fields, one in Bradley County and one in Drew County, AR. Thirty-eight isolates were received from other researchers (Table 1). Two isolates of *F. oxysporum* Schlechtend.:Fr. f. sp. *radicis-lycopersici* W. R. Jarvis & Shoemaker (causal agent of crown rot), BM1 and BM3, were included for comparison.

Vegetative compatibility tests. The vegetative compatibility of isolates recovered in Arkansas was determined according to the protocols described by Puhalla (33) and Correll et al. (10).

Briefly, nitrate nonutilizing (*nit*) mutants were generated on a minimal agar medium amended with 1.5% potassium chlorate ($KClO_3$) (MMC) (10). After 10 to 14 days, chlorate-resistant sectors were transferred to basal agar medium amended with sodium nitrate as the nitrogen source (MM). Colonies were examined after approximately 5 days. Those exhibiting thin, expansive growth with little or no aerial mycelium were selected as *nit* mutants. *Nit* mutants were characterized according to their phenotype on minimal agar medium amended with one of several different nitrogen sources (10). The *nit* mutant tester strains from several reference isolates were provided by R. W. Schneider (Louisiana State University). *Nit* mutants were paired in all combinations in order to assign isolates to the VCGs of *F. oxysporum* f. sp. *lycopersici* (14,33). All pairing tests were conducted a minimum of three times to confirm VCG identity.

Race identification and virulence tests. To determine forma specialis and race identity, pathogenicity tests were performed on a differential set of tomato cultivars with isolates recovered from Arkansas as well as reference isolates of *F. oxy-*

Table 1. Isolates of *Fusarium oxysporum* f. sp. *lycopersici* used in this study

Vegetative compatibility group (VCG)	Isolate[a]	Race[b]	Geographic origin[c]
0030	HMS2 (#66)	(1)	CA
	UM1 (FRC 0-1118)	1	CA
	F118	(1)	CA
	F136	(2)	CA
	F149	(3)	CA
	RS1 (DFO-63)	(3)	CA
	RS2 (DF1-11)	(3)	CA
	RS3 (DF1-62)	(3)	CA
	RS4 (DF2-100)	(3)	CA
	RS5 (DF2-16)	(3)	CA
	FOLCA	3	CA
	JBF5	(2)	FL
	FRC 0-1078	(2)	FL
	BE1 (5397)	(3)	FL
	JBF6	(3)	FL
	LSU3	1	LA
	RO3 (BRIP14844)	(3)	Australia
	RO4 (21922)	(3)	Australia
	DL1	(3)	Mexico
	DL3	(3)	Mexico
	DL4	(3)	Mexico
0031	F189	(2)	CA
	OSU451	2	OH
0032	MM59	2	AR-1
	MM61	2	AR-1
	MM62	2	AR-1
	MM64	2	AR-1
	MM66	2	AR-1
	MM67	2	AR-1
	MM57	...[d]	AR-1
	MM58	...	AR-1
	MM60	...	AR-1
	MM63	...	AR-1
	MM65	...	AR-1
	LSU7	(2)	LA
0033	MM2	3	AR-2
	MM8	3	AR-2
	MM10	3	AR-2
	MM14	3	AR-2
	MM15	3	AR-2
	MM25	3	AR-2
	MM30	3	AR-2
	MM36	3	AR-2
	MM39	3	AR-2
	(48 additional isolates)	...	AR
	GG1	3	AR-3
	TE1	...	AR-4
	TE6	...	AR-4
	TE7	...	AR-4
	RE1	...	AR-5
	RE2	...	AR-5
	RE3	...	AR-5
	DC1 (24-91)	3	FL
	DC2 (27-91)	3	FL
	DC3 (28-91)	3	FL
	RG1	3	NC
	RG2	3	NC
	RG3	3	NC
	RG4	3	NC
003-[e]	90-50	(1)	CA
	JBF2 (626K-1)	(1)	FL
	BM1 (FORL-B)	CR[f]	FL
	BM2 (47161b)	(CR)	FL
	BM3 (FORL Is. E)	(CR)	FL
	BM4 (FORL Is. H)	(CR)	FL
	BM5 (FORL 12)	(CR)	FL

[a] Previous isolate designations in parentheses (15).
[b] Race designation determined on differential tomato cvs. Bonny Best (susceptible to races 1,2, and 3), UC82-L (resistant to race 1), MH-1 (resistant to races 1 and 2), and I_3R-1 (resistant to races 1,2, and 3). Race designations in parentheses were those reported or previously determined.
[c] In the U.S. or Australia. Numbers following AR locations indicate different growers' fields.
[d] Race determinations not performed.
[e] VCG 003- isolates vegetatively incompatible with VCGs 0030, 0031, 0032, or 0033 of *F. oxysporum* f. sp. *lycopersici*.
[f] Isolates designated CR are crown-rot pathogens, *F. oxysporum* f. sp. *radicis-lycopersici*.

sporum f. sp. *lycopersici* that had previously been described for race. The isolates used in the pathogenicity tests included LSU3, OSU451, MM59, FOLCA, MM2, MM14, and GG1, and were selected based on their VCG identity and geographic origin. Each pathogenicity test was conducted twice. The differential tomato cultivars used for pathogenicity tests were Bonny Best (susceptible to races 1, 2, and 3), UC82-L (resistant to race 1), MH-1 (resistant to races 1 and 2), and I_3R-1 (resistant to races 1, 2, and 3) (seed courtesy of Petoseed, Woodland, CA).

Tomato seedlings were grown in commercial potting mix (Fissurs Sunshine Mix, Vancouver, BC) for approximately 2 weeks or until the first two true leaves had emerged. A soil drench of 0.001% Ridomil-2E (0.3 ml of Ridomil 2E/1 liter of water) solution was applied to all plants 24 and 48 h after inoculation.

Conidia of all isolates were recovered from 1-week-old cultures grown on PDA. Conidial suspensions were adjusted to 1×10^6 conidia per ml. Seedlings were removed from the growing medium and the excess soil was shaken from the roots. Roots were trimmed to a length of approximately 2.5 cm. The roots of each seedling were submerged in the inoculum suspension for approximately 5 min and transplanted into Sunshine mix. Three inoculated seedlings were planted into each 15-cm-diameter pot. Seedlings dipped in deionized water served as controls. The plants were incubated in the greenhouse, where day and night temperatures averaged 37 and 20°C, respectively.

Disease severity was assessed daily starting 10 days after inoculation. Disease was rated on 1 to 5 scale, as follows: 1 = no symptoms; 2 = slight chlorosis, wilting, or stunting of plant; 3 = moderate chlorosis, wilting, or stunting of plant; 4 = severe chlorosis, wilting, or stunting of plant; and 5 = dead plant. Cultivars with average disease ratings greater than 2.5 were considered susceptible. Final assessments were made 21 days after inoculation.

In addition to the pathogenicity tests, virulence tests were performed on susceptible tomato cv. Mt. Pride to compare the virulence, or aggressiveness, of race 3 isolates of *F. oxysporum* f. sp. *lycopersici* from different VCGs. Eight race 3 isolates in VCG 0033 (MM2, MM10, MM14, MM15, MM25, MM30, MM39, and GG1) recovered from Arkansas were compared with a race 3 isolate in VCG 0030 (FOLCA) from California. Three race 2 isolates (MM59, MM61, MM64) from Arkansas, a race 2 isolate (OSU451) from Ohio, a race 1 isolate (LSU) from Louisiana, and a crown-rot isolate (BM1) of *F. oxysporum* f. sp. *radicis-lycopersici* also were included in the virulence tests. The inoculation procedure and disease rating scale were the same as described for the pathogenicity tests.

Experimental design. The inoculation experiments were completely randomized, with three to five replications (pots) per isolate per cultivar and three plants per pot. Statistical analyses were performed with the general linear models (GLM) procedure of SAS (SAS Institute, Cary, NC). Differences in virulence between isolates were determined according to the least significant differences of the mean disease severity ratings. The pathogenicity and virulence tests were conducted twice.

mtDNA RFLP analysis. Fungal cultures for DNA extraction were grown by transferring mycelial plugs from cultures on PDA to 0.5-liter flasks containing 200 ml of complete medium broth (9). Total DNA was extracted according to a modified miniprep procedure (9,27). Powdered mycelium was mixed in 2.0 ml of extraction buffer, shaken for 30 min, incubated at 65°C for 1 h, and centrifuged for 10 min at maximum speed in a microcentrifuge. The supernatant was extracted with an equal volume of phenol/chloroform (1:1, vol/vol) and chloroform/isoamyl alcohol (24:1, vol/vol). The supernatant/extraction liquid was mixed by inversion and centrifuged for 30 min at 4°C. The supernatant was collected and extracted two more times with chloroform/isoamyl alcohol. Total DNA was precipitated by adding an equal volume of sodium acetate and isopropanol and centrifuging for 1 min. The DNA pellet was resuspended in 400 µl of TE (1× TE = 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and 200 µl of NH_4OAc. The supernatant was centrifuged for 20 min at 4°C, then precipitated with 1.0 ml of 95% ethanol, and centrifuged for 2 min; the resulting pellet was rinsed with 95% ethanol. The nucleic acid pellet was resuspended in 250 µl of TE and incubated at room temperature for 1 h, then at 37°C for 20 min, and again at room temperature until fully dissolved. The nucleic acid pellet was dried in a desiccator under vacuum for 60 min and then resuspended in 100 µl of TE at room temperature until fully dissolved. The amount of DNA was quantified with a TKO 100 Fluorometer with Hoechst dye. DNA was stored at –20°C.

Genomic DNA from 17 isolates was digested with five restriction enzymes (*Hha*I, *Msp*I, *Hae*III, *Pvu*II, and *Bgl*II) according to the manufacturer's recommendations (Stratagene, La Jolla, CA). Digested DNA (1.5 µg per lane) was separated in 0.7% agarose in Tris-borate-EDTA (TBE) buffer (0.5×) for 16 h at 20 V in a 14 × 16 cm gel electrophoresis apparatus (Biorad, Richmond, CA). Capillary transfer (bidirectional) of DNA to nylon$^+$ membrane (Hybond-N$^+$, Amersham, Arlington Heights, IL) was conducted overnight.

Table 2. Disease reactions of differential tomato cultivars to isolates of *Fusarium oxysporum* f. sp. *lycopersici*

		Cultivar[a]							
		Bonny Best		UC82-L		MH-1		I_3R-1	
Isolate	Race	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Control		1.0[b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
LSU3	1	4.7	5.0	1.0	1.6	1.0	1.0	1.0	1.0
OSU451	2	4.6	5.0	3.6	4.6	1.1	1.1	1.0	1.0
MM59	2	5.0	5.0	3.9	5.0	1.1	1.0	1.0	1.0
FOLCA	3	4.9	5.0	4.1	4.9	4.0	4.2	1.0	1.0
MM2	3	5.0	5.0	3.9	4.8	3.9	4.1	1.0	1.1
MM14	3	4.7	5.0	3.9	4.6	3.6	3.2	1.0	1.0
GG1	3	4.4	5.0	3.8	4.7	3.6	3.1	1.0	1.0

[a] Differential cultivars used for race designations were Bonny Best (susceptible to races 1, 2, and 3), UC82-L (resistant to race 1), MH-1 (resistant to races 1 and 2), and I_3R-1 (resistant to races 1, 2, and 3). Seed for differential cultivars was provided by Petoseed, Woodland, CA.

[b] Disease severity was assessed on a scale of 1 to 5, as follows: 1 = no symptoms; 2 = slight wilting, chlorosis, or stunting; 3 = moderate chlorosis, wilting, or stunting; 4 = severe chlorosis, wilting, or stunting; and 5 = dead plant. Cultivars with a mean disease rating >2.5 were considered susceptible.

Table 3. Disease reactions of the tomato cv. Mt. Pride to isolates of *Fusarium oxysporum* f. sp. *lycopersici*

		Disease rating[a]	
Isolate	Race	Test 1	Test 2
Control		1.0	1.0
BM1	CR[b]	1.0	1.0
LSU3	1	1.0	1.2
OSU451	2	1.0	1.0
MM59	2	1.0	1.0
MM61	2	1.0	1.6
MM64	2	1.0	1.0
FOLCA	3	4.3	3.4
MM2	3	3.9	3.0
MM10	3	4.0	3.3
MM14	3	4.2	2.7
MM15	3	4.3	3.1
MM25	3	3.5	3.2
MM30	3	3.6	3.0
MM39	3	4.2	2.7
GG1	3	3.9	2.9
LSD ($P = 0.05$)[c]		(0.3)	(0.6)

[a] Disease severity was assessed on a scale of 1 to 5 as follows: 1 = no symptoms; 2 = slight wilting, chlorosis, or stunting; 3 = moderate chlorosis, wilting, or stunting; 4 = severe chlorosis, wilting, or stunting; and 5 = dead plant. Cultivars with a mean disease rating >2.5 were considered susceptible.

[b] Isolate BM1 is a crown-rot pathogen, *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

[c] Least significant difference.

Two large, non-overlapping, mtDNA clones, 4U40 (13.7 kb) and 2U18 (10.1 kb), from *Colletotrichum orbiculare* (Berk. & Mont.) Arx were used for the hybridizations. These two mtDNA clones have been effective in detecting 85 to 100% of the mitochondrial genome of *F. oxysporum* Schlechtend.:Fr. f. sp. *spinaciae* (Sherb.) W. C. Snyder & H. N. Hans. (35) and *Pyricularia grisea* (Cooke) Sacc. (J. C. Correll, *unpublished*). The two clones were combined in equimolar concentrations and used to probe blots.

An enhanced chemiluminescent gene detection kit (Amersham, Arlington Heights, IL) was used to label the two pooled mtDNA probes and the lambda marker. Prehybridization and hybridization reactions were performed according to the manufacturer's instructions in a rotisserie oven (Hybaid, Woodbridge, NJ) at 42°C. The mtDNA probes were covalently labeled with horseradish peroxidase with glutaraldehyde. Hybridizations were allowed to proceed for 12 to 16 h. Membranes were washed twice at 42 C in primary wash buffer (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 36% urea, and 0.4% sodium dodecyl sulfate) for 20 min each time, followed by two 5-min washes in 2× SSC at room temperature. Film (Hyperfilm-ECL, Amersham, Arlington Heights, IL) was placed on the membranes and exposed for 10 to 45 min depending on the strength of the enzyme reaction signal. mtDNA RFLP haplotypes were assigned based on the visual restriction patterns observed among the various isolates for each of the restriction enzymes used.

mtDNA fragments were scored for their presence or absence. Relative relatedness among isolates was determined by multivariate analysis of similarity coefficients (SIMQUAL) (NTSYS-PC, Exeter Publishing Co., Setarket, NY). A matrix of similarity coefficients for each pair of isolates was constructed by the formula $S_m = (a + b)/n$, where a = the number of mtDNA bands in common with any two isolates, b = the number of bands in one isolate and absent in the second, and n = the total number of mtDNA fragments examined for the two isolates. To determine relative relatedness, a cluster analysis on the similarity coefficients was performed with the unweighted pair-grouping method with arithmetic averages (UPGMA).

Isozyme analysis. Six race 3 isolates of *F. oxysporum* f. sp. *lycopersici* were examined for isozyme electrophoretic phenotypes. Three isolates each were selected from VCGs 0030 and 0033 to represent

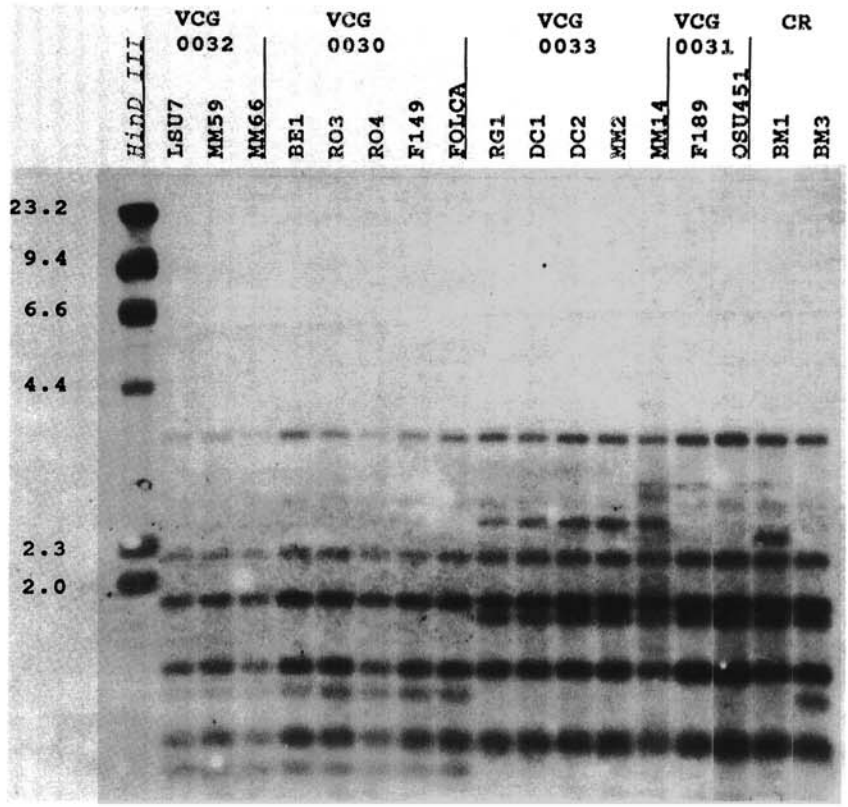

Fig. 1. Mitochondrial DNA (mtDNA) restriction fragment length polymorphisms among isolates of *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (isolates BM1 and BM3). A Southern blot of total DNA was digested with *Hha*I and probed with two mtDNA probes (11). Lane 1 contains lambda DNA digested with *Hin*dIII as a size marker in kilobases.

Table 4. Vegetative compatibility group (VCG), race, and mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) haplotypes among isolates of *Fusarium oxysporum* f. sp. *lycopersici*

Isolate	VCG	Race	Origin	Genomic DNA RFLP haplotype[a]				
				*Hha*I	*Msp*I	*Hae*III	*Pvu*II	*Bgl*II
LSU7	0032	2	LA	A	A	A	A	A
MM59	0032	2	AR	A	A	A	A	A
MM66	0032	2	AR	A	A	A	A	A
BE1	0030	3	FL	A	A	A	A	A
RO3	0030	3	Australia	A	A	A	A	A
RO4	0030	3	Australia	A	A	A	A	A
F149	0030	3	CA	A	A	A	A	A
FOLCA	0030	3	CA	A	A	A	A	A
RG1	0033	3	NC	B	B	B	B	B
DC1	0033	3	FL	B	B	B	B	B
DC2	0033	3	FL	B	B	B	B	B
MM2	0033	3	AR	B	B	B	B	B
MM14	0033	3	AR	B	B	B	B	B
F189	0031	2	CA	C	C	B	B	B
OSU451	0031	2	OH	C	C	B	B	B
BM1	NC[b]	CR[c]	FL	D	D	C	C	C
BM3	NC	CR	FL	E	A	D	D	A

[a] Letters within a column indicate the same mtDNA RFLP haplotype.
[b] Not characterized into a vegetative compatibility group.
[c] Isolates designated CR are crown-rot pathogens, *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

isolates from different geographical origins. Isolates FOL-CA, BE-1, and RO-3 in VCG 0030 were from California, Florida, and Australia, respectively, and isolates MM14, DC-2, and RG1 in VCG 0033 were from Arkansas, Florida, and North Carolina, respectively (Table 1).

Protein extractions and staining procedures were performed following previously described protocols (4,5). Proteins were separated by discontinuous native polyacrylamide gel electrophoresis (7% resolving gel, pH 8.9; 4% stacking gel, pH 8.8) at 10°C at 25 mA (12). Staining procedures for 13 enzymes were performed. Isolates were compared for relatedness as described for the mtDNA RFLP analysis.

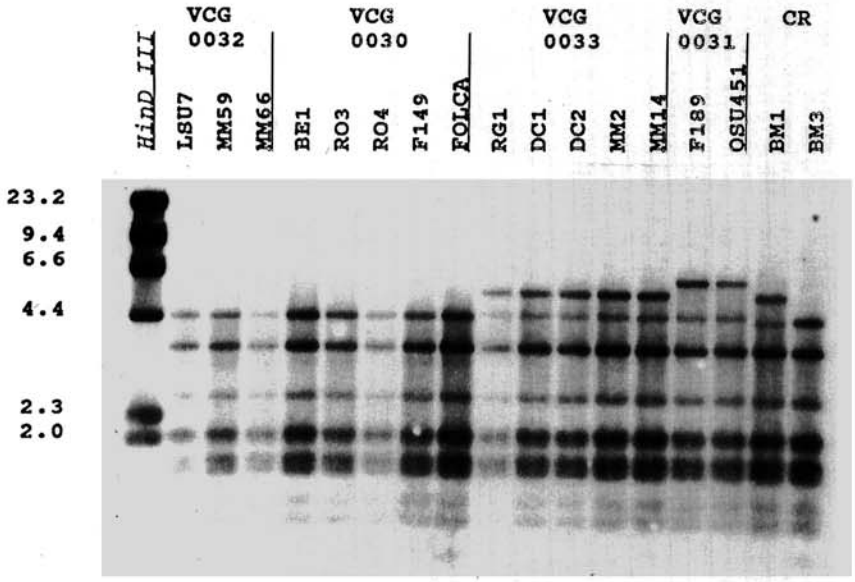

Fig. 2. Mitochondrial DNA (mtDNA) restriction fragment length polymorphisms among isolates of *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (isolates BM1 and BM3). A Southern blot of total DNA was digested with *Msp*I and probed with two mtDNA probes (11). Lane 1 contains lambda DNA digested with *Hin*dIII as a size marker in kilobases.

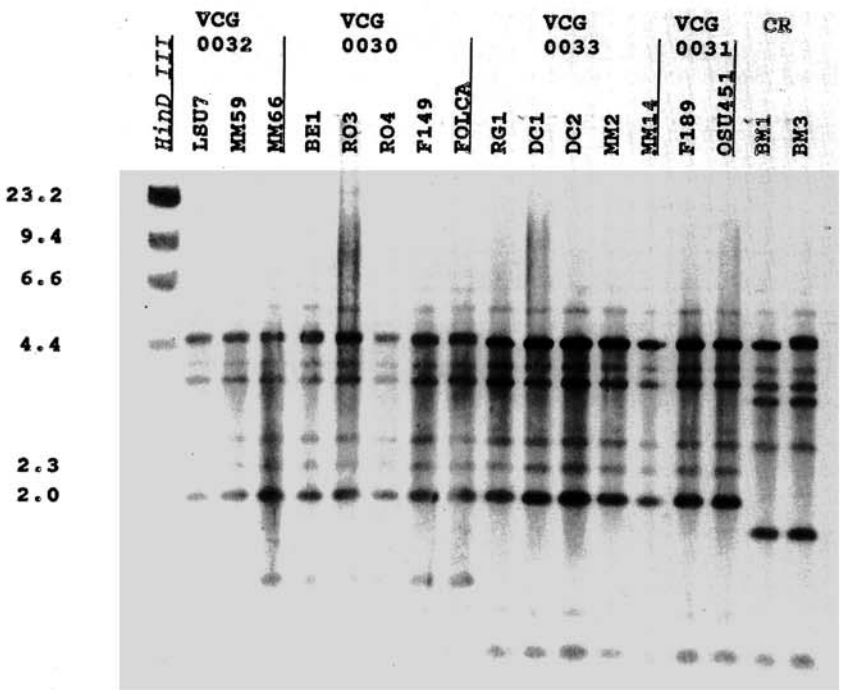

Fig. 3. Mitochondrial DNA (mtDNA) restriction fragment length polymorphisms among isolates of *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (isolates BM1 and BM3). A Southern blot of total DNA was digested with *Hae*III and probed with two mtDNA probes (11). Lane 1 contains lambda DNA digested with *Hin*dIII as a size marker in kilobases.

RESULTS

Vegetative compatibility tests. Four VCGs were identified among the 113 isolates examined (Table 1). Three VCGs (0030, 0031, and 0032) have previously been described (14,33). Isolates in VCG 0030 were collected in California, Louisiana, Florida, and Australia, and included races 1, 2, and 3. Two isolates belonged to VCG 0031 and originated from Ohio and California. Eleven isolates, recovered from cv. Bradley in Arkansas, were vegetatively compatible with an isolate from Louisiana in VCG 0032. The 64 isolates recovered from cvs. Mt. Delight and Mt. Spring from Arkansas belonged to a single previously undescribed VCG (designated VCG 0033). Four recently recovered isolates from North Carolina and three from Florida also belonged to VCG 0033. Seven isolates, including five crown-rot isolates of *F. oxysporum* f. sp. *radicis-lycopersici* (22), were not vegetatively compatible with any other isolates used in the current study.

Race identification and virulence tests. A total of 27 isolates of *F. oxysporum* f. sp. *lycopersici* were used in pathogenicity tests on the differential tomato cultivars (Table 2; disease reactions to seven isolates are shown). All six isolates in VCG 0032 recovered from cv. Bradley that were tested had a race 2 virulence phenotype (Tables 1 and 2). All 17 isolates in VCG 0033 recovered from cvs. Mt. Delight or Mt. Spring from Arkansas, and from North Carolina and Florida, had a race 3 virulence phenotype (Tables 1 and 2). The expected disease reactions to the race 1, 2, and 3 reference isolates were confirmed (Table 2). In the virulence tests with cv. Mt. Pride, no differences in disease reactions were observed among plants with the race 3 isolates regardless of their VCG origin (Table 3).

mtDNA RFLP haplotypes. Restriction digests of genomic DNA of *F. oxysporum* f. sp. *lycopersici* probed with mtDNA clones from *C. orbiculare* (11) indicated that the minimum mtDNA genome size of *F. oxysporum* f. sp. *lycopersici* was approximately 35.5 kb (based on mtDNA fragment sizes detected with *Hae*III). The estimate of the mtDNA genome size with other enzymes was less than 35.5 kb; this may have been due to the inability to distinguish two distinct but co-migrating mtDNA fragments.

Restriction digests with the enzyme *Hha*I revealed the greatest diversity; five distinct mtDNA RFLP haplotypes, designated A, B, C, D, and E, were identified among the 17 isolates tested (Table 4; Fig. 1). Race 2 isolates of *F. oxysporum* f. sp. *lycopersici* in VCG 0032 from Arkansas and Louisiana, and race 3 isolates in VCG 0030 from California, Florida, and Australia had an identical mtDNA haplotype (haplotype A) with all five restriction enzymes examined. Race 3 isolates in VCG 0033 from Arkansas, Florida, and North

Carolina also shared an identical mtDNA haplotype (haplotype B) with all five restriction enzymes examined.

Four additional restriction enzymes, *Msp*I, *Hae*III, *Pvu*II, and *Bgl*II, discriminated three or four mtDNA RFLP haplotypes (Table 4; Figs. 2 and 3; data for *Pvu*II and *Bgl*II not shown). mtDNA haplotypes B and C detected with the enzymes *Hha*I and *Msp*I, representing VCGs 0033 and 0031, respectively, were not distinguished with enzymes *Hae*III, *Pvu*II, or *Bgl*II. One of the two crown-rot isolates examined, BM3, could not be distinguished from mtDNA haplotype A with the enzymes *Msp*I or *Bgl*II (Table 4; Fig. 2). However, the crown-rot isolates could be distinguished from isolates of *F. oxysporum* f. sp. *lycopersici* with the enzymes *Hha*I, *Pvu*II, and *Hae*III.

Relative genetic relatedness, as determined by cluster analysis of the mtDNA RFLPs detected with five enzymes, indicated that there may be two genetically distinct lineages among the isolates of *F. oxysporum* f. sp. *lycopersici* examined (Fig. 4). One group contained isolates in VCGs 0030 and 0032, whereas the second group contained isolates in VCGs 0031 and 0033 (Fig. 4).

Isozyme analysis. No differences in enzyme mobility were detected among the six isolates examined with nine of the 13 enzymes detected (Table 5). However, two isozyme electrophoretic phenotypes were detected with each of four enzymes (EST, ME, PGM, and MDH) (Table 5). The three isolates in VCG 0030 belonged to one phenotype and the three isolates in VCG 0033 belonged to the second phenotype.

DISCUSSION

Race 3 of *F. oxysporum* f. sp. *lycopersici* was recovered from five locations in the commercial tomato-growing region of southeastern Arkansas between 1992 and 1994. The race 3 isolates recovered in Arkansas were all vegetatively compatible with one another and in the same VCG as several race 3 isolates from North Carolina and Florida. This is a newly described VCG (VCG 0033) of *F. oxysporum* f. sp. *lycopersici*. Previously described race 3 isolates, from California, Florida, and Australia, belonged to VCG 0030. Although the two VCGs are genetically different, race 3 isolates from the two VCGs could not be distinguished based on virulence (aggressiveness) on susceptible cv. Mt. Pride in a greenhouse inoculation test. Thus, the I_3R gene, which confers resistance to *F. oxysporum* f. sp. *lycopersici* race 3 previously identified (35), apparently is effective against race 3 isolates in both VCG 0030 and 0033.

The origin of race 3 of *F. oxysporum* f. sp. *lycopersici* in Arkansas is unknown. However, it is common for seasonal workers to move from commercial tomato fields in Florida to Arkansas. Thus, it is possible that the movement of infested soil on equipment, packing boxes, or other items could have introduced the pathogen into Arkansas. Five of the six race 3 isolates from Florida examined in an earlier study belonged to VCG 0030 (14). It is possible that isolate FDA-3, the sole race 3 isolate from Florida not in VCG 0030, represented a race 3 isolate in VCG 0033. However, this isolate was inadvertently destroyed and direct comparisons could not be made. Circumstantial evidence based on isozyme analysis indicate that this isolate may have been a VCG 0033 isolate.

Two independent techniques, examination of mtDNA RFLPs and isozymes, indicated that race 3 isolates of *F. oxysporum* f. sp. *lycopersici* in VCGs 0030 and 0033 represent two genetically distinct populations. In addition, isolates in VCG 0032 shared a common mtDNA RFLP haplotype with isolates in VCG 0030 whereas isolates in VCG 0031 shared a similar mtDNA RFLP haplotype with isolates in VCG 0033.

The common mtDNA haplotype among isolates in VCGs 0030 and 0032 indicates that they may share a common evolutionary lineage. Similarity in mtDNA RFLP haplotypes among isolates in VCGs 0031 and 0033 also indicates that these two VCGs may share a second evolutionary lineage among isolates of *F. oxysporum* f. sp. *lycopersici*. Using isozymes, Elias and Schneider (15) also found two genetically distinct groups among the race 1, 2, and 3 isolates they examined. One group included VCG 0030 and 0032 isolates and the second included VCG 0031 isolates. Two groups also were identified in a study of relatedness among race 1 and 2 isolates of *F. oxysporum* f. sp. *lycopersici* with nu-

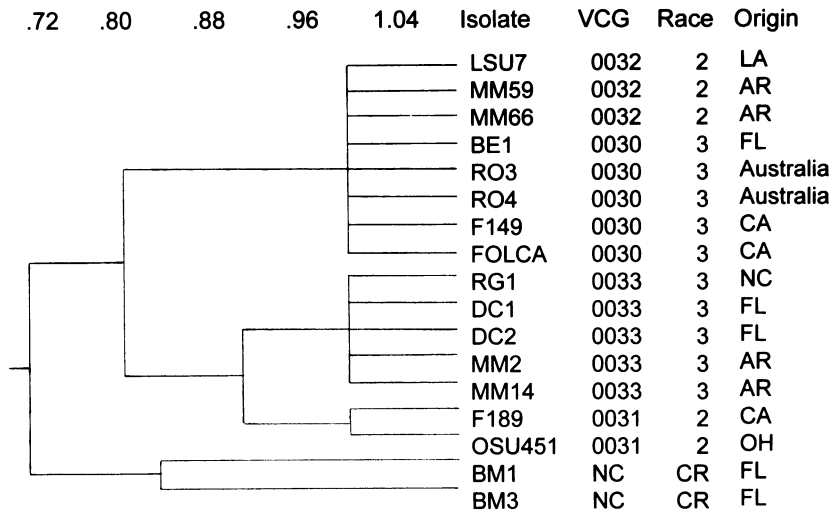

Fig. 4. Cluster analysis of mitochondrial DNA (mtDNA) restriction fragment length polymorphism comparisons by the unweighted pair grouping method with arithmetic averages (UPGMA). The figure includes isolate designations, vegetative compatibility group, and geographic origin.

Table 5. Electrophoretic phenotype of *Fusarium oxysporum* f. sp. *lycopersici* based on isozyme analysis

	Isolate (vegetative compatibility group)					
Enzyme[a]	**BE-1 (0030)**	**FOL-CA (0030)**	**RO-3 (0030)**	**MM14 (0033)**	**RG1 (0033)**	**DC-2 (0033)**
AAT	A[b]	A	A	A	A	A
G6PDH	A	A	A	A	A	A
6PGD	A	A	A	A	A	A
SOD	A	A	A	A	A	A
HK	A	A	A	A	A	A
NADHDH	A	A	A	A	A	A
NADPHDH	A	A	A	A	A	A
DIA	A	A	A	A	A	A
MR	A	A	A	A	A	A
EST	A	A	A	B	B	B
ME	A	A	A	B	B	B
PGM	A	A	A	B	B	B
MDH	A	A	A	B	B	B

[a] Enzymes and EC numbers: AAT = Aspartate aminotransferase (2.6.1.1); G6PDH = Glucose-6-phosphate dehydrogenase (1.1.1.49); 6PGD = 6-phosphogluconate dehydrogenase (1.1.1.44); SOD = Superoxide dismutase (1.15.1.1); HK = Hexokinase (2.7.1.1); NADHDH = Nico.aden.dinucleo. dehydrogenase (1.6.99.1); NADPHDH = Nico.aden.dinuc.phos.dehydrogenase (1.6.99.1); DIA = Diaphorase (1.6.4.3); MR = Menadione reductase (1.6.99.2); EST = Esterase (3.1.1.1); ME = Malic enzyme (1.1.1.40); PGM = Phosphoglucomutase (2.7.5.1); MDH = Malate dehydrogenase (1.1.1.37).

[b] Electrophoretic type detected. Same letter within a row indicates no difference in enzyme mobility.

clear DNA RFLPs (16). The correspondence between molecular genotypes identified with nuclear DNA RFLP markers in *F. oxysporum* f. sp. *lycopersici* (16) and mtDNA RFLP markers used in the current study also has been observed with other fungi (11) and this non-random association of independent genetic markers provides strong evidence of an asexual mode of reproduction for plant pathogenic fungi (36).

Evidence in the current study, as well as data from others (16), supports the hypothesis that microevolutionary events (e.g., changes in virulence) occur among isolates of *F. oxysporum* f. sp. *lycopersici* belonging to genetically distinct lineages. Changes in virulence within genetically isolated subpopulations, such as VCGs, may occur due to selection pressure imposed by resistance genes deployed on a large scale in commercial tomato cultivars. Similar relationships between virulence, VCGs, and molecular genotypes have been observed in other formae speciales of *F. oxysporum* (2,8,18,20,23,24). For example, with *F. oxysporum* f. sp. *cubense*, it was hypothesized that isolates pathogenic on banana have coevolved with edible and wild banana hosts (32). Subsequent microevolution of isolates of *F. oxysporum* pathogenic on banana has led to the association of various races and VCGs with distinct molecular haplotypes (3,26), as we have observed with *F. oxysporum* f. sp. *lycopersici*.

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