Origin of Race 3 of *Fusarium oxysporum* f. sp. *lycopersici* at a Single Site in California

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Accepted for publication 7 April 2003.

ABSTRACT

Cai, G., Gale, L. R., Schneider, R. W., Kistler, H. C., Davis, R. M., Elias, K. S., and Miyao, E. M. 2003. Origin of race 3 of *Fusarium oxysporum* f. sp. *lycopersici* at a single site in California. Phytopathology 93:1014-1022.

Thirty-nine isolates of *Fusarium oxysporum* were collected from tomato plants displaying wilt symptoms in a field in California 2 years after *F. oxysporum* f. sp. *lycopersici* race 3 was first observed at that location. These and other isolates of *F. oxysporum* f. sp. *lycopersici* were characterized by pathogenicity, race, and vegetative compatibility group (VCG). Of the 39 California isolates, 22 were in VCG 0030, 11 in VCG 0031, and six in the newly described VCG 0035. Among the isolates in VCG 0030, 13 were race 3, and nine were race 2. Of the isolates in VCG 0031, seven were race 2, one was race 1, and three were nonpathogenic to tomato. All six isolates in VCG 0035 were race 2. Restriction fragment length polymorphisms (RFLPs) and sequencing of the intergenic spacer (IGS) region of rDNA identified five IGS RFLP haplotypes, which coincided with VCGs, among 60 isolates of *F. oxysporum* from tomato. Five race 3 isolates from California were of the same genomic DNA RFLP haplotype as a race 2 isolate from the same location, and all 13 race 3 isolates clustered together into a subgroup in the neighbor joining tree. Collective evidence suggests that race 3 in California originated from the local race 2 population.

Fusarium wilt of tomato, caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans., is a devastating disease in major tomato-growing regions worldwide (44) and has been reported in at least 32 countries (20). Three races of *F. oxysporum* f. sp. *lycopersici* have been reported. They are distinguished by their virulence to tomato cultivars that contain single resistance genes (29,37). Race 1 was initially described in 1886 (7), and race 2 was first reported in 1945 in Ohio (1). Race 3 was observed in Australia in 1978 (19) and was subsequently reported in several U.S. states: California (13), Florida (43), Georgia (10), Arkansas and North Carolina (28), and Tennessee (8). It also has also been found in Mexico (41). Currently, few commercial cultivars with race 3 resistance are available (20).

Vegetative compatibility is typically used to characterize pathogenic populations in the asexual fungus *F. oxysporum* (25). Four vegetative compatibility groups (VCGs), 0030 to 0033, have been reported in *F. oxysporum* f. sp. *lycopersici* (14,28). Races 1 and 2 occur in VCGs 0030 to 0032 (14), and race 3 in VCGs 0030 and 0033 (14,28). Isozyme analyses (15), mitochondrial DNA restriction fragment length polymorphisms (RFLPs) (28), and nuclear DNA RFLPs (17) have shown that different races in the same VCG are closely related, while isolates of the same race in different VCGs are distinct from each other. These studies suggest that VCG is an indicator of genomic evolutionary origin, whereas race most likely evolves independently within different VCGs.

Investigations of the origin of new races in *F. oxysporum* have been hampered by the lack of suitable collections from sites where new races were recently discovered, which would include the established races and the newly discovered race. These isolates could then be analyzed for genetic relatedness to determine if the new race arose from the existing ancestral population or if it was introduced. Such a site was available in a single tomato field in California where race 3 was first discovered in 1987 (13). Two years after this discovery, this field was sampled for *F. oxysporum* f. sp. *lycopersici* by isolating *F. oxysporum* from roots and stems of several tomato varieties. Isolates were characterized by race, virulence, and vegetative compatibility (36). The present study examined genetic relatedness among VCGs and races 1, 2, and 3 in that collection with the objective of determining the origin of the newly discovered race. Isolates from other locations, including the three races of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici,* were also included. A preliminary report was published (9).

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Publication no. P-2003-0523-02R

MATERIALS AND METHODS

Isolates. In 1989, several tomato cultivars, resistant and susceptible to races 1 and 2 and susceptible to race 3, were planted in the field in California where race 3 of *F. oxysporum* f. sp. *lycopersici* was discovered in 1987 (13). Isolations were made from stems and taproots of tomato plants that showed vascular wilt symptoms. Isolates from tomato cultivars susceptible to all races were numbered with the prefix DF0-; those from cultivars resistant to race 1 but susceptible to races 2 and 3, with the prefix DF1-; and those from cultivars resistant to races 1 and 2 but susceptible to race 3, with the prefix DF2-.

Thirty-nine isolates from this California collection and 18 isolates of *F. oxysporum* f. sp. *lycopersici* from diverse locations were included in this study (Table 1). Also included were two isolates of *F. oxysporum* f. sp. *radicis-lycopersici* and isolate CF75, from a Korean population that was initially reported to be a member of VCG 0034 of *F. oxysporum* f. sp. *lycopersici* but is now presumed to belong to VCG 0094 of *F. oxysporum* f. sp. *radicis-*

TABLE 1. Isolates of *Fusarium oxysporum* f. sp. *lycopersici* and related strains used in this study

VCG[a]	Isolate[b]	Race[c]	Geographic origin	IGS RFLP haplotype[d]			Genomic DNA RFLP haplotype
				*Eco*RI	*Rsa* I	*Hae* III	
0030	*DF0-3	3	California	A	B	A	H1
	DF0-25	2	California	A	B	A	H2
	DF0-36	3	California	A	B	A	H3
	DF0-40	2	California	A	B	A	H4
	DF0-41	3	California	A	B	A	H3
	DF0-42	2	California	A	B	A	H5
	DF0-47	2	California	A	B	A	H6
	DF0-63	3	California	A	B	A	H3
	DF0-65	3	California	A	B	A	H7
	DF0-70	2	California	A	B	A	H8
	*DF0-77	2	California	A	B	A	H3
	DF0-110	2	California	A	B	A	H9
	*DF1-6	3	California	A	B	A	H7
	DF1-19	3	California	A	B	A	H10
	DF1-36	3	California	A	B	A	H3
	DF1-37	3	California	A	B	A	H3
	DF1-41	2	California	A	B	A	H11
	DF1-66	2	California	A	B	A	H12
	*DF2-8	3	California	A	B	A	H13
	DF2-16	3	California	A	B	A	H14
	DF2-18	3	California	A	B	A	H15
	DF2-20	3	California	A	B	A	H16
	JBF-6	(3)	Florida	A	B	A	H17
	*5397	(3)	Florida	A	B	A	H17
	*BRIP14844	(3)	Australia	A	B	A	H18
	21991	(3)	Australia	A	B	A	H19
	*21992	(3)	Australia	A	B	A	H20
	Mx395	(3)	Mexico	A	B	A	H21
	Mx2	(3)	Mexico	A	B	A	H22
	Mx4	(3)	Mexico	A	B	A	H23
	*Mx6	(3)	Mexico	A	B	A	H22
0031	*DF0-9	2	California	B	C	C	H24
	DF0-16	2	California	B	C	C	H25
	DF0-29	2	California	B	C	C	H26
	*DF0-38	2	California	B	C	C	H27
	DF0-67	2	California	B	C	C	H28
	*DF0-68	1	California	B	C	C	H29
	DF0-89	2	California	B	C	C	H30
	*DF1-7	2	California	B	C	C	H31
	*BFOL-51	(1)	Louisiana	B	C	C	H32
	OSU-451	(2)	Ohio	B	C	C	H33
	*DF0-62	N[e]	California	B	C	C	H34
	*DF0-79	N[e]	California	B	C	C	H35
	DF0-106	N[e]	California	B	C	C	H36
0032	*MM59	(2)	Arkansas	A	B	A	H37
	*MM66	(2)	Arkansas	A	B	A	H38
0033	*DC1	(3)	Florida	A	A	E	H39
	DC2	(3)	Florida	A	A	E	H40
	RG1	(3)	North Carolina	A	A	E	H41
	*MM2	(3)	Arkansas	A	A	E	H41
	MM14	(3)	Arkansas	A	A	E	H42
0035	*DF0-23	2	California	A	C	B	H44
	*DF0-35	2	California	A	C	B	H45
	DF0-54	2	California	A	C	B	H46
	*DF1-12	2	California	A	C	B	H47
	DF1-48	2	California	A	C	B	H45
	DF1-60	2	California	A	C	B	H45
Other isolates[f]							
F. oxysporum f. sp. *radicis-lycopersici*	*CF75[g]	…	Korea	A	C	D	H43
	*BM1	…	Florida	A	C	D	H48
	*BM2	…	Florida	A	C	D	H49
F. moniliforme	*A-149	…	…	…	…	…	…
	*F-728	…	…	…	…	…	…

[a] Vegetative compatibility group (VCG) designations of isolates JBF-6, 5397, BRIP14844, 21991, 21992, BFOL-51, OSU-451, MM59, MM66, DC1, DC2, RG1, MM2, and MM14 were previously reported (14,28). Results in this study agreed with previous VCG designations, with the exception of MM59, which was found to be a bridging isolate that can also form heterokaryons with some members of VCG 0030.

[b] Isolates selected for sequencing of the 5′ intergenic spacer region of rDNA are marked with an asterisk.

[c] Race designations in parentheses were previously reported (14,15,28,41), and the results in this study agree with previous designations.

[d] Haplotypes based on restriction fragment length polymorphisms (RFLPs) of intergenic spacer (IGS) region of rDNA.

[e] Nonpathogenic isolate.

[f] The pathogenicity of these isolates was not verified in this study. The pathogenicity of BM1 and BM2 was determined in a previous study (28).

[g] Isolate CF75 was first reported as race 1 of *F. oxysporum* f. sp. *lycopersici* but is now considered to belong to VCG 0094 of *F. oxysporum* f. sp. *radicis-lycopersici* (22).

lycopersici (22). Two isolates of *F. moniliforme* (48) were chosen as the out-group in phylogenetic analyses. All 62 isolates were single-spored and stored as dried agar cultures.

Pathogenicity tests. To confirm the identification of the forma specialis and race of putative *F. oxysporum* f. sp. *lycopersici* isolates from the California collection and isolates from other locations, greenhouse pathogenicity tests were performed with the differential tomato cultivars Bonny Best (no resistance), VFN-8 (resistant to race 1), and Walter (resistant to races 1 and 2). Microconidia from 5-day-old cultures on potato-dextrose agar were rinsed by centrifugation in deionized water. Conidial suspensions were adjusted to about 1×10^6 spores per ml. Tomato seedlings were grown in a soilless substrate (Jiffy Mix, Jiffy Products of America, Batavia, IL) for about 2 weeks, until the first true leaf was fully expanded. The root dip method of inoculation (46) was utilized as described below. Seedlings were uprooted and shaken to remove excess soil. Roots were dipped in conidial suspensions for about 5 min and then transplanted to plastic cell trays containing a sterile 1:1 mixture of sand and soil, with one seedling per cell. Seedlings dipped in deionized water served as controls. Six seedlings of each cultivar were tested for each isolate and for the control.

Disease severity was assessed daily, starting 10 days after inoculation, on the following scale (28): 1 = no symptoms; 2 = slight chlorosis, stunting, or wilting; 3 = moderate chlorosis, stunting, or wilting; 4 = severe chlorosis, stunting, or wilting; 5 = death. The races of isolates were generally determined 15 days after inoculation, but the plants were kept for six more days for final confirmation of the races. Pathogenicity tests were conducted at least twice for each isolate.

Vegetative compatibility tests. Vegetative compatibility tests were conducted, following the protocols described by Puhalla (33) and Correll et al. (12). Nitrate nonutilizing (*nit*) mutants were generated on KPS agar containing 1.5% $KClO_3$ and were assigned to different physiological phenotypes (*nit*1, NitM, and *nit*3) on the basis of their growth on minimal medium (MM) amended with different nitrogen sources. Two *nit* mutants, *nit*1 and NitM, were chosen for each isolate.

Nit testers of known VCG were used to assign isolates to different VCGs. *Nit* testers for VCGs 0030, 0031, and 0032 were generated from isolates UM-1, OSU-451, and LSU-7, respectively (14). *Nit* testers for VCG 0033 were generated from isolate MM2 (28). *Nit* mutants were also generated from isolate CF75 and paired with *nit* mutants of all isolates of *F. oxysporum* f. sp. *lycopersici.* As mentioned earlier, this isolate was reported to be a member of VCG 0034 of *F. oxysporum* f. sp. *lycopersici* (49) but is now presumed to belong to VCG 0094 of *F. oxysporum* f. sp. *radicis-lycopersici* (22).

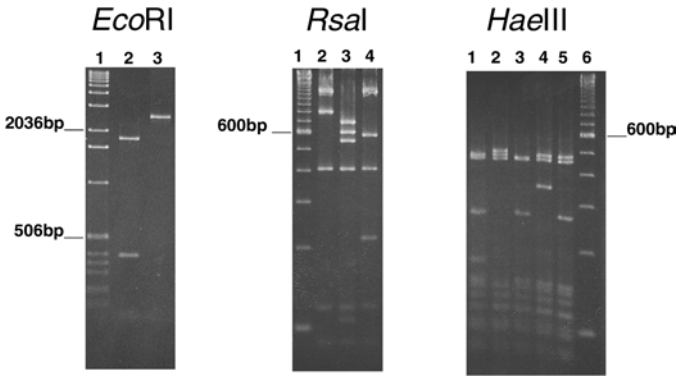

Fig. 1. Restriction fragment length polymorphism patterns resulting from the digestion of the intergenic spacer region of rDNA from isolates of *Fusarium oxysporum* f. sp. *lycopersici* and other *F. oxysporum* strains with enzymes *Eco*RI, *Rsa*I, and *Hae*III. *Eco*RI lanes 1 to 3: 1-kb DNA ladder and patterns A and B, respectively. *Rsa*I lanes 1 to 4: 100-bp DNA ladder and patterns A, B, and C, respectively. *Hae*III lanes 1 to 6: patterns A, B, C, D, and E and 100-bp DNA ladder, respectively.

Nit mutants from all isolates were paired with *nit* testers and with each other on MM with nitrate as the sole source of nitrogen. *Nit* mutants from each isolate were also paired to determine self-compatibility. Plates were incubated at 28°C under fluorescent light for 12 h followed by darkness for 12 h. Each pairing was conducted at least twice.

DNA extraction. DNA extraction followed the protocol outlined by Lee and Taylor (27) with some modifications. Stationary cultures were grown in potato-dextrose broth for 7 days at room temperature. About 0.2 g of mycelium (fresh weight) was ground in liquid nitrogen and mixed with 400 µl of lysis buffer. After incubation at 65°C for 1 h, the mixture was centrifuged for 10 min at top speed in a microcentrifuge. The supernatant was extracted with equal volumes of a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, vol/vol) followed by an equal volume of a mixture of chloroform and isoamyl alcohol (24:1, vol/vol). DNA was precipitated by adding one-half volume of 7.5 M NH_4OAc and two volumes of 95% ethanol and centrifuging for 10 min. DNA pellets were washed with 70% ethanol and dissolved in 50 µl of Tris-EDTA (pH 8.0).

Polymerase chain reaction. The intergenic spacer (IGS) region of rDNA was amplified with the primers CNL12 (CTGAACGCCTCTAAGTCAG) and CNS1 (GAGACAAGCATATGACTACTG) (2,42,45). Amplification reactions were conducted in a 50-µl reaction mixture containing $MgCl_2$ (1.5 mM); dATP, dCTP, dGTP, and dTTP (0.2 mM each); CNL12 and CNS1 (0.5 µM each); *Taq* DNA polymerase reaction buffer; 1.5 units of *Taq* DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA); and approximately 1 ng of template DNA. Amplifications were performed in a programmable thermal cycler (Amplitron II, Barnstead Thermolyne, Dubuque, IA). An initial denaturation step of 95°C for 2 min was followed by 35 amplification cycles of denaturation (95°C for 35 s), annealing (58°C for 55 s), and extension (72°C for 45 s in the first cycle and 5 s more after each cycle). After amplification, samples were incubated for an additional 10 min at 72°C. A negative control (no DNA template) was always included. The sizes of the amplified IGS fragments were estimated by comparison to a 1-kb DNA ladder (GIBCO, Invitrogen Life Technologies, Rockville, MD) in 1% agarose gels.

Restriction digestions. Polymerase chain reaction (PCR) products were individually digested with the restriction enzymes *Eco*RI, *Rsa*I, and *Hae*III according to the manufacturer's directions (Boehringer Mannheim, Indianapolis, IN). *Eco*RI-, *Rsa*I-, and *Hae*III-digested DNA was subjected to electrophoresis at 3 V/cm in, respectively, 1.5, 3, and 4% NuSieve GTG agarose gels (FMC BioProducts, Rockland, ME) and stained with ethidium bromide.

DNA sequencing and phylogeny. Isolates for sequencing were chosen on the basis of VCG, race, geographic origin, and IGS haplotype. Appel and Gordon (6) designed a primer, U49:67 (AATACAAGCACGCCGACAC), based on the IGS sequence of *F. oxysporum* f. sp. *melonis.* This primer, in combination with CNL12, amplified a fragment of about 1,100 bp from all *F. oxysporum* isolates listed in Table 1. The PCR products of the two *F. moniliforme* isolates were slightly longer. These two primers were used to sequence the 5′ region of the IGS. PCR products were purified with a Prep-A-Gene purification kit (BioRad, Hercules, CA) according to the manufacturer's directions and sequenced in both directions at the DNA Sequencing Core Laboratory, University of Florida, Gainesville.

Sequences were aligned with ClustalX1.8 (40) and modified manually. Parsimony analyses were performed with PAUP, version 4.0b2 (D. L. Swofford, Sinauer Associates, Sunderland, MA), including and not including the single deletion event found in the aligned sequences. When it was included in the analysis, it was counted as a single character. Bootstrap analyses using the heuristic search option were performed with 1,000 replicates while retaining groups with more than 50% frequency to test the statistical support of each branch. Neighbor joining analyses were conducted

with ClustalX1.8, including and not including the deletion event, and maximum likelihood analyses were conducted with PHYLIP, version 3.572c (Joseph Felsenstein, Department of Genome Sciences, University of Washington, Seattle), including and not including the deletion event and with transition/transversion ratios of 1 or 2.

Generation of genomic DNA RFLP data. Two probes, pRL-82.8 and pRL172, from a genomic library of an isolate of *F. oxysporum* f. sp. *radicis-lycopersici* VCG 0094 (34), were used in separate hybridizations with *Eco*RV-digested DNA. Both probes revealed variable RFLP patterns when probed to DNA of both *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici.*

DNA extraction and generation of RFLP data followed previously published protocols (34,35). X-ray films were exposed at –80°C for 1 to 4 days, depending on the radioactive signal, and then developed.

Genomic DNA RFLP data analysis. Restriction fragments revealed by each probe were treated as distinct loci, and their presence and absence were scored as 1 and 0, respectively, to create binary data sets representing individual haplotypes.

The mean number of pairwise differences between RFLP haplotypes and its standard deviation were calculated according to Tajima (39). Average gene diversity and its standard deviation within each group were calculated according to Nei (31) and Tajima (38). To determine relatedness among isolates, RFLP

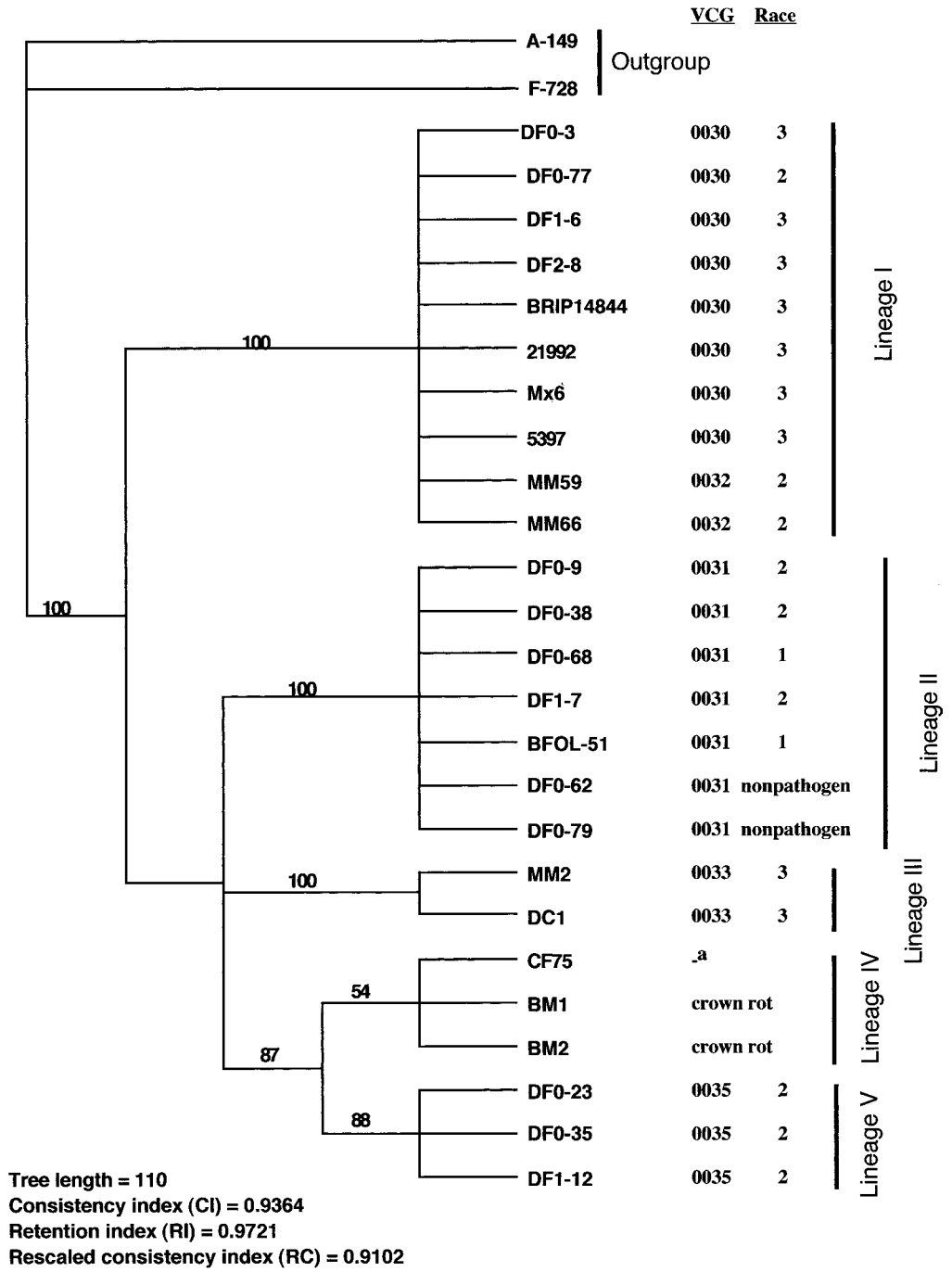

Fig. 2. Consensus tree of the three most parsimonious trees based on sequences of the 5′ intergenic spacer region of rDNA from isolates of *Fusarium oxysporum* f. sp. *lycopersici* and related strains. The computer program PAUP 4.0b2, beta version, was used. Insertion and deletion events were not included in the analysis. Bootstrap values are shown as percentages of 1,000 replicates. VCG = vegetative compatibility group. [a]Isolate CF75 was first reported as a member of a new VCG, 0034, of *F. oxysporum* f. sp. *lycopersici* but is now considered to belong to VCG 0094 of *F. oxysporum* f. sp. *radicis-lycopersici* (22).

haplotypes were subjected to phenetic analysis with neighbor joining search in PAUP, version 4.0b2.

RESULTS

Pathogenicity tests. Race determination was unambiguous. Disease severity ratings for isolates of specific races were between 4 and 5 on susceptible cultivars, while resistant cultivars did not show any symptoms. Of the 39 isolates in the California collection, one isolate was assigned to race 1, 22 isolates were assigned to race 2, and 13 isolates were assigned to race 3 (Table 1). The remaining three isolates caused no symptoms in any cultivars tested and were judged to be parasitic but nonpathogenic isolates. Pathogenicity tests for these three isolates were repeated at least three times with consistent results. The pathogenicity of the 18 isolates of *F. oxysporum* f. sp. *lycopersici* from other locations was previously reported, and our results confirmed these characterizations (Table 1).

Vegetative compatibility tests. Vegetatively compatible isolates formed a zone of wild-type growth (aerial mycelium) where the borders of expanding *nit* mutant colonies met, while vegetatively incompatible isolates did not. All isolates were self-compatible. Of the 39 isolates from the California collection, 22 were assigned to VCG 0030, and 11 were assigned to VCG 0031 (Table 1). VCG 0030 included race 2 and race 3 isolates, and VCG 0031 included race 1 and race 2 isolates. The three nonpathogenic isolates were vegetatively compatible with isolates of VCG 0031. Six isolates, all race 2, were incompatible with *nit* mutants of any of the isolates from known VCGs of FOL. These isolates were compatible with each other and caused typical vascular wilt symptoms in susceptible tomato cultivars. They were assigned to a new VCG, 0035, of *F. oxysporum* f. sp. *lycopersici.* Thirteen of the 18 isolates of *F. oxysporum* f. sp. *lycopersici* from other locations had previously been assigned to VCGs (14,28). Results of this study were in agreement with previous reports. The remaining five isolates (21991, Mx395, Mx2, Mx4, and Mx6) were in VCG 0030.

MM59, an isolate previously reported to be in VCG 0032, was confirmed to be vegetatively compatible with *nit* testers and with MM66, the other isolate in VCG 0032 in this study. However, it also formed heterokaryons with some members of VCG 0030 (Mx395, Mx2, Mx6, and 21992). In four repeated pairings, the interfaces between MM59 and these isolates formed discontinuous or continuous zones of wild-type growth, while isolates from the same VCG consistently formed continuous zones of wild-type growth.

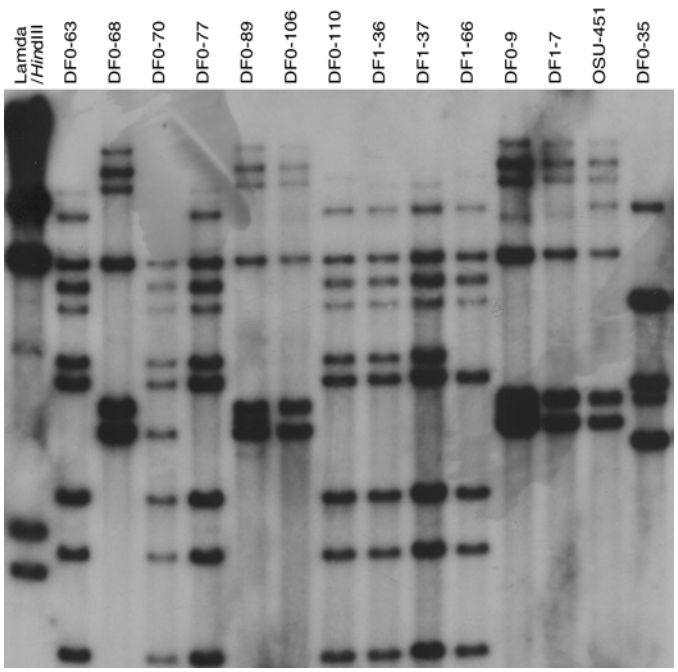

Fig. 3. Examples of restriction fragment length polymorphism patterns in genomic DNA of isolates of *Fusarium oxysporum* f. sp. *lycopersici* and related strains, revealed by probe pRL82.8.

PCR RFLP analysis of the IGS region. A single fragment, of approximately 2.6 kb, was amplified from the 60 isolates of *F. oxysporum* by PCR with primers CNL12 and CNS1. Restriction digestion with *Eco*RI, *Rsa*I, and *Hae*III produced two, three, and five restriction fragment patterns, respectively, and each unique pattern was designated by a letter (Fig. 1). A three-letter code was assigned to each isolate, and each code was considered to be represent a different IGS haplotype (Table 1). Five IGS haplotypes were identified among the 60 isolates of *F. oxysporum.*

The total size of the amplified fragment, estimated by summing the sizes of constituent restriction fragments, did not always add up to 2.6 kb. For example, pattern A produced by *Eco*RI consisted of two fragments of approximately 1.8 and 0.4 kb (Fig. 1). This discrepancy could have been caused by failure to detect very small fragments or by co-migrating fragments that could not be resolved (5).

The IGS haplotype was correlated with VCG (Table 1). Each VCG had a distinct IGS haplotype, except for VCG 0030 and VCG 0032, which had the same haplotype. The three nonpathogenic isolates in VCG 0031 had the same IGS haplotype as pathogenic isolates in this VCG. CF75, previously identified as *F. oxysporum* f. sp. *lycopersici,* had the same haplotype as the two isolates of *F. oxysporum* f. sp. *radicis-lycopersici.*

DNA sequencing and phylogeny. The 5′ IGS region of 25 isolates of *F. oxysporum* and two isolates of *F. moniliforme* were sequenced (Table 1). Approximately 1,000 bp were obtained from each isolate. Among the 25 isolates of *F. oxysporum,* five distinct sequences were found, which correlated with the five IGS haplotypes. No sequence differences were detected within an IGS haplotype. Single base substitutions at 33 positions were detected among the five distinct sequences of *F. oxysporum* in addition to a 15-bp deletion in VCG 0031. Of the substitutions, 23 were transitions and 11 were transversions (one position had both a transition and a transversion). Sequences were submitted to GenBank (available online at the GenBank website), and accession numbers AY118114 to AY118139 were assigned to isolates A-149, F-728, DF0-3, DF1-6, DF2-8, BRIP14844, 21992, Mx6, 5397, MM59, MM66, DF0-9, DF0-38, DF0-68, DF1-7, BFOL-51, DF0-62, DF0-79, MM2, DC1, DF0-23, DF0-35, DF1-12, CF75, BM1, and BM2, respectively, and accession number AY263991 was assigned to isolate DF0-77. The aligned sequences were also sent to TreeBASE (available online at the TreeBASE website), in which the study accession number is S879.

Parsimony analysis without counting the deletion event generated three most parsimonious trees. The strict consensus tree of the three most parsimonious trees with bootstrap values is shown in Fig. 2. Of the five resolved lineages in *F. oxysporum,* lineage IV (consisting of isolate CF75 and the two isolates of *F. oxysporum* f. sp. *radicis-lycopersici*) and lineage V (VCG 0035) were grouped together with strong bootstrap support (87%) (Fig. 2). Although lineage I (VCG 0030) was resolved from the clade consisting of lineages II (VCG 0031), III (VCG 0033), IV, and V, it was not well supported by bootstrap analysis. The bootstrap value grouping lineages II, III, IV, and V in a clade was below 50% and is not shown in Fig. 2. When the deletion event was included, parsimony analysis generated a consensus tree with similar branching patterns except that lineages II, III, IV, and V were not grouped in a clade. Neighbor joining trees and maximum likelihood trees confirmed that lineages IV and V are closely related to each other and that the relationship between lineages I, II, and III and the clade consisting of lineages IV and V was not consistently resolved.

Genomic DNA RFLPs. Probes pRL82.8 and pRL172 revealed a total of 26 and 51 restriction fragments, respectively. Examples of RFLP patterns revealed by probe pRL82.8 are shown in Fig. 3. Among all 60 *F. oxysporum* isolates, 49 haplotypes, designated H1 to H49, were identified (Table 1). H3 was the most frequent haplotype. It was shared by five race 3 isolates from California (DF0-36, DF0-41, DF0-63, DF1-36, and DF1-37) and one race 2 isolate from the same field (DF0-77). Shared haplotypes were not found among isolates from different VCGs or of different geographic origins.

To determine relatedness among isolates, RFLP haplotypes were subjected to phenetic analysis. Neighbor joining analysis of the genetic data in PAUP 4.0b2, beta version, produced a midpoint rooted tree (Fig. 4). Bootstrap values are shown as percentages of 1,000 replicates. A basal dichotomy with strong bootstrap support (89%) was observed. *F. oxysporum* f. sp. *lycopersici* isolates belonging to VCGs 0030 and 0032 formed one major branch, while *F. oxysporum* f. sp. *lycopersici* isolates belonging to VCGs 0031, 0033, and 0035, the two isolates of *F. oxysporum* f. sp. *radicis-lycopersici,* and isolate CF75 formed the other branch. Isolates in

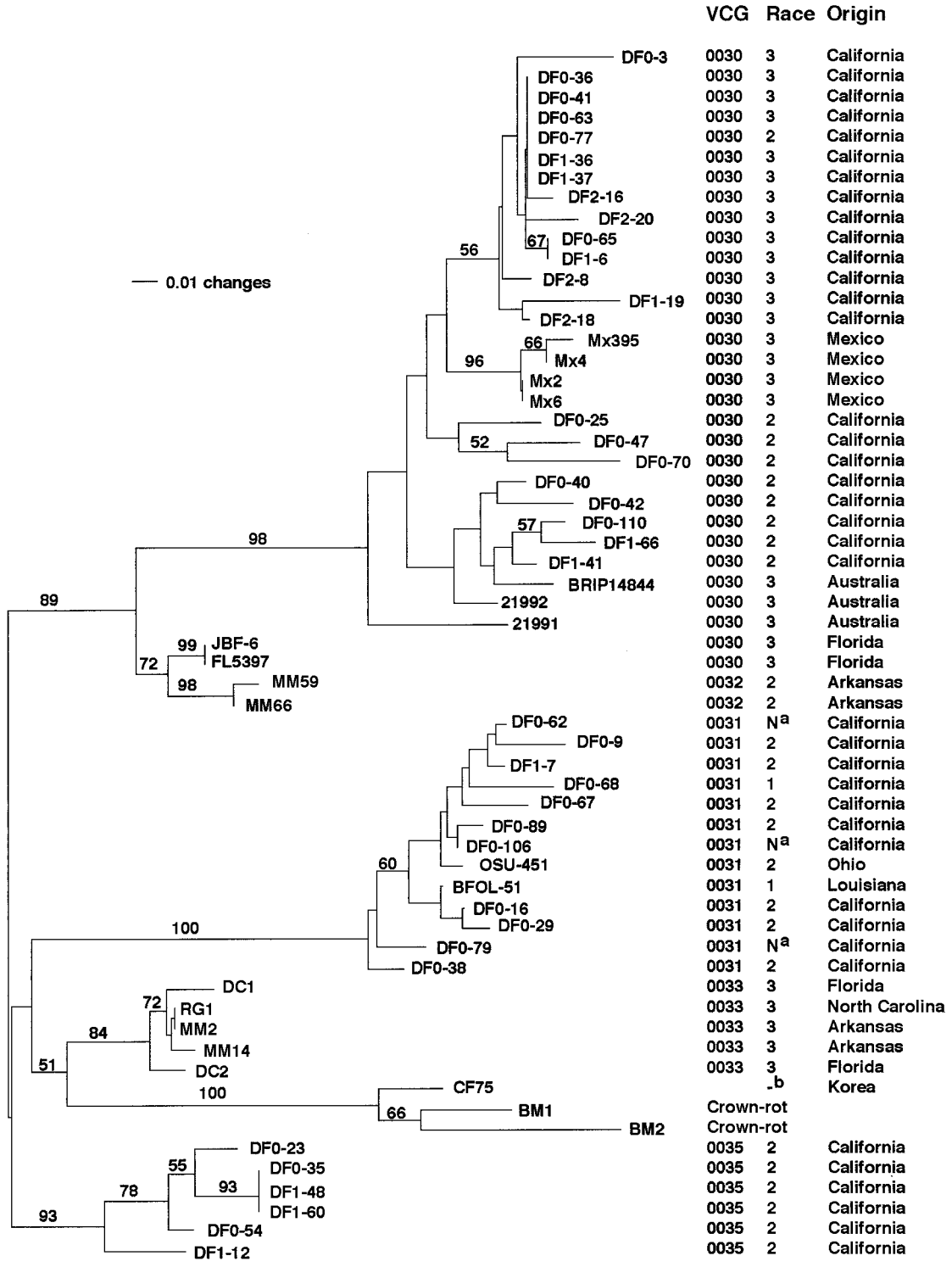

Fig. 4. Midpoint rooted neighbor joining tree based on restriction fragment length polymorphism haplotypes of genomic DNA of isolates of *Fusarium oxysporum* f. sp. *lycopersici* and related strains. Bootstrap values are shown as percentages from 1,000 repeats. The computer program PAUP 4.0b2, beta version, was used. VCG = vegetative compatibility group. [a]Nonpathogenic isolates. [b]Isolate CF75 was first reported as a member of a new VCG, 0034, of *F. oxysporum* f. sp. *lycopersici* but is now considered to belong to VCG 0094 of *F. oxysporum* f. sp. *radicis-lycopersici* (22).

these two branches were further divided into six groups with moderate to strong bootstrap support (72% or higher). The six groups correlated with VCG, with the only exception that two race 3 isolates in VCG 0030 from Florida, JBF-6 and 5397, formed a group with VCG 0032, supported by a 72% bootstrap value. The nonpathogenic isolates associated with VCG 0031 formed a group with pathogenic isolates in VCG 0031, with 100% bootstrap support. Isolate CF75 formed a group with BM1 and BM2, the two isolates of *F. oxysporum* f. sp. *radicis-lycopersici.*

In the California collection, race 3 isolates were in VCG 0030. Five of the 13 race 3 isolates had the same haplotype, H3 (Table 1), and all 13 isolates clustered together into a subgroup with a race 2 isolate, DF0-77, from the same collection, with 56% bootstrap support (Fig. 4). The mean number of pairwise differences (39) and average gene diversity (31,38) in the race 2 and race 3 isolates in VCG 0030 from California are shown in Table 2. Genetic diversity in race 3 isolates was about 32% of that in race 2 isolates.

DISCUSSION

Several studies showed that VCG is a good indicator of the evolutionary lineage of *F. oxysporum* f. sp. *lycopersici* (15,17,28) and other formae speciales of *F. oxysporum* (6,26). Results of this study, including the IGS haplotype and its partial sequence analysis and the genomic DNA RFLPs, support this conclusion. The exceptions to this general rule were the two isolates of race 3, in VCG 0030, from Florida, which were more closely related to isolates in VCG 0032 than to other isolates in VCG 0030. A close relationship between VCG 0030 and VCG 0032 has been demonstrated by analysis of a variety of markers, i.e., isozymes (15), mitochondrial DNA haplotypes (28), genomic DNA haplotypes (17), and random amplified polymorphic DNA (30). In the current study, isolates representing these two VCGs had identical IGS haplotypes and sequences, and analysis based on genomic DNA RFLPs grouped these two VCGs, with 89% bootstrap support. These lines of evidence indicate that these two VCGs have the most recent shared common ancestry, as has previously been suggested (17,30).

The isolates from California used in this study were recovered from a field in the Sacramento Valley in which race 3 of *F. oxysporum* f. sp. *lycopersici* had first been observed 2 years before, while race 2 was known to occur at that location for a much longer period of time. In a comparison of VCG 0030 isolates from the same field, the genetic diversity of race 3 isolates was found to be about one-third of that of race 2 isolates. This result supports the contention that race 3 was a more recently established population than race 2 at the time of sampling. We assume that after race 3 first appeared in this field, additional genetic changes accumulated in descendants of the original race 3 strain, leading to some genetic variation within the race 3 population. Considering the short time elapsed since race 3 was first observed at that location, we deem it reasonable to conclude that the highest proportion of the extant race 3 population would still be indistinguishable from the original race 3 clone. Of the 13 race 3 isolates examined from this field, five shared the same genomic DNA haplotype, H3, as revealed by probes pRL82.8 and pRL172. Though the remaining eight isolates were distributed among seven haplotypes, all 13 isolates were closely related and clustered together into a single subgroup in the neighbor joining tree. On the basis of these data, we suggest that race 3 isolates in this field are very likely to be descendants of an ancestor with the H3 haplotype. A less satisfactory explanation for the dominance of the H3 haplotype would be that the local environment favored isolates with this haplotype. However, we found that while the H3 haplotype also occurred among race 2 isolates in the field, its frequency was low; therefore, it is likely that a higher fitness for H3 haplotype isolates can be excluded.

Either race 3 in California was introduced from another location, or it originated from the local population of *F. oxysporum* f. sp. *lycopersici.* Before 1987, race 3 had been described in Queensland, Australia, in 1978 (19) and in Florida in 1982 (43). Isolates from both locations and race 3 isolates from Mexico (41) were included in the current study. While Australian isolates of race 3 in VCG 0030 were found to be highly diverse (the mean number of pairwise differences between haplotypes of the three race 3 isolates was 9.333), they were not found to be closely related to their counterparts in California (Fig. 3), and the race 3 isolates from Florida were either found to be more closely related to isolates in VCG 0032 than to other isolates in VCG 0030 or found to belong to the newly described VCG 0033 (28). On the other hand, the H3 haplotype was shared by a race 2 isolate, DF0-77, from the same field in California where race 3 isolates were collected.

Isolates of *F. oxysporum* f. sp. *lycopersici* can be disseminated in seed, transplants, or soil (7). While the dissemination of race 3 to California from another site cannot be completely excluded, it is highly unlikely. At the time when the isolations were made, the nearest reported occurrence of race 3 was nearly 5,000 km away, in Florida. Meanwhile, in California, in the 1980s, the tomato crop was direct-seeded with seeds that were produced locally. In addition, tomato seeds are extracted with hydrochloric acid, a process known to essentially eliminate *F. oxysporum* f. sp. *lycopersici* (3). Collectively, these findings strongly suggest that race 3 isolates recovered from this field in California originated from the local race 2 population, probably from a clone of DF0-77. An independent, local origin of race 3 may also be suggested for two other race 3 populations included in this study. The fact that race 3 isolates from Florida, associated with VCG 0030, are more closely related to isolates in VCG 0032 than to isolates in VCG 0030 suggests that race 3 in Florida had an independent origin. The Mexican population of race 3 isolates was also found to be distinct from other race 3 isolates in VCG 0030, which again suggests a local origin.

In this study we described a new VCG, 0035, of *F. oxysporum* f. sp. *lycopersici.* In a preliminary report (9), we named it VCG 0034. Although the Korean population that was proposed to be VCG 0034 of *F. oxysporum* f. sp. *lycopersici* (49) is now considered to belong to VCG 0094 of *F. oxysporum* f. sp. *radicis-lycopersici* (22), to avoid confusion 0034 should not be assigned to other VCGs of *F. oxysporum* f. sp. *lycopersici* (22). According to the numbering system for VCGs in *F. oxysporum* (25,33), and considering the current status of VCGs in *F. oxysporum* (21,22), we assigned these isolates to VCG 0035. Isolate MM59 in VCG 0032 was vegetatively compatible with some members of VCG 0030. Likewise, bridging isolates were reported in previous studies (24,30).

Very rarely has a relationship between pathogenic and nonpathogenic isolates of *F. oxysporum* been described in the literature. For example, Elias et al. (16) examined 471 isolates of *F. oxysporum* recovered from symptomless tomato roots and found that these nonpathogenic isolates were not vegetatively compatible with the previously established VCGs, 0030 to 0032, of *F.*

TABLE 2. Mean number of pairwise differences and average gene diversity of haplotypes based on genomic DNA restriction fragment length polymorphisms in race 2 and race 3 isolates in vegetative compatibility group 0030 of *F. oxysporum* f. sp. *lycopersici* from California

Race	Sample size	Mean number of pairwise differences and SD[a]	Gene diversity and SD[b]
2	9	8.500 (7.135)	0.110 (0.064)
3	13	2.718 (2.136)	0.035 (0.023)

[a] Mean number of pairwise differences and standard deviation according to Tajima (39).

[b] Average gene diversity and standard deviation according to Nei (31) and Tajima (38).

oxysporum f. sp. *lycopersici.* Similarly, in *F. oxysporum* f. sp. *vasinfectum* (23) and *F. oxysporum* f. sp. *cyclaminis* (47), co-occurring nonpathogenic isolates were not vegetatively compatible with pathogenic isolates. On the other hand, two nonpathogenic isolates of *F. oxysporum* f. sp. *spinaciae* (18) and four nonpathogenic isolates of *F. oxysporum* f. sp. *melonis* (4) were associated with VCGs containing the pathogens. However, in *F. oxysporum* f. sp. *melonis,* the four nonpathogenic isolates were differentiated from pathogenic isolates by sequence analysis of the 5′ IGS region (6).

We report here that three nonpathogenic isolates from California were vegetatively compatible with pathogenic isolates in VCG 0031. These nonpathogenic isolates had the same IGS haplotype and partial sequences as pathogenic isolates in this VCG, and genomic DNA RFLPs did not differentiate them from pathogenic isolates in the same VCG. This is in contrast to previous reports discussed above. Correll (11) suggested that the highly diverse and parasitic but nonpathogenic portion of the population of *F. oxysporum* represents the base population of this species. Mutation to virulence may occur, and if the mutation occurs in an isolate that is in close proximity to a susceptible host, this isolate may proliferate, eventually being recognized as an established VCG within a forma specialis. An alternative hypothesis is that nonpathogenic isolates within an established VCG represent revertants (from virulence back to avirulence).

On the basis of DNA sequences of translation elongation factor 1a and mitochondrial small-subunit ribosomal RNA genes, O'Donnell et al. (32) showed that several formae speciales of *F. oxysporum,* including *F. oxysporum* f. sp. *lycopersici,* were not monophyletic. In other words, isolates of a forma specialis might be more closely related to some isolates from other formae speciales and nonpathogenic isolates of *F. oxysporum* than to isolates from the same forma specialis. In this study, on the basis of partial IGS sequences, isolates in VCG 0035 were found to be more closely related to the isolates of *F. oxysporum* f. sp. *radicis-lycopersici* used in this study than to isolates in the other VCGs of *F. oxysporum* f. sp. *lycopersici.* This would indicate that VCG 0035 did not evolve from known VCGs of *F. oxysporum* f. sp. *lycopersici* and would further support a polyphyletic origin of this forma specialis.

ACKNOWLEDGMENTS

We thank J. C. Correll, V. Jurgens, D. Lawn, J. W. Scott, and S. H. Yu for providing isolates used in this study. We acknowledge the research support of the United States–Israel Binational Agricultural Research and Development (BARD) program in independent grants to the third and fourth authors. The advice of Talma Katan during the course of this investigation is gratefully acknowledged.

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