Population Structure of *Fusarium oxysporum* f. sp. *lycopersici:* Restriction Fragment Length Polymorphisms Provide Genetic Evidence That Vegetative Compatibility Group Is an Indicator of Evolutionary Origin

Karol S. Elias,[1] D. Zamir,[2] Tzili Lichtman-Pleban,[2] and Talma Katan[1]

[1]Department of Plant Pathology, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel, and [2]Department of Field and Vegetable Crops and Otto Warburg Center for Biotechnology in Agriculture, The Hebrew University of Jerusalem, Faculty of Agriculture, P.O. Box 12, Rehovot 76100, Israel.

Received 25 November 1991. Accepted 21 May 1993.

A worldwide collection of 47 isolates of *Fusarium oxysporum* f. sp. *lycopersici*, of two physiological races, and eight isolates of other formae speciales of *F. oxysporum* were subjected to DNA restricion fragment length polymorphism (RFLP) analysis. Fifty genomic clones from a library produced from an isolate of *F. o.* f. sp. *lycopersici* (FRC 0-1078, race 2) were used individually as probes in Southern hybridizations with DNA of the 55 isolates digested with four restriction enzymes. From the hybridization pattern observed after a high-stringency wash, it was estimated that the genome of *F. o.* f. sp. *lycopersici* is composed of 68, 12, and 20% single-copy, multiple-copy, and repetitive DNAs, respectively. Two clones were identified that were specific to vegetative compatibility group (VCG) 0030, the major VCG of *F. o.* f. sp. *lycopersici*, and another two clones differentiated *F. o.* f. sp. *lycopersici* from the other formae speciales. RFLP patterns were recorded as phenotypes, and a dendrogram was constructed based on cluster analysis of similarity coefficients using the unweighted pair-group method with arithmetic average. A principal components analysis revealed the relatedness of each isolate. Results indicate that isolates within each VCG are clonal derivatives of a common ancestor, and that races arose independently within each VCG. In addition, there is evidence to suggest that mutations have occurred in vegetative incompatibility loci, resulting in many single-member VCG isolates (VCG 003–) that cluster with VCGs. RFLP analysis of total genomic DNA reveals that the VCG can be a reliable indicator of evolutionary origin and population structure of *F. oxysporum*.

Additional keywords: genetic diversity, *Lycopersicon esculentum*, population genetics.

This research was supported by grant no. US-1408-87 and by postdoctoral fellowship (KSE) no. SU-0079-87 from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD).
We thank all contributors for supplying fungal isolates (Table 3).
Current address of first author: Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 19687, New Orleans, LA 70179.

MPMI, Vol. 6, No. 5, 1993, pp. 565-572

Fusarium wilt of tomato (*Lycopersicon esculentum* Mill.), caused by the soilborne vascular wilt pathogen *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W. C. Snyder & H. N. Hans. (Walker 1971), is a devastating disease that occurs in major tomato-growing regions of the world. The three known physiological races of *F. o.* f. sp. *lycopersici* are distinguished by their pathogenicity to tomato cultivars with specific dominant resistance genes. Races 1 and 2 are found in virtually all major tomato-growing regions, whereas race 3 is presently limited to Australia (Grattidge and O'Brien 1982), Florida (Volin and Jones 1982), and California (Davis *et al.* 1988). The mechanisms by which physiological races have developed in *F. oxysporum* are not known, but mutations at avirulence gene loci and recombination of such genes are possible explanations (van Kan *et al.* 1991). *F. oxysporum* is an imperfect fungus. Thus, in the absence of a known sexual cycle, it is not amenable to genetic analysis by conventional methods. Consequently, it is impossible to determine by crosses whether or not genes controlling the physiological race phenotypes of different strains are identical, allelic, or linked.

Whereas mutations can occur at virulence or avirulence loci of all strains, hyphal anastomosis is a prerequisite for gene flow and recombination in *F. oxysporum*. Hyphal anastomosis and the subsequent formation of stable heterokaryons can occur only between vegetatively compatible strains, belonging to the same vegetative compatibility

group (VCG), and strains that cannot form heterokaryons with each other are assigned to different VCGs (Puhalla 1985). Elias and Schneider (1991) utilized nitrogen metabolism mutants to study heterokaryosis between isolates of *F. o.* f. sp. *lycopersici* from various geographic locations. Among 115 isolates representing the three known physiological races, they identified one major VCG (0030), two minor VCGs (0031 and 0032), and many single-member VCGs (003–). Physiological race did not correlate with VCG, in that both race 1 and race 2 isolates occurred in all three VCGs, and race 3 isolates occurred both in VCG 0030 and as a single-member VCG. These findings can be explained in one of two ways: either 1) the development of races occurred before the formation of VCGs or 2) subsequent to the development of VCGs, the races evolved independently in each VCG.

Table 1. Characterization of the genomic library from *Fusarium oxysporum* f. sp. *lycopersici*

Clone	Size (bp)	Class[a]	VCG-specific RFLPs[b]
FG2	600	S	+
FG4	650	S	–
FG7	1,100	S	–
FG21	900	R	–
FG25	1,100	S	+
FG30	550	S	–
FG34	800	M	+
FG36	500	S	+
FG41	1,600	S	+
FG43	2,000	R	+
FG44	1,400	R	+
FG46	650	S	–
FG48	900	S	–
FG49	650	M	+
FG52	900	M	+
FG62	700	S	+
FG65	1,000	S	–
FG74	700	S	–
FG85	650	R	–
FG91	700	S	+
FG93	800	S	+
FG103	1,000	S	+
FG120	2,000	S	+
FG135	800	S	–
FG136	500	R	+
FG143	500	R	+
FG155	600	R	+
FG162	1,500	S	+
FG163	2,000	S	+
FG171	650	S	+
FG177	500	S	+
FG185	800	S	–
FG187	900	S	+
FG194	1,000	M	+
FG201	500	S	–
FG204	1,500	S	+
FG211	800	S	–
FG213	950	S	+
FG215	700	S	–
FG225	700	S	+
FG228	350	S	+
FG230	1,100	M	–
FG236	2,000	R	–
FG260	900	S	+
FG261	1,500	S	+
FG265	1,000	M	+
FG278	1,300	S	+
FG281	2,000	R	+
FG284	2,000	R	+
FG302	1,400	S	+
Mean	1,006		

[a] S = single-copy clone; M = multiple-copy clone; R = repetitive clone.

[b] Presence (+) and absence (–) of restriction fragment length polymorphisms (RFLPs) that differentiate vegetative compatibility groups (VCGs).

The phenotypic traits of vegetative compatibility and race depend on the expression of the respective genes, and race determination also depends on the expression of host genes. In that VCGs are considered genetically isolated populations, vegetative compatibility may serve as an indicator of relatedness or common ancestry among isolates. However, it does not reveal how closely related are isolates belonging to the same VCG or how distantly related are isolates belonging to different VCGs. Fortunately, other approaches can be used to investigate genetic relationships between fungal populations. First, isozyme polymorphisms have been used to examine genetic relationships between fungal populations. Bosland and Williams (1987), who examined isolates of *F. oxysporum* pathogenic on crucifers, found enzyme polymorphisms for three of 18 enzymes examined, and the polymorphisms were correlated with formae speciales and VCGs but not races. In addition, Elias and Schneider (1992), who used isozyme polymorphisms to examine genetic diversity in *F. o.* f. sp. *lycopersici*, found the distribution of isolates correlated with VCG rather than race, geographic origin, or forma specialis, and they concluded that *F. o.* f. sp. *lycopersici* arose from at least two progenitor populations. Second, variation in mitochondrial and nuclear DNA has been used to examine genetic relationships between fungal populations. Kistler *et al.* (1987) found mtDNA restriction fragment length polymorphisms (RFLPs) that corresponded directly to formae speciales of *F. oxysporum* pathogenic on crucifers. Jacobson and Gordon (1990), who examined isolates of *F. o.* f. sp. *melonis*, found distinct mtDNA RFLP patterns that corresponded to VCGs. In addition, Jabaji-Hare *et al.* (1990) and Koch *et al.* (1991) identified nuclear DNA RFLP patterns that differentiated anastomosis groups of *Rhizoctonia solani* and aggressive and nonaggressive isolates of *Leptosphaeria maculans*, respectively.

The objective of the study reported here was to obtain a quantitative estimate, at the genomic level, of the genetic diversity among isolates of *F. o.* f. sp. *lycopersici*. In particular, RFLP analysis of total genomic DNA was used to determine if there is greater similarity between isolates in the same VCG (with VCGs being considered to be clonal derivatives of a common ancestor) or between isolates of the same physiological race that share virulence traits. A preliminary report has been published (Elias *et al.* 1990).

RESULTS

Genome composition of *F. o.* f. sp. *lycopersici*.

Three hundred six putative recombinant genomic clones were isolated from the *Sau*3A library of *F. o.* f. sp. *lycopersici* (FRC 0-1078). DNA inserts ranged in size from

less than 100 to 2,000 bp. Fifty *F. o.* f. sp. *lycopersici* genomic clones (FG) with the largest inserts (average size 1 kb) (Table 1) were radiolabeled and hybridized individually to membranes that contained digested DNAs from 58 fungal isolates, including isolate FRC 0-1078, which had been used for the construction of the library.

The clones were classified as either single-copy, multiple-copy, or repetitive (Table 1) under high-stringency wash conditions, in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A clone was classified as single-copy if it hybridized to only a single DNA fragment with at least one of the four restriction enzymes. A clone was classified as multiple-copy if a minimum of two bands were detected with all restriction enzymes. A clone was classified as repetitive if a minimum of 10 bands were detected with all restriction enzymes or if a single very intense band with a continuous smear was detected after a very short film exposure time (2–4 hr). Of the 50 clones selected, 34 were classified as single-copy, 6 as multiple-copy, and 10 as repetitive (Table 1).

Several FG clones displayed specificity in Southern hybridization analysis. All the clones hybridized to the original isolate used to construct the library and to all isolates in VCG 0030, while only 48 hybridized to isolates in VCG 0031 or VCG 0032. Thus, the other two clones (the single-copy FG25 and FG36) contained DNA unique to VCG 0030. Four of the clones (FG2, FG25, FG36, and FG213) contained DNA unique to *F. o.* f. sp. *lycopersici*, in that no homology was detected with the other formae speciales tested. In addition, four repetitive clones (FG21, FG136, FG236, and FG284) and one single-copy clone (FG62) hybridized to the isolates of other fungal genera.

RFLP analysis.

Autoradiograms obtained from hybridizations of the genomic clones to DNAs of the 58 fungal isolates were scored on their overall banding patterns. Isolates that displayed identical banding patterns were assigned the same phenotype value for many FG clones, but no two fungal isolates were identical overall. Because four restriction enzymes and 50 genomic clones were used, there could have been 200 phenotype values (banding patterns) per isolate to contribute to the analysis; however, only 181 phenotype values (banding patterns) were amenable to unequivocal scoring and were included in our analysis. The number of phenotypes observed for a single clone varied from one to 14. The most informative enzyme in revealing polymorphisms was *Eco*RI, followed by *Eco*RV, *Dra*I, and *Hae*III. Thirty-four clones revealed RFLPs that differentiated the VCGs in *F. o.* f. sp. *lycopersici* (Table 1), while the three clones FG155, FG278, and FG281 showed RFLPs that differentiated *F. o.* f. sp. *lycopersici* from the other formae speciales of *F. oxysporum* examined.

A simple matching coefficient of similarity was calculated for each pair of fungal isolates to allow a quantitative comparison of the RFLP phenotype values. However, because of the large size of this table, an abbreviated similarity matrix is presented (Table 2), giving the means of similarities within and between VCGs of *F. o.* f. sp. *lycopersici* and two other formae speciales of *F. oxysporum*. The coefficients of similarity in Table 2 demonstrate the following: isolates were genetically more similar to other isolates in the same VCG than to those in other VCGs; isolates in VCG 0032 were more similar to isolates in VCG 0030 (coefficient of similarity 0.644) than to those in VCG 0031 (0.321); and isolates in VCG 0031 were more similar to the other formae speciales (0.325 and 0.283) than to isolates in VCGs 0030 and 0032 (0.235 and 0.321).

The dendrogram generated by cluster analysis using the unweighted pair–group method with arithmetic average (UPGMA) is presented in Figure 1. In order to reduce the size of the tree, only two fungal isolates from other formae speciales of *F. oxysporum* are included. The additional six isolates from other formae speciales and three other fungi had even smaller simple matching coefficients of similarity (<0.200) than the strains included. The clustering patterns revealed in the dendrogram are similar to those demonstrated in the similarity matrix. In total, five isolates (PHW-554, T-1, IA-9, JBF-1, and FRC 0-1082) from single-member VCGs clustered with VCG 0030, four (FOLR5-6, BFOL-57, FA-14, and BFOL-56) with VCG 0032, and three (PHW-555, F-23, and UCD-1) with VCG 0031. The VCG 0032 cluster was more similar to VCG 0030 (coefficient of similarity 0.770) than to VCG 0031 (0.440), and no clustering of isolates by race was observed.

Principal components analysis (PCA) provided more information regarding the relative position of each isolate with respect to all isolates examined. The first five principal components accounted for 32.2, 15.6, 7.4, 6.5, and 5.1% of the variability, respectively. No other factors ac-

Table 2. Simple matching coefficients of similarity based on restriction fragment length polymorphisms for strains of *Fusarium oxysporum* f. sp. *lycopersici* and other formae speciales of *F. oxysporum*[a]

	VCG[b]			*F. o.* f. sp. *melonis*	*F. o.* f. sp. *dianthi*
	0030	**0031**	**0032**	**FOM 331/2[c]**	**CSU-1[c]**
VCG 0030	0.802 (0.073)	0.235 (0.039)	0.644 (0.055)	0.194 (0.030)	0.202 (0.023)
VCG 0031		0.722 (0.111)	0.321 (0.034)	0.325 (0.032)	0.283 (0.028)
VCG 0032			0.826 (0.049)	0.255 (0.023)	0.274 (0.023)
F. o. f. sp. *melonis* FOM 331/2				...	0.558
F. o. f. sp. *dianthi* CSU-1					...

[a] Average similarity, with standard error in parentheses.
[b] Vegetative compatibility groups are numbered according to Puhalla (1985).
[c] Strain numbers from Table 3.

counted for more than 5.0% of the variability. A plot of the first and second principal components is presented in Figure 2. The patterns of clustering found in the similarity matrix (Table 2) and the dendrogram (Fig. 1) can also be found in the PCA. Isolates are clustered by VCG, and VCG 0032 is clustered more tightly with VCG 0030 than with VCG 0031. Several isolates from single-member VCGs clustered with each VCG cluster. Isolates clustered by VCG rather than by physiological race or geographic origin, and isolates from other formae speciales of *F. oxysporum* did not cluster with *F. o.* f. sp. *lycopersici*.

DISCUSSION

Despite the fact that *F. oxysporum* is not amenable to conventional genetic analysis, here we provide a first

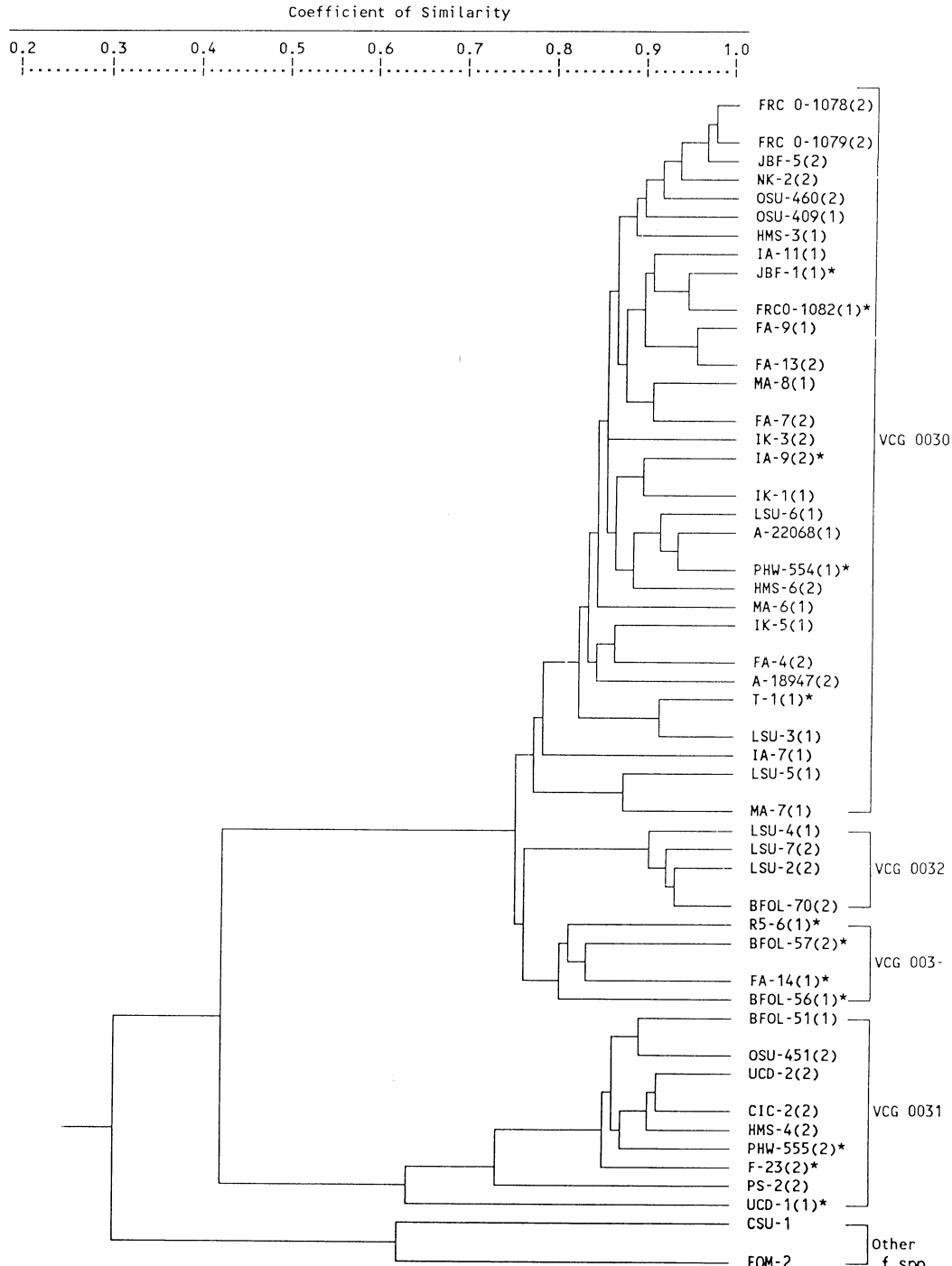

Fig. 1. A dendrogram generated by cluster analysis of pairwise simple matching coefficients of similarity between isolates of *Fusarium oxysporum* f. sp. *lycopersici* and other formae speciales of *F. oxysporum*, using the unweighted paired-group method with arithmetic averaging (UPGMA). The scale shows the genetic similarity, which can range from 1 (isolates are identical) to 0 (no restriction fragment length polymorphism patterns in common between isolates). Race designation is given in parentheses. Asterisks indicate isolates that are single members of a vegetative compatibility group.

sketch of genome organization in *F. o.* f. sp. *lycopersici*. The proportion of single-copy, multiple-copy, and repetitive DNA clones reported here (68, 12, and 20%, respectively) should be representative of the entire genome, in that a random genomic library was utilized. However, in that it is unlikely that our repetitive clones are composed entirely of repetitive DNA sequences, the figure 20% is the upper-limit estimate of the amount of repetitive DNA in the genome. While we are aware of no studies that have characterized a fungal genome in this manner, the genomic compositions of several plant species have been similarly determined: tomato (Zamir and Tanksley 1988), wheat (Flavell 1980), and pea (Murray *et al.* 1981). The proportion of single-copy sequences in those genomes ranged from less than 20 to 78%, and our estimates for *F. o.* f. sp. *lycopersici*, with 68% single-copy sequences, fall within this range. In addition, Zamir and Tanksley (1988) estimated that 4% of the tomato genome is repetitive under high-stringency conditions, whereas a relatively high portion of the genome of *F. o.* f. sp. *lycopersici* (20%) was repetitive under the same stringency conditions. Furthermore, using DNA reassociation kinetics, Timberlake (1978) estimated that 97–98% of the nuclear DNA of *Aspergillus nidulans* consists of unique sequences, while the remaining 2–3% is repetitive and probably coded for rRNA, and Francis *et al.* (1990) found that about 65% of the nuclear DNA of *Bremia lactucae* is repetitive. Thus, our estimates presented here fall within the ranges observed for other plant and fungal systems.

This study provides an example of how RFLPs can be used to answer questions concerning the evolution of specific traits (VCG and physiological race) and reveal the population structure of imperfect fungi. While our collection of *F. o.* f. sp. *lycopersici* is not considered representative of the world population, much detail of the population structure of *F. o.* f. sp. *lycopersici* has been revealed, in that the isolates used (especially those in the major VCG, 0030) originated from five continents. First, we have demonstrated that VCGs, which are defined on the basis of the ability to form heterokaryons, do constitute genetically distinct subpopulations of *F. o.* f. sp. *lycopersici*. Brasier (1987) put forth the concept that in entirely non-outcrossing or in imperfect fungi, vegetative incompatibility automatically becomes the primary isolating mechanism. In this study, a high frequency of variation at the DNA level (RFLPs) was revealed when isolates from different VCGs and formae speciales were compared. This variation did not correlate with the geographic origin or physiological race of isolates, and less variation was observed between isolates in the same VCG, even though it encompassed isolates of diverse geographic origin and more than one physiological race. No hybrid isolates displaying restriction fragment patterns characteristic of more than one VCG were found. Were there no isolation between VCGs, the pattern in which isolates clustered would have revealed a population structure linked to some other factor (i.e., geography, physiological race, etc.). In a similar study, Jacobson and Gordon (1990) examined mtDNA restriction fragment patterns to define genetically isolated populations of *F. o.* f. sp. *melonis* that correlated with VCGs. In addition, RFLPs have been used to confirm that biological species of *Armillaria mellea* (Anderson *et al.* 1987) and subgroups in the *Phytophthora megasperma* complex (Forster *et al.* 1989) are distinct entities within which genetic divergence has occurred. They have also been used to confirm genetic divergence between and within anastomosis groups of *Rhizoctonia solani* (Jabaji-Hare *et al.* 1990) and between aggressive and nonaggressive groups of *Leptosphaeria maculans* (Koch *et al.* 1991).

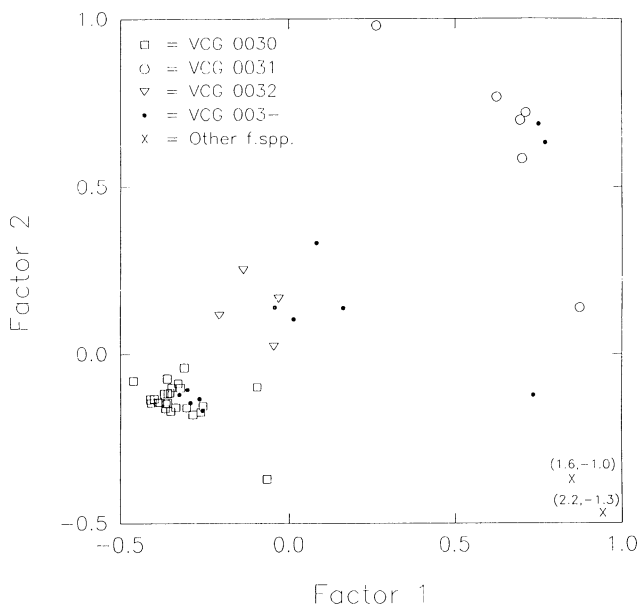

Fig. 2. A plot of 47 isolates of *Fusarium oxysporum* f. sp. *lycopersici* and two other formae speciales of *F. oxysporum*, projected onto the first and second principal component axes. Factors 1 and 2 account for 32.2 and 15.6% of the variability, respectively. Each symbol represents one isolate. Numbers in parentheses indicate coordinates for isolates that were off the scale.

Second, isolates within a VCG appear to have arisen from a common ancestral progenitor. In that isolates in VCGs 0030 and 0032 often exhibited identical RFLPs (for many probes), it is possible that VCG 0032 diverged from VCG 0030 more recently than VCG 0031 did. Manicom *et al.* (1987, 1990) found a correlation between RFLP pattern and VCG, which corresponded to race in *F. oxysporum* f. sp. *dianthi*. In the formae speciales of *F. oxysporum* that attack crucifers, a common ancestry was inferred from RFLP analysis of isolates in each VCG and forma specialis, regardless of physiological race, which was consistent with other criteria for relatedness (Kistler *et al.* 1991).

Third, in *F. o.* f. sp. *lycopersici*, the races seem to have arisen independently within each VCG. This suggests that not all isolates of a given physiological race are necessarily identical or clonal derivatives, notwithstanding their common phenotype on a given set of differential cultivars. In that they probably have multiple origins, they may also differ genetically. In addition, we provide indirect evidence to suggest that mutations have occurred in putative vegetative incompatibility genes. Several single-member VCG 003– isolates cluster tightly with multiple-member

VCGs, indicating a high degree of DNA homology and a common origin. Given what we know about vegetative incompatibility systems in other fungi (Anagnostakis 1982; Perkins *et al.* 1982; Puhalla and Spieth 1983), it is plausible that single-gene mutations in putative vegetative incompatibility genes could be responsible for the large number of VCG 003– isolates.

DNA probes showing different degrees of variability or specificity should be useful in future studies. Probes that specifically hybridize only with *F. oxysporum* can be used as naturally occurring genetic markers in other studies of *F. oxysporum*. Induced genetic markers in *F. oxysporum* are obtained only after considerable time and effort, and the effect of mutagenic agents on the overall fitness of an organism is unknown. Specificity for *F. o.* f. sp. *lycopersici* (as revealed by probes FG2 and FG213) and RFLPs that differentiate *F. o.* f. sp. *lycopersici* from other formae speciales of *F. oxysporum* (as revealed by probes FG155, FG278, and FG281) can serve as genetic markers for ecological or population dynamics studies. Probes that distinguish between VCGs or that specifically hybridize to isolates of one VCG can be used to assign new isolates to VCGs. It is unfortunate that probes that are diagnostic for races were not identified. Additionally, by comparing observed RFLPs of strains of *F. o.* f. sp. *lycopersici*, the ancestry of most isolates in single-member VCGs (VCG 003–) can be postulated. Finally, this RFLP analysis technique provides a way to identify the genetic relationships between pathogenic races, formae speciales, and nonpathogenic populations of *F. oxysporum*. Although this approach provides another classification scheme for strains, it appears to be compatible with the currently recognized and used scheme for naming formae speciales (and races), which relies on the specificity of host species (and cultivars). Although this is just the beginning of the use of DNA probes to study the population genetics of *F. o.* f. sp. *lycopersici*, this work supports and validates the natural taxonomic groups provided by the formae speciales–VCG naming system (Elias and Schneider 1988, 1991, 1992).

Table 3. Fungal strains used in this study

Fungus	VCG[a]	Strain[b]	Race[c]	Source[d]	Origin
Fusarium oxysporum f. sp. *lycopersici*	0030	LSU-3	1	a	Louisiana
		LSU-5	1	a	Louisiana
		LSU-6	1	a	Louisiana
		OSU-409	1	b	Ohio
		OSU-460	2	b	Ohio
		HMS-3 (#74)	1	c	California
		HMS-6 (#80)	2	c	California
		FRC 0-1078	2	d	Florida
		FRC 0-1079	2	d	Florida
		NK-2	2	e	Florida
		JBF-5 (8)	2	f	Florida
		A-22068	1	g	Australia
		A-18947	2	g	Australia
		IK-1 (ATCC 66044)	1	h	Israel
		IK-3 (FOL-R)	2	h	Israel
		IK-5 (FOL-650)	1	h	Israel
		MA-6 (FK3)	1	i	Morocco
		MA-7 (MB6)	1	i	Morocco
		MA-8 (M6)	1	i	Morocco
		FA-4 (FOL15)	2	i	Tunisia
		FA-7 (FOL27)	2	i	France
		FA-9 (FOL29)	1	i	France
		FA-13 (FOL33)	2	i	France
		IA-7 (FOL77)	1	i	Italy
		IA-11 (FOLV)	1	i	Italy
	0031	BFOL-51	1	j	Louisiana
		OSU-451	2	b	Ohio
		HMS-4 (#65)	2	c	California
		PS-2	2	k	California
		UCD-2 (1776)	2	l	California
		CIC-2	2	m	California
Fusarium oxysporum f. sp. *lycopersici* (cont.)	0032	LSU-2	2	a	Louisiana
		LSU-4	1	a	Louisiana
		LSU-7	2	a	Louisiana
		BFOL-70	2	j	Louisiana
	003–	JBF-1 (626-6B)	1	f	Florida
		FRC 0-1082	1	d	Florida
		UCD-1 (1775)	1	l	California
		F-23	2	n	Maryland
		BFOL-56	1	j	Arkansas
		BFOL-57	2	j	Arkansas
		FOL R5-6	1	o	Wisconsin
		PHW-554	1	p	Wisconsin
		PHW-555	2	p	Wisconsin
		T-1	1	q	Taiwan
		FA-14 (FOL62)	1	i	Senegal
		IA-9 (FOL1)	2	i	Italy
F. o. f. sp. *asparagi*	...	FOA-10	...	r	Michigan
	...	FOA-22	...	r	Michigan
F. o. f. sp. *dianthi*	...	CSU-1	...	s	Colorado
	...	FOD-E114 (ATCC 66048)	...	h	Israel
F. o. f. sp. *radicis-lycopersici*	...	FA-1 (FORL-19)	...	i	France
	...	FORL-C63F	...	h	Israel
F. o. f. sp. *melonis*	...	FOM-318 (ATCC 66052)	...	h	Israel
	...	FOM-331/2	...	h	Israel
Pythium aphanidermatum	...	P-1	...	t	Israel
Trichoderma harzianum	...	TH-35	...	t	Israel
Rhizoctonia solani	...	RS	...	t	Israel

[a] Vegetative compatibility groups (VCGs) are numbered according to Puhalla (1985). VCG 003– is an artificial group containing isolates that are single members of a VCG.

[b] Original strain number in parentheses.

[c] Race of strains was determined with the appropriate tomato differential cultivars Walter (resistant to races 1 and 2), Supersonic (resistant to race 1), and Fantastic (susceptible to all three races).

[d] a = Author (KSE); b = R. Rowe; c = K. Kimble; d = Fusarium Research Center, Pennsylvania State University, University Park; e = R. Volin; f = J. P. Jones; g = R. G. O'Brien; h = T. Katan; i = C. Alabouvette; j = L. Black; k = J. Watterson; l = R. G. Grogan; m = H. Bolkan; n = T. H. Barksdale; o = T. Isakeit; p = P. Bosland; q = S. K. Sun; r = W. Elmer; s = R. Baker; t = I. Chet.

MATERIALS AND METHODS

Fungal strains.

Forty-seven strains of *F. o.* f. sp. *lycopersici* (Table 3) were chosen from the collection of K. S. Elias and R. W. Schneider, Louisiana State University, Baton Rouge. These strains encompass the diversity observed in *F. o.* f. sp. *lycopersici* with respect to geographic origin, VCG, and physiological race (excluding race 3) (Elias and Schneider 1991). The race designation of all strains was verified by greenhouse pathogenicity tests on the appropriate differential tomato cultivars. In addition, eight isolates of *F. oxysporum* from other formae speciales and three isolates from other fungal genera were included for comparison (Table 3). Single spores or hyphal tips of each isolate were transferred to potato-dextrose agar, and isolates were then stored on filter paper.

Tissue preparation.

Each isolate was grown in five 250-ml Erlenmeyer flasks, each containing 100 ml of potato-dextrose broth, for 5 days on an orbital shaker (125 rpm) at 28° C. Mycelium was collected on Whatman No. 1 filter paper by vacuum filtration and rinsed with sterile distilled water. The mycelium was transferred to a petri dish, frozen (–20° C), lyophilized, and stored at –70° C until needed for extraction of DNA.

DNA extraction.

Total genomic DNA was extracted by a procedure modified from Lee *et al.* (1988) and Zolan and Pukkila (1986). A 300-mg aliquot of lyophilized mycelium was ground with an equal amount of sterile acid-washed sand, placed in a 1.5-ml microfuge tube with 700 µl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate [SDS], plus 7.5 µl of 1 M dithiothreitol), and incubated at 65° C for 1 hr. The lysate was extracted with 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) followed by 600 µl of chloroform/isoamyl alcohol (24:1 v/v). The DNA solution was treated with 50 µg of RNase A for 30 min at 37° C and then extracted again with phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol. DNA was precipitated with 20 µl of 3 M NaOAc and 700 µl of ice-cold isopropanol. After the DNA was allowed to dissolve overnight in 400 µl of TE (10 mM Tris-HCl plus 1 mM EDTA, pH 8.0), a 2-µl aliquot was electrophoresed simultaneously with 100 ng of *Hin*dIII-digested lambda DNA to determine the quality and concentration.

Cloning of random DNA fragments.

Standard protocols were used throughout (Maniatis *et al.* 1982). Total DNA (50 µg) from isolate FRC 0-1078 (race 2, VCG 0030) of *F. o.* f. sp. *lycopersici* was partially digested with *Sau*3A and ligated into the *Bam*HI site of the plasmid pBluescript+SK (Stratagene, La Jolla, CA). The ligation mixture was transformed into competent *Escherichia coli* XL-1-Blue cells (Stratagene), and colonies that contained inserts were selected by screening for ampicillin resistance and lack of β-galactosidase activity according to the manufacturer's instructions. All putative transformants were selected and grown on Luria-Bertani medium overnight, and plasmids were extracted by the alkaline lysis plasmid preparation procedure (Maniatis *et al.* 1982). The presence of an insert of DNA from *F. o.* f. sp. *lycopersici* was verified and the insert size was determined by electrophoresis in 2% agarose gels, which included *Hin*dIII-digested lambda DNA as a size standard.

Southern blotting and hybridization.

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and reactions were done according to the manufacturer's recommendations. Four restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, and *Hae*III) were routinely used to detect DNA RFLPs. DNA in 10-µg aliquots was incubated with 30 units of enzyme for 6–8 hr at 37° C. Electrophoresis of DNA was in 20 × 25 × 0.4-cm 0.9% agarose gel (for *Dra*I, *Eco*RI, and *Eco*RV) and in 1.4% agarose gel (for *Hae*III) in neutral electrophoresis buffer (100 mM Tris-HCl, 0.25 mM EDTA, plus 12.5 mM NaOAc, pH 8.1) (Palmer and Zamir 1982) at 75 mA for 18 hr. A 1-kb ladder (Bethesda Research Laboratories, Gaithersburg, MD) was included in gels, so that restriction fragment sizes could be estimated. The DNA was visualized by ultraviolet illumination (302 nm) after the gels had been stained with ethidium bromide (0.5 µg/ml). After the gels were photographed, a modified alkaline blotting method (Maniatis *et al.* 1982) was used for capillary transfer of DNA from gels to nylon Genescreen Plus hybridization membranes (NEN Research Products, Boston, MA). In that a total of 60 DNAs (all fungal isolates and standards) were examined and a single gel could accommodate 20 samples, three gels were requisite for a single restriction enzyme. Four restriction enzymes were used separately, and so 12 gels blotted onto 12 membranes accommodated all DNAs and all restriction enzymes. Eight replicate sets of membranes (12 membranes per set) were prepared, so that eight DNA probes could be utilized per hybridization experiment. Plasmid probes were labeled with [α-^{32}P]dCTP by random hexamer labeling (Feinberg and Vogelstein 1983). Prehybridization and hybridization reactions were done in plastic trays at 68° C according to the membrane manufacturer's recommendations. Membranes were washed at 65° C for 30 min in 2× SSC plus 0.1% SDS, then in 1× SSC plus 0.1% SDS, and finally in 0.5× SSC plus 0.1% SDS. The membranes were wrapped in plastic and exposed to Kodak X-Omat AR film in cassettes with intensifying screens for 4 hr to 4 days at –70° C. Labeled DNA probes were removed from the membranes before reprobing according to the manufacturer's recommendations. In this way, each membrane could be probed at least 10 times, allowing the analysis of many DNA probes.

Data analysis.

The copy number for each DNA probe, restriction fragment sizes, and polymorphisms were determined from developed autoradiographs. The presence or absence of a

particular polymorphism was recorded and RFLP pattern phenotypes were assigned for each of the 50 clones hybridized to DNAs cut with each of the four restriction enzymes. The relative intensity of bands was ignored. For analysis of the RFLP data, a computer program, NTSYS-PC version 1.60 (Exeter Publishing, New York), was used. Simple matching coefficients (S_{sm}) for each pair of isolates were calculated as described by Sneath and Sokal (1973) by the formula

$$S_{sm} = m/(m+u)$$

where m is the number of bands found in common between the two isolates, and u is the total number of bands unique to each isolate. The matrix of similarity coefficients was then subjected to cluster analysis using UPGMA (Sneath and Sokal 1973) in order to construct a dendrogram. PCA (Gower 1966) was also employed in an attempt to resolve phylogenetic groups. The data matrix was first standardized by subtracting the mean of each variable and then dividing by the standard deviation. Product-moment correlation coefficients between variables were calculated based on the standardized data. Eigenvectors were extracted from the correlation matrix, and the standardized data were projected onto the eigenvectors. The percentage of variance that each principal component contributed was used to determine the number of principal components to be represented graphically, and no attempts were made to name the factors.

LITERATURE CITED

Anagnostakis, S. L. 1982. Genetic analyses of *Endothia parasitica:* Linkage data for four single genes and three vegetative compatibility types. Genetics 102:25-28.

Anderson, J. B., Petsche, D. M., and Smith, M. L. 1987. Restriction fragment polymorphisms in *Armillaria mellea*. Mycologia 79:69-76.

Bosland, P. W., and Williams, P. H. 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogencity, isozyme polymorphism, vegetative compatibility, and geographic origins. Can J. Bot. 65:2067-2073.

Brasier, C. M., 1987. The dynamics of fungal speciation. Pages 231-260 in: Evolutionary Biology of the Fungi. A. D. M. Rayner, C. M. Brasier, and D. Moore, eds., Cambridge University Press, Cambridge.

Davis, R. M., Kimble, K. A., and Farrar, J. J. 1988. A third race of *Fusarium oxysporum* f. sp. *lycopersici* identified in California. Plant Dis. 72:453.

Elias, K. S., Katan, T., and Zamir, D. 1990. RFLP based phylogeny of *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*) reveals no association between race and genetically isolated populations. (Abstr.) Phytopathology 80:980.

Elias, K. S., and Schneider, R. W. 1988. Isozyme analysis of races and vegetative compatibility groups of *Fusarium oxysporum* f. sp. *lycopersici*. (Abstr.) Phytopathology 78:1543.

Elias, K. S., and Schneider, R. W. 1991. Vegetative compatibility groups in *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology 81:159-162.

Elias, K. S., and Schneider, R. W. 1992. Genetic diversity within and among races and vegetative compatibility groups of *Fusarium oxysporum* f. sp. *lycopersici* as determined by isozyme analysis. Phytopathology 82:1421-1427.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Flavell, R. B. 1980. The molecular characterization and organization of plant chromosomal DNA sequences. Annu. Rev. Plant Physiol. 31:569-596.

Forster, H., Kinscherf, T. G., Leong, S. A., and Maxwell, D. P. 1989. Restriction fragment length polymorphisms of the mitochondrial DNA of *Phytophthora megasperma* isolated from soybean, alfalfa, and fruit trees. Can. J. Bot. 67:529-537.

Francis, D. M., Hulbert, S. H., and Michelmore, R. W. 1990. Genome size and complexity of the obligate fungal pathogen, *Bremia lactucae*. Exp. Mycol. 14:299-309.

Gower, J. C. 1966. Some distance properties of latent root and vector methods in multivariate analysis. Biometrika 53:325-338.

Grattidge, R., and O'Brien, R. G. 1982. Occurrence of a third race of Fusarium wilt of tomato in Queensland. Plant Dis. 66:165-166.

Jabaji-Hare, S. H., Meller, Y., Gill, S., and Charest, P. M. 1990. Investigation of genetic relatedness among anastomosis groups of *Rhizoctonia solani* using cloned DNA probes. Can. J. Plant Pathol. 12:393-404.

Jacobson, D. J., and Gordon, T. R. 1990. Variability of mitochondrial DNA as an indicator of relationships between populations of *Fusarium oxysporum* f. sp. *melonis*. Mycol. Res. 94:734-744.

Kistler, H. C., Bosland, P. W., Benny, U., Leong, S., and Williams, P. H. 1987. Relatedness of strains of *Fusarium oxsporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. Phytopathlogy 77:1289-1293.

Kistler, H. C., Momol, E. A., and Benny, U. 1991. Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. Phytopathology 81:331-336.

Koch, E., Song, K., Osborn, T. C., and Williams, P. H. 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol. Plant-Microbe Interact. 4:341-349.

Lee, S. B., Milgroom, M. G., and Taylor, J. W. 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Genet. Newsl. 35:23-24.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Manicom, B. Q., Bar-Joseph, M., Kotze, J. M., and Becker, M. M. 1990. A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxyporum* f. sp. *dianthi*. Phytopathology 80:336-339.

Manicom, B. Q., Bar-Joseph, M., Rosner, A., Vigodsky-Hass, H., and Kotze, J. M. 1987. Potential applications of random DNA probes and restriction fragment length polymorphisms in the taxonomy of the fusaria. Phytopathology 77:669-672.

Murray, M. G., Peters, D. L., and Thompson, W. F. 1981. Ancient repeated sequences in pea and mung bean genomes and implications for genome evolution. J. Mol. Evol. 17:31-42.

Palmer, J. D., and Zamir, D. 1982. Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. Proc. Natl. Acad. Sci. U.S.A. 79:5006-5010.

Perkins, D. D., Radford, A., Newmeyer, D., and Bjorkman, M. 1982. Chromsomal loci of *Neurospora crassa*. Microbiol. Rev. 46:426-570.

Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63:179-183.

Puhalla, J. E., and Spieth, P. T. 1983. Heterokaryosis in *Fusarium moniliforme*. Exp. Mycol. 7:328-335.

Sneath, P. H., and Sokal, R. R. 1973. Numerical Taxonomy. W. H. Freeman, San Francisco.

Timberlake, W. E. 1978. Low repetitive DNA content in *Aspergillus nidulans*. Science 202:973-975.

van Kan, J. A. L., van den Ackerveken, G. F. J. M., and de Wet, P. J. G. M. 1991. Cloning and characterization of cDNA of avirulence gene *avr*9 of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. Mol. Plant-Microbe Interact. 4:52-59.

Volin, R. B., and Jones, J. P. 1982. A new race of Fusarium wilt of tomato in Florida and sources of resistance. Proc. Fla. State Hortic. Soc. 95:268-270.

Walker, J. C. 1971. Fusarium Wilt of Tomato. Monogr. 6. American Phytopathological Society, St. Paul, MN.

Zamir, D., and Tanksley, S. D. 1988. Tomato genome is comprised largely of fast-evolving, low copy-number sequences. Mol. Gen. Genet. 213:254-261.

Zolan, M. E., and Pukkila, P. J. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. 6:195-200.