Molecular Characterization of Races and Vegetative Compatibility Groups in *Fusarium oxysporum* f. sp. *vasinfectum*

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Restriction fragment length polymorphism (RFLP) and vegetative compatibility analyses were undertaken to assess genetic relationships among 52 isolates of *Fusarium oxysporum* f. sp. *vasinfectum* of worldwide origin and representing race A, 3, or 4 on cotton plants. Ten distinct vegetative compatibility groups (VCGs) were obtained, and isolates belonging to distinct races were never in the same VCG. Race A isolates were separated into eight VCGs, whereas isolates of race 3 were classified into a single VCG (0113), as were those of race 4 (0114). Ribosomal and mitochondrial DNA (rDNA and mtDNA) RFLPs separated four rDNA haplotypes and seven mtDNA haplotypes. Race A isolates displayed the most polymorphism, with three rDNA haplotypes and four mtDNA haplotypes; race 4 isolates formed a single rDNA group but exhibited three mtDNA haplotypes, while race 3 isolates had unique rDNA and mtDNA haplotypes. Two mtDNA molecules with distinct sizes were identified; the first (45-kb mtDNA) was found in all race A isolates and seven race 4 isolates, and the second (55-kb mtDNA) was found in all race 3 isolates and in two isolates of race 4. These two mtDNA molecules were closely related to mtDNAs of *F. oxysporum* isolates belonging to other formae speciales (*conglutinans*, *lycopersici*, *matthioli*, and *raphani*). Isolates within a VCG shared the same rDNA and mtDNA haplotypes, with the exception of VCG0114, in which three distinct mtDNA haplotypes were observed. Genetic relationships among isolates inferred from rDNA or mtDNA site restriction data were different, and there was not a strict correlation between race and RFLPs. However, the races in *F. oxysporum* f. sp. *vasinfectum* constituted three distinct genetic groups with regard to VCG and nuclear and mtDNA RFLPs.

In many soil-borne fungal pathogens, the apparent absence of a sexual stage may be compensated by other processes in ensuring genetic exchanges and diversity buildup (23, 27). In *Fusarium oxysporum* Schlechtend.:Fr., in which genetic exchanges may occur via hyphal anastomosis between strains within vegetative compatibility groups (VCGs), several models of evolution of formae speciales, races, and VCGs have been proposed (8, 17, 24). The relative importance of host specialization and genetic isolation (as represented by a VCG) was considered to explain the observed diversification within *F. oxysporum*.

Extensive DNA analyses have recently been conducted with several formae speciales of *F. oxysporum* to assess these models. Most of the studies failed to show a correlation between races and the genomic evolution as determined by restriction fragment length polymorphism (RFLP) analyses of nuclear or mitochondrial DNA (mtDNA) (19, 22, 25, 28, 29). However, examination of DNA variation among isolates belonging to different VCGs provided new insight into *F. oxysporum* population structure. Correspondences between RFLP patterns and VCGs were obtained with randomly cloned DNA probes in *F. oxysporum* formae speciales *dianthi*, *gladioli*, and *pisi* (29, 31, 40) and with mtDNA in *F. oxysporum* f. sp. *melonis* (16, 17). Recently, Elias et al. (13) used 50 randomly cloned fragments representing single-copy as well as repetitive DNA in *F. oxysporum* f. sp. *lycopersici*. They showed that (i) VCGs are genetically distinct subpopulations (clonal lineages) and that (ii) races arose independently in each VCG. In addition,

Boehm et al. (6) showed that the variation in electrophoretic karyotypes among 11 VCGs in *F. oxysporum* f. sp. *cubense* was highly correlated with VCGs.

These results led us to study whether such genetic relatedness among individuals within a VCG actually exists in *F. oxysporum* f. sp. *vasinfectum* (Atk.) Snyder & Hansen, the causal agent of *Fusarium* wilt of cotton (*Gossypium* spp.). This fungus has a wide host range and displays variation in virulence on distinct host species and genera (2, 3). In a previous study, we characterized 46 *F. oxysporum* f. sp. *vasinfectum* isolates of worldwide origin on the basis of the pathogenicity of the isolates on differential cotton species (*Gossypium hirsutum*, *Gossypium barbadense*, and *Gossypium arboreum*) and by random amplified polymorphic DNA (RAPD) analysis (4). Three distinct races were identified which however are not defined in a gene-for-gene system: race A, race 3, and race 4, corresponding to those previously characterized (race A regrouped the former races 1, 2, and 6, which were not distinguishable by their pathogenicity on cotton, but only on secondary host plants) (2, 3). These races are distinctly separated geographically; race A is found in America and most of Africa; race 3 is found in Egypt, Sudan, and Israel; and race 4 is found in India, China, and Uzbekistan.

The RAPD analysis separated the 46 *F. oxysporum* f. sp. *vasinfectum* isolates into three groups corresponding to the races A, 3, and 4 and provided useful markers for race identification (4). Such genetic differentiation of races is not common in *F. oxysporum* (19, 22, 25, 28, 29) and may be attributed to the large host species range of *F. oxysporum* f. sp. *vasinfectum* and to their geographical separation.

In the present study, we assessed *F. oxysporum* f. sp. *vasinfectum* population structure and tested whether isolates within a VCG displayed genetic similarity. We analyzed the vegetative compatibility of *F. oxysporum* f. sp. *vasinfectum* isolates, and we

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evaluated the genetic relatedness of the distinct VCGs by RFLP analysis of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA). Additionally, we examined relationships between *F. oxysporum* f. sp. *vasinfectum* and other *F. oxysporum* formae speciales on the basis of published mtDNA molecular data (16, 19, 20–22, 30, 39).

MATERIALS AND METHODS

Isolates. Fifty-two isolates of *F. oxysporum* f. sp. *vasinfectum* were collected from different cotton-growing regions throughout the world; there were 31, 12, and 9 isolates of race A, race 3, and race 4, respectively. This collection included type isolates of races 1, 2, 3, 4, and 6 deposited by Armstrong and Armstrong (2, 3) in the American Type Culture Collection (ATCC), Rockville, Md. The geographical origin and race for each isolate are listed in Table 1. All cultures were single spored, and mycelia were maintained on potato dextrose agar slants for short-term storage and frozen in liquid nitrogen for long-term storage.

Vegetative compatibility analysis. Vegetative compatibility groups were determined according to the methods described by Puhalla (37) and Correll et al. (9), using spontaneous nitrate nonutilizing (*nit*) mutants. According to Puhalla (37), groups were numbered as VCG011-, where the first three digits identify the forma specialis (here, 011 for *F. oxysporum* f. sp. *vasinfectum*) and the last digit identifies subgroup within the forma specialis.

Genomic DNA extraction. Fungal mycelium was cultivated in flasks containing 200 ml of GYP medium (2% glucose, 0.5% yeast extract, 0.5% peptone) for 5 days at 25°C. The mycelium was harvested by filtration, washed with sterile distilled water, and lyophilized for 48 h. Total DNA extraction was performed by a miniprep procedure (26).

Restriction endonuclease digestion, electrophoresis, and blotting. For each isolate, approximately 3 to 5 μg of total genomic DNA was digested with 20 to 30 U of the restriction enzymes (Boehringer Mannheim, Meylan, France), with the addition of 5 mM spermidine per reaction mixture, for 6 to 16 h at 37°C. Restriction fragments were separated by electrophoresis in 0.8 or 1.2% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) at 1 V cm^{-1} overnight. Gels stained with ethidium bromide (0.5 μg ml^{-1}) were photographed on a UV transilluminator. DNA fragments were blotted onto NylonN$^+$ membranes (Amersham, Les Ullis, France) by alkaline vacuum transfer (TE 80 TransVac; Hoefer Scientific Instruments, San Francisco, Calif.).

Labelling of probes and hybridization conditions. The hybridization probes used in this study are listed in Table 2. The rDNA probe was labelled and detected by chemiluminescence (ECL kit; Amersham). mtDNA probes were labelled with [^{32}P]dCTP by random priming, hybridized to membrane-bound DNA fragments, and detected by autoradiography according to the specifications of the manufacturer (Megaprime kit and Rapid-Hyb buffer; Amersham).

RFLP data analysis. Restriction fragments obtained in each enzyme-probe combination were scored, and each isolate was characterized with a specific rDNA and mtDNA haplotype obtained by gathering together the data with different enzymes. Cluster analyses were performed using mapped restriction site data with the computer program Restsite (32), which calculates the number of nucleotide substitutions per site on the basis of Nei's distance (34). Dendrograms were derived from the distance matrix by using the UPGMA algorithm (38).

TABLE 1. Codes, geographic origins, and race identifications of the isolates of *F. oxysporum* f. sp. *vasinfectum* tested and their corresponding VCGs as determined in this study

Isolate	Geographic origin	Race[a]	VCG no.
ATCC16421	United States	A (race 1)	0111
3F90[b]	United States	A	0119
91-1[b]	United States	A	0119
91-4[b]	United States	A	0119
91-8[b]	United States	A	0119
91-11[b]	United States	A	0119
91-13[b]	United States	A	0119
91-15[b]	United States	A	0119
ATCC16611	United States	A (race 2)	0112
Pu[c]	Peru	A	0112
9[d]	Tanzania	A	0112
11[d]	Tanzania	A	0112
13[d]	Tanzania	A	0112
15[d]	Tanzania	A	0112
34[d]	Tanzania	A	0112
189[d]	Tanzania	A	0112
J218[c]	Ivory Coast	A	0112
Fm8	Ivory Coast	A	0112
Ci[c]	Ivory Coast	A	0112
Ci5[c]	Ivory Coast	A	0112
Cip[c]	Ivory Coast	A	0112
Cian[c]	Ivory Coast	A	01110
Cysa	Ivory Coast	A	0115
Okra	Ivory Coast	A	0115
Apl	Benin	A	0117
Bn[c]	Benin	A	0117
ATCC36198	Brazil	A (race 6)	0116
Arg[c]	Argentina	A	0116
Pa[c]	Paraguay	A	0116
48[d]	Zimbabwe	A	0116
Bir[e]	Unknown	A	0118
ATCC16612	Egypt	3	0113
Gis[f]	Sudan	3	0113
Bar1[f]	Sudan	3	0113
Bar821[f]	Sudan	3	0113
EB731[f]	Sudan	3	0113
GS821[f]	Sudan	3	0113
Mary[f]	Sudan	3	0113
Olb[f]	Sudan	3	0113
Pim[f]	Sudan	3	0113
I348[g]	Israel	3	0113
Mh3[g]	Israel	3	0113
Fil69[g]	Israel	3	0113
ATCC16613	India	4	0114
40[h]	Uzbekistan	4	0114
CH1[i]	China	4	0114
CH3[i]	China	4	0114
CH4[i]	China	4	0114
CH5[i]	China	4	0114
CH6[i]	China	4	0114
CH7[i]	China	4	0114
CH8[i]	China	4	0114

[a] Race identifications as determined by Assigbetse et al. (4).
[b] Donated by S. N. Smith, University of California, Berkeley, Calif.
[c] Donated by J.-C. Follin, CIRAD-CA, Montpellier, France.
[d] Donated by M. A. Rutherford, International Mycological Institute, Egham, United Kingdom.
[e] Donated by C. Croft, School of Biological Sciences, Birmingham, United Kingdom.
[f] Donated by G. Ibrahim, Agricultural Research Corporation, Wad Medani, Sudan.
[g] Donated by J. Katan, Hebrew University, Jerusalem, Israel.
[h] Donated by L. G. Portenko, Academy of Sciences, Dushanbe, Tadjikistan.
[i] Donated by G. H. Dai, Hebei Academy of Agricultural Science, Beijing, China.

TABLE 2. DNA probes tested in this study

Probe	Characteristic(s)
Nuclear rDNA[a]	*Sordaria macrospora* 8.5-kb-rDNA repeat unit in pBR329
mtDNA[b] (from *F. oxysporum* f. sp. *conglutinans*)	
Whole purified mtDNA	52-kb molecule
pUF1-9	3.7-kb *Eco*RI fragment in pUC119
pUF1-11	5.8-kb *Eco*RI fragment in pUC119
pUF1-22	3.3-kb *Eco*RI fragment in pUC119
pUF1-38	5.0-kb *Eco*RI fragment in pUC119

[a] Provided by Landry and Lechevanton, Orsay, France.
[b] Provided by Kistler and Benny (21).

RESULTS

Vegetative compatibility analysis. Among 52 isolates of our collection, 10 VCGs were identified (Table 1). All isolates were self-compatible (10). Race A isolates were separated into eight VCGs, whereas those of race 3 were all in VCG0113 and those of race 4 were all in VCG0114. No isolates of distinct races were compatible.

Half of the race A isolates (the majority being from Africa) were in VCG0112. The other seven VCGs found among race A isolates each contained one to seven isolates. The three type isolates representative of the previously described races 1, 2, and 6 (2, 3) were classified into three separate VCGs as follows: ATCC16421, representing race 1, which was the only member of VCG0111; ATCC16611, representing race 2, which was a member of the relatively large VCG0112; and ATCC36198, representing race 6, which was grouped with three isolates from South America and Africa in VCG0116. The most variability was found among isolates from the Ivory Coast and the United States, each of which had representatives in three VCGs.

rDNA RFLPs. Among the 11 restriction enzymes tested, only 3 detected polymorphisms among the isolates (*Eco*RI, *Xho*I, and *Kpn*I); no polymorphism was shown with *Bam*HI, *Bgl*I, *Eco*RV, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I, and *Sma*I. The length of the ribosomal repeat unit of *F. oxysporum* f. sp. *vasinfectum* was calculated as 8.0 kb on the basis of the sizes of the DNA fragments hybridizing with the rDNA probe, and no evidence of size polymorphism among the isolates was apparent. On the basis of the number of *Eco*RI, *Xho*I, and *Kpn*I sites present in the ribosomal unit, four rDNA haplotypes (designated I, II, III, and IV) were identified (Table 3). Isolates within a VCG had the same rDNA haplotype (Table 4). Cluster analysis separated the 52 *F. oxysporum* f. sp. *vasinfectum* isolates into four groups, which corresponded to the four rDNA haplotypes; for convenience, Fig. 1 presents the resulting dendrogram showing relationships among VCGs. rDNA group I was composed of the isolates of VCG0111, VCG0112, VCG0119,

TABLE 3. Determination of rDNA haplotypes based on the numbers of *Eco*RI, *Xho*I, and *Kpn*I restriction sites in the ribosomal units of *F. oxysporum* f. sp. *vasinfectum* isolates

rDNA haplotype	Restriction site number		
	*Eco*RI	*Xho*I	*Kpn*I
I	4	1	0
II	4	1	1
III	4	0	1
IV	3	0	1

TABLE 4. mtDNA and rDNA haplotypes of the 10 *F. oxysporum* f. sp. *vasinfectum* VCGs determined in this study

VCG no.	No. of isolates	mtDNA haplotype	rDNA haplotype	Race[a]
0111, 0112, 0119, and 01110	16	I	I	A
0115	2	I	II	A
0117	2	II	II	A
0118	1	III	III	A
0116	4	IV	III	A
0113	12	V	III	3
0114	1 (CH7)	VI	IV	4
0114	1 (CH8)	VII	IV	4
0114	7	III	IV	4

[a] Determined by Assigbetse et al. (4).

and VCG0110 (race A); group II contained isolates of VCG 0115 and VCG0117 (race A), and group III contained isolates of VCG0116 and VCG0118 (race A) and isolates of VCG0113 (race 3), while isolates of VCG0114 (race 4) clustered apart. The dendrogram was divided into two main branches, separating the isolates belonging to rDNA haplotypes I and II and those belonging to rDNA haplotypes III and IV.

mtDNA RFLPs. The whole mtDNA of *F. oxysporum* f. sp. *conglutinans* was labelled and used to probe filters containing enzyme digests of total DNA of the 52 strains of *F. oxysporum* f. sp. *vasinfectum*. mtDNA polymorphisms were detected with several enzymes (*Bgl*II, *Cfo*I, *Eco*RI, *Hae*III, *Hin*fI, *Kpn*I, *Msp*I, *Pst*I, and *Xho*I). On the basis of the restriction patterns observed with the enzymes *Bgl*II, *Eco*RI, and *Hae*III (Fig. 2 and 3), seven mtDNA RFLP haplotypes were defined (from I to VII [Table 5]). Four haplotypes were detected among isolates of race A and three were detected among isolates of race 4, but only a single haplotype was detected among isolates of race 3. Apart from VCG0114, all of the isolates within a VCG had the same mtDNA haplotype (Table 4).

Site polymorphisms (presence or absence) as well as mtDNA size differences were observed (Fig. 3). Discrepancies in mtDNA total size values were obtained with different restriction enzymes, particularly with *Hae*III. Repeated experiments did not allow us to identify fragments that might not
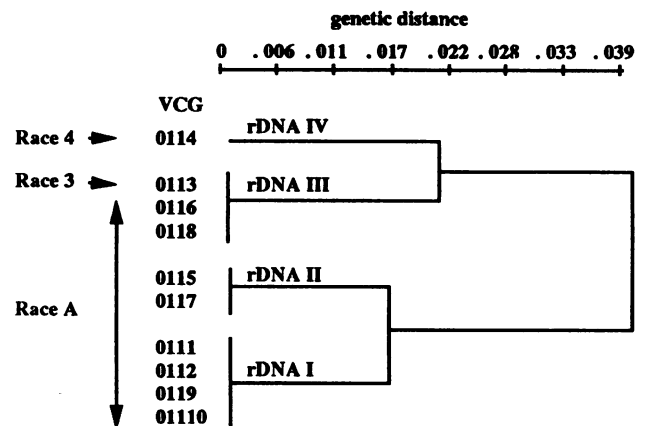

FIG. 1. Dendrogram showing the genetic relationships between *F. oxysporum* f. sp. *vasinfectum* VCGs, which were derived from genetic distances obtained by rDNA RFLP analysis with restriction enzymes *Eco*RI, *Xho*I, and *Kpn*I. rDNA haplotypes identified are indicated on each branch of the dendrogram.
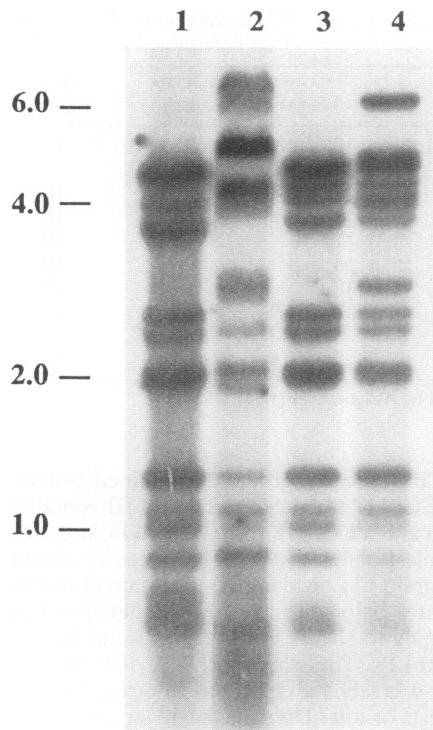
FIG. 2. HaeIII restriction patterns of mtDNA of F. oxysporum f. sp. vasinfectum isolates. Lanes 1 to 4: ATCC16421, ATCC16612, ATCC 16613, and CH8, respectively. Molecular weights of the fragments are indicated on the left.

have been detected, suggesting that some bands that were considered single are doublets. However, mtDNAs from haplotypes V to VII were always larger than mtDNAs from haplotypes I to IV with the three enzymes tested.

F. oxysporum f. sp. vasinfectum mtDNA characterization. A single isolate representative of each RFLP haplotype was selected. The four mtDNA clones listed in Table 2 were tested on total DNAs of F. oxysporum f. sp. vasinfectum isolates digested with restriction enzymes previously mapped in the formae speciales of F. oxysporum that are pathogenic for members of the family Cruciferae: EcoRI, XhoI, BamHI, KpnI, SalI, SacI, and SmaI (21). Probes pUF1-9 and pUF1-38 strongly hybridized to all of the isolates, whereas probes pUF1-11 and pUF1-22 hybridized strongly only to the DNAs of the race 3 isolates and to DNAs of two of the race 4 isolates, CH7 and CH8 (Fig. 4). Thus, the largest part of the mtDNA sequences contained in plasmids pUF1-11 and pUF1-22 does not seem to be present in the mtDNAs of race A isolates or in those of most of the race 4 isolates. The same selective hybridization of probe pUF1-11 was also observed among the F. oxysporum formae speciales that are pathogenic to the Cruciferae, differentiating F. oxysporum f. sp. raphani from F. oxysporum f. sp. conglutinans and F. oxysporum f. sp. matthioli (22).

Restriction site positions were deduced from the hybridization results with each probe and were compared with those in the F. oxysporum f. sp. conglutinans mtDNA (21); however, we have not constructed an accurate restriction map for F. oxysporum f. sp. vasinfectum mtDNA. The positions of the sites for each enzyme allowed us to conclude that in our F. oxysporum f. sp. vasinfectum collection, two distinct sizes of mtDNA

molecules are present. The first type, designated 45kb-mtDNA, is about 45 kb in length and is found in the isolates of race A and in most race 4 isolates; the numbers of the restriction sites for the seven enzymes and their respective positions are identical to those of F. oxysporum f. sp. raphani (21). The second type, designated 55kb-mtDNA, is about 55 kb in length and is carried by race 3 isolates and by two race 4 isolates (CH7 and CH8). The mtDNA restriction patterns of race 3 isolates were close to those of F. oxysporum f. sp. conglutinans (21), differing by the presence of one additional EcoRI site and one additional XhoI site and the absence of two SacI sites; the mtDNAs carried by the two race 4 isolates CH7 and CH8 presented restriction patterns close to those of F. oxysporum f. sp. matthioli (21), differing by an additional KpnI site, a different position for two SacI sites, and the absence of one EcoRI site.

Cluster analyses were conducted on the basis of the mtDNA restriction site data of F. oxysporum f. sp. vasinfectum isolates. In a first analysis, we used EcoRI, XhoI, BamHI, KpnI, SalI, SacI, and SmaI site data, and we included those from F. oxysporum f. sp. lycopersici (30) and F. oxysporum formae speciales of the Cruciferae (21). F. oxysporum f. sp. vasinfectum isolates were separated into three groups: isolates carrying 45kb-mtDNA, isolates CH7 and CH8, and race 3 isolates (Fig. 5). Isolates of each F. oxysporum f. sp. vasinfectum group were closer to isolates of another forma specialis than to isolates of another F. oxysporum f. sp. vasinfectum group. This analysis evaluates the average number of nucleotide changes between individuals on the basis of shared restriction sites. Since we coded each mtDNA for the presence or absence of restriction sites, this analysis thus underestimates genetic distances between isolates when mutations such as insertions or deletions occur between two restriction sites in their mtDNAs (7). A second cluster analysis (dendrogram not shown) based on BglII, EcoRI, and HaeIII site data was conducted to assess relationships among 45kb-mtDNA and 55kb-mtDNA isolates separately, in order to avoid errors in distance calculation due to length changes. Isolates were separated into several groups corresponding to haplotypes; however, distances separating the groups were small (less than 0.019). Overall, the results were consistent with the dendrogram presented in Fig. 5.

DISCUSSION

In this study, we have characterized vegetative compatibility groups in the cotton wilt fungus F. oxysporum f. sp. vasinfectum. We investigated the genetic relatedness among them and among the different races which had been characterized previously (4). We found 10 distinct VCGs in our collection of F. oxysporum f. sp. vasinfectum isolates, 4 rDNA haplotypes, and 7 mtDNA haplotypes.

Isolates belonging to the three different races on cotton plants constituted distinct VCGs, and we never observed that isolates of different races were vegetatively compatible. Race A isolates could be separated into eight VCGs, whereas all isolates of race 3 were in VCG0113 and all isolates of race 4 were in VCG0114 (Table 1). Most isolates within the same VCG had the same rDNA and mtDNA haplotypes (Table 4). However, in one VCG (VCG0114), three distinct mtDNA haplotypes occurred, and among race A isolates, the same haplotypes were observed in four distinct VCGs. Our findings for F. oxysporum f. sp. vasinfectum are in good agreement with those obtained for other pathogenic F. oxysporum formae speciales, in which VCGs and RFLP groups corresponded (13, 17, 28, 40); however, the correspondence that we observed

| | | *Hae*III | | | *Eco*RI | | | | *Bgl*II | | | | |
				Restriction enzyme patterns									
1	2	3	4	1	2	3	1	2	3	4	5	6
		6.2		23.0			15.0	15.0	15.0	15.0	15.0	15.0
			6.0									
		5.8			21.0	21.0	12.0	12.0	12.0	12.0	12.0	12.0
		4.8										
		4.8	4.8	13.0		13.0				7.8	7.8	7.8
4.6	4.6		4.6									
4.6	4.6		4.6		10.0		4.0	4.0	4.0	4.0	4.0	4.0
	4.3		4.3		5.8	6.0	3.8	3.8		3.8	3.8	3.8
4.0	4.0	4.0	4.0	5.0	5.0	5.0				3.6	3.6	3.6
												3.4
		3.9	3.9						2.5			
3.7	3.7	3.7	3.7				2.4	2.4	2.4	2.4	2.4	2.45
3.7							2.3	2.3	2.3	2.3	2.3	
		2.9	2.9	3.7	3.7	3.7		2.2				
		2.8			3.3	3.3	2.1		2.1	2.1		2.15
2.6	2.6		2.6	3.1								
2.5	2.5	2.5	2.5									
2.1	2.1	2.1	2.1		1.8	1.8	1.5	1.5	1.5	1.5	1.5	1.5
2.0	2.0		2.0						1.3			
		1.9					1.2	1.2	1.2	1.25	1.25	1.25
1.3	1.3	1.3	1.3								1.1	
1.1	1.1	1.1	1.1								1.05	
1.0	1.0	1.0	1.0				1.0	1.0	1.0	1.0	1.0	1.0
0.8	0.8	0.8	0.8									
		0.7	0.7									
0.6												
0.5	0.5	0.5	0.5									
0.2	0.2	0.2	0.2									
0.1	0.1	0.1	0.1									
Total length 35.4	35.4	51.1	53.7	44.7	53.7	54.0	45.4	45.5	45.4	56.7	56.7	57.8

FIG. 3. Restriction pattern numbers, fragment sizes, and total lengths of *F. oxysporum* f. sp. *vasinfectum* mtDNAs observed with *Hae*III, *Eco*RI, and *Bgl*II enzymes (fragment sizes are in kilobases).

between race and VCG was not always obtained in other formae speciales (1, 12, 16, 36, 40).

Use of several probes in RFLP experiments showed that race 3 isolates constituted a genetically homogeneous group, whereas race A and race 4 isolates formed two heterogeneous

TABLE 5. *F. oxysporum* f. sp. *vasinfectum* mtDNA haplotypes based on *Hae*III, *Eco*RI, and *Bgl*II restriction patterns

mtDNA haplotype	mtDNA pattern no.[a]		
	*Hae*III	*Eco*RI	*Bgl*II
I	1	1	1
II	1	1	2
III	2	1	1
IV	1	1	3
V	3	2	6
VI	4	3	4
VII	4	3	5

[a] See Fig. 3 for a description of mtDNA patterns.

groups. There was no strict correlation between race and genomic variability, but isolates within each race could be differentiated by combining rDNA and mtDNA RFLP data (Table 4). Genetic relationships among the isolates based on RFLP data were different, depending on whether they were inferred from nuclear rDNA data or mtDNA data. rDNA analysis (Fig. 1) differentiated VCG0114 (race 4) and VCG 0115 and VCG0117 (race A) from VCG0111, VCG0112, VCG 0119, and VCG01110 (race A), whereas they were all grouped by mtDNA analysis (except for CH7 and CH8) (Fig. 4). In addition, rDNA data grouped VCG0113 (race 3) and VCG 0116 and VCG0118 (race A) together, whereas mtDNA analysis separated VCG0113 and grouped VCG0116 and VCG 0118 with the other VCGs.

Levels of variation within rDNA and mtDNAs differ widely among filamentous fungi, providing useful criteria for the identification of either species or populations (7). Variations in rDNA among closely related taxa are usually found in the internal transcribed spacer (ITS [subdivided into ITS1 and ITS2]) and in the intergenic spacer (IGS) which separates the
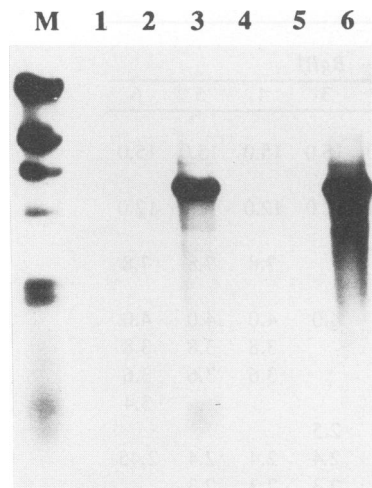
FIG. 4. Selective hybridization of probe pUF1-11 on EcoRI-digested DNAs of F. oxysporum f. sp. vasinfectum isolates. Lanes 1 to 6, ATCC16421, Bn, ATCC16612, ATCC16613, ATCC16198, and CH7, respectively. M, molecular weight marker (radiolabelled HindIII-digested lambda phage DNA). Note that the same amount of digested DNA was loaded into each well.

repeated ribosomal units (35). In our collection of F. oxysporum f. sp. vasinfectum, RFLPs were detected in the ribosomal unit, separating the isolates into four RFLP groups. Amplification by PCR of the ITS1 gave rise to a unique 220-bp fragment for all of the isolates, and no RFLP was detected with several restriction enzymes (14). Sequencing of ITS and IGS of F. oxysporum belonging to several formae speciales revealed that most of the nucleotide variation is found in the IGS (5).

Analysis of the mitochondrial genome revealed polymorphisms, allowing us to differentiate subgroups within F. oxysporum f. sp. vasinfectum; however, those polymorphisms were not specific to the forma specialis. The following mtDNA molecules with distinct sizes were characterized: 55kb-mtDNA and 45kb-mtDNA, which, respectively, differentiated race 3
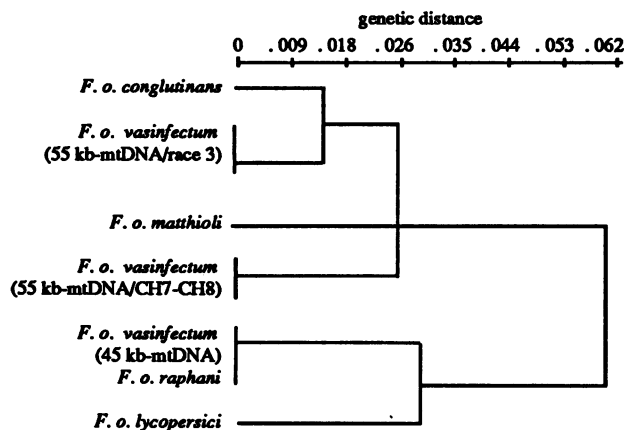

FIG. 5. Dendrogram showing the genetic relationships among F. oxysporum f. sp. vasinfectum isolates (45kb-mtDNA and 55kb-mtDNA) and other F. oxysporum isolates, derived from genetic distances obtained by mtDNA RFLP analysis with enzymes EcoRI, XhoI, BamHI, KpnI, SalI, SacI, and SmaI (this study and references 21 and 30).

isolates and two isolates of race 4 (CH7 and CH8) from the other race 4 and race A isolates. The restriction profiles of 45kb-mtDNA with seven enzymes were identical to those of F. oxysporum f. sp. raphani (21) and were conserved with several enzymes with those of F. oxysporum formae speciales lycopersici (30), elaeidis (14), niveum (19), and melonis (16). The 55kb-mtDNA was genetically closer to those of F. oxysporum formae speciales conglutinans, matthioli (21), and albedinis (39) than to the mtDNAs of the other F. oxysporum f. sp. vasinfectum strains (Fig. 5). Such mtDNA-based relationships were also observed in the formae speciales of F. oxysporum that are pathogenic to members of the family Cucurbitaceae, in which isolates of distinct formae speciales appeared to be more genetically related than isolates of the same forma specialis (20). However, use of nuclear repetitive DNA sequences has recently provided evidence that all of these cucurbit-infecting formae speciales differed in nuclear DNA content and organization (33).

These findings suggest that the genetic evolution inferred from mtDNA variation patterns does not necessarily reflect the whole genome differentiation and emphasize the need for composite genetic analyses of F. oxysporum. Both RAPD and VCG studies can be regarded as multilocus analyses of variation. By comparing results obtained from RAPD, VCG, and RFLP analyses, the following three levels of genetic variation were found when each race of F. oxysporum f. sp. vasinfectum was considered separately.

(i) Race 3 isolates constitute a single homogeneous group with regard to rDNA and mtDNA RFLP haplotypes and VCGs. The isolates originated from three adjacent countries in which G. barbadense is preferentially grown: Sudan, Egypt, and Israel. In Israel, Katan and Katan (18) found a unique VCG (VCG0113) among 386 isolates of race 3 that were tested. Our isolates (three of them were isolated by Katan and Katan) also belonged to VCG0113. Identical patterns were obtained for all of them by RFLP and RAPD analyses, regardless of the probe or primer used (this study and reference 4). The race 3 isolates also had a unique mtDNA molecule, distinct from that of the other F. oxysporum f. sp. vasinfectum isolates, which suggests that these two groups may have different evolutionary origins. Furthermore, RAPD analysis that was extended to 30 other Sudanese isolates unambiguously grouped them with race 3 isolates, and no polymorphism was detected among them (11). These close genetic relationships among isolates are suggestive of the clonality of race 3.

(ii) Race 4 isolates originate from three Asiatic adjacent countries (China, India, and Uzbekistan). They constitute a unique VCG (VCG0114) and rDNA haplotype; however, they do not carry the same mtDNA. Occurrence of several mtDNA haplotypes in the same VCG was also reported for nonpathogenic isolates of F. oxysporum (15). The authors concluded that weak vegetative interactions among isolates of distinct VCGs may permit the transfer of mitochondria. Bridging isolates in F. oxysporum f. sp. cubense (36) and weak interactions between isolates from distinct VCGs in F. oxysporum f. sp. pisi (40) also have been reported. Since race 4 isolates exhibited the same rDNA haplotype (distinct from those of other isolates) and were grouped together by RAPD analysis (4), such horizontal transfer of mtDNA could explain the presence of distinct mtDNA molecules within VCG0114.

(iii) Race A isolates displayed the most variability, with eight VCGs and three rDNA and four mtDNA haplotypes being detected among 31 isolates (Tables 1 and 4). There was a strong correlation between VCGs and DNA haplotypes (Table 4). Isolates from Paraguay, Argentina, Brazil (formerly described as race 6), and Zimbabwe belonged to the same VCG

(VCG0116) and had the same rDNA patterns as the race 3 isolates. Isolates from the Ivory Coast displayed polymorphisms and were separated into three VCGs corresponding to three RFLP haplotypes. The two isolates from Benin clustered apart (VCG0117), exhibiting particular rDNA and mtDNA haplotypes. Isolates from the United States (together with isolates of the formerly described races 1 and 2), Peru, and Tanzania constituted three VCGs but always clustered together in RFLP experiments.

Heterogeneity within race A isolates was expected, since RAPD analysis could separate these isolates into distinct subgroups (4). The high level of variation of race A isolates compared with isolates from races 3 and 4 may be attributed to several factors. First, the wide geographic distribution of race A (America and Africa) could account for a higher genetic diversification by local differentiation of genotypes. Second, *F. oxysporum* f. sp. *vasinfectum* is thought to have been introduced into Africa along with infected plant materials originating from the Americas. This hypothesis is supported by the VCG and RFLP analyses that group American isolates together with some African isolates. Such migration of pathogens may contribute to gene flow between populations and lead to the emergence of new genotypes. Third, differences in the pathogenicity of race A isolates toward secondary host plants were described, differentiating them into races 1, 2, and 6 (2, 3). The three type isolates representing these former races were grouped into three distinct VCGs (VCG0111, VCG0112, and VCG0116, respectively), suggesting genetic differences between them. Isolates of races 1 and 2 were never differentiated by RFLP or by RAPD analysis (4), while race 6 isolates exhibited distinct RFLP haplotypes and RAPD patterns (4).

Our results obtained in *F. oxysporum* f. sp. *vasinfectum* reinforce the concept of VCGs as independently evolving lineages within *F. oxysporum* (13). Models of evolution of formae speciales, races, and VCGs have been proposed in several reviews (8, 17, 24), and it is likely that data accumulating in distinct formae speciales will help to test them. Our results also provide evidence that analysis of genetic variation within a single forma specialis must be undertaken at several genetic levels. For instance, it is striking that isolates CH7 and CH8 were not distinguished from other race 4 isolates either by RAPD analysis (4) or by rDNA RFLPs but did possess a mtDNA distinct from those of the other isolates. The VCG and RFLP studies were consistent with the RAPD analysis, which separated the *F. oxysporum* f. sp. *vasinfectum* isolates into three main groups corresponding to their pathological specialization (4). Although they were insufficient to make complete distinctions, separate RFLP analyses of rDNA and mtDNA did provide useful data for estimating genetic relationships among the races and among the VCGs in *F. oxysporum* f. sp. *vasinfectum*.

ACKNOWLEDGMENTS

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