Restriction Fragment Length Polymorphism Groups and Physical Map of Mitochondrial DNA from *Fusarium oxysporum* f. sp. *niveum*

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ABSTRACT

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Variation in *Fusarium oxysporum* f. sp. *niveum* was examined by identifying restriction fragment length polymorphisms (RFLPs) in mitochondrial DNA (mtDNA). Six RFLP groups (RFLPG) were detected among 50 pathogenic isolates by Southern hybridization with cloned *Pst*I mtDNA fragment probes (pFON2a [9.1 kb]–pFON8b [1.0 kb]). A restriction map of mtDNA from a race 0 isolate (FL-60-3A) was constructed by digestion of the mtDNA with four restriction endonucleases: *Eco*RI, *Hin*dIII, *Hpa*I, and *Pst*I, either singly or in selected pairs, as well as by identifying bands in each digestion pattern with each of the pFON clones. Intra-mtDNA sequence homology was revealed by hybridization between the *Pst*I fragment pFON7 (1.5 kb) and two other areas of the mtDNA: pFON2a (9.1 kb) and pFON6 (2.0 kb). Most of the fragment differences that discriminated RFLP groups were detected by the pFON7 probe. Relatedness between RFLP groups was analyzed by the unweighted paired group method using arithmetic means with Jaccard's similarity matrix based on the presence or absence of hybridization bands. The cophenetic correlation coefficient in the dendrogram generated was 0.997. RFLPG I was the most common group containing 34 isolates and was clustered to RFLPG IV with a similarity of 90%. RFLPG II was the second most common group containing seven of the isolates and was clustered to RFLPG III with a similarity of 90% resulting in two main clusters. Both main clusters were grouped to each other with a similarity of 84%. RFLPG VI was the most distant from the other RFLP groups with a similarity of 65%, whereas RFLPG V was intermediate to the other four RFLPGs (I, II, III, and IV) with a similarity of 75%. Two nonpathogenic isolates (SC-A[87] and OK-256[87]) were outgrouped from all pathogenic isolates with a similarity of only 21 and 8%, respectively. The RFLP groups appeared primarily to be a result of base substitutions and/or length mutations within the mtDNA; however, a heteroplasmy of mtDNA was also detected in RFLPG IV. The relationship between RFLP groups of the pathogenic isolates was analyzed by simple empirical analysis to produce an unrooted most parsimonious gene tree with the fewest possible stepwise changes. In the gene tree produced, RFLPG II was located at the center with the fewest numbers of steps to all other groups. RFLPG V was the most distant group and required the most steps (four) to get to RFLPG II.

Additional keywords: Fusarium wilt of watermelon.

The soilborne phytopathogenic fungus, *Fusarium oxysporum* Schlechtend.:Fr. can cause vascular wilt in a wide variety of crops and may be a limiting factor in crop production (27). *F. oxysporum* is further specialized into formae speciales based on host specificity (1,35).

Most isolates of *F. o. niveum* (E.F. Sm.) W. C. Snyder & H. N. Hans., the causal agent of Fusarium wilt of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), are host specific. This host specificity is further exemplified by the existence of three cultivar-specific races: race 0, race 1, and race 2. However, exceptions to the rule of host specificity exist within *F. o. niveum*. Several studies have shown cross-infectivity (i.e., inoculation of seedlings and young plants of different species with a given isolate can produce disease) (8,13,26,28,30), suggesting that there is more pathological variation in *F. o. niveum* than the three currently described host-specific races. In addition to pathogenic differences, other genetic variations in *F. oxysporum* have been detected by vegetative compatibility (33), isozyme analysis (3,4), RNA sequence comparison (16), karyotype (31), and restriction fragment length polymorphisms (RFLP) (7,18,20,21). Among these procedures, RFLP has the advantage of potentially detecting numerous polymorphisms at the DNA level. Mitochondrial DNA (mtDNA) is a compact, circular DNA molecule of relatively small size and shows a high rate of mutation and a high rate of fixation of those mutations in vertebrates (6,11). From an evolutionary point of view and based on the molecular clock hypothesis (22,38), mtDNA is potentially useful to study genetic relatedness and the phylogenetic relationship of closely related populations or isolates.

In a previous study (21), three mtDNA RFLP groups were detected among 13 isolates of *F. o. niveum*; 12 collected from around the United States and one from Israel. However, specific pathological race classification of the isolates did not correspond to a given RFLP group. In the present study, variation in the mtDNA RFLP and relatedness among members of a larger collection of *F. o. niveum* isolates has been examined. To determine where and how these variations in the mitochondrial genome occurred, a physical restriction map of the mtDNA was constructed. In addition, a gene tree was also constructed to investigate the phylogeny of mtDNA RFLP groups based on the restriction map.

MATERIALS AND METHODS

Fungal strains. A total of 50 isolates of *F. o. niveum* encompassing the three known races and collected from widely separated geographic regions, along with two nonpathogenic *F. oxysporum* isolates recovered from wilted watermelon plants, were used (Table 1). Forty-two isolates were from the United States, eight from China, one from Taiwan, and one from Israel. All isolates were single-spored, increased, and stored in sterile soil tubes (29). Race identity of each isolate was confirmed before DNA extraction by a greenhouse pathogenicity test using the differential cultivars Black Diamond, Charleston Gray, and Calhoun Gray (27). An active culture of each isolate was obtained by plating a small aliquot of soil culture on potato-dextrose agar and incubating at 25 C. After 2 or 3 days, actively growing hyphal regions were cut using a No. 1 cork borer (0.4 mm diameter), and six agar blocks were placed into 250 ml of potato-dextrose broth, gyrated at 80–100 rpm for 7–8 days at 22 ± 2 C, and then filtered through Miracloth (Calbiochem, San Diego, CA) in a Buchner funnel. The mycelial mat was frozen in liquid nitrogen and stored at −70 C until used for DNA extraction.

DNA extraction. DNA was extracted using a minipreparation procedure modified from Zolan and Pukkila (39). Three grams

of frozen mycelial mat of each culture was ground to a fine powder in liquid nitrogen with a mortar and pestle, suspended in 10 ml of CTAB extraction buffer (700 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% 2-mercaptoethanol [v/v], and 1% hexadecyltrimethylammonium bromide [CTAB] [w/v]) in a centrifuge tube and placed in a 60 C water bath for 45 min with gentle mixing every 15 min. An equal volume of chloroform/isoamylalcohol (24:1) was added and mixed by gentle inversion followed by centrifugation at 10,000 rpm for 10 min. The upper aqueous phase was recovered and again treated with chloroform/isoamylalcohol. DNA was precipitated by the addition of an equal volume of isopropanol and centrifugation at 10,000 rpm for 10 min. The DNA was resuspended in 2 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and further purified by successive extractions with the following reagents: an equal volume of Tris-saturated phenol; phenol/chloroform/isoamylalcohol (25:24:1) (2×); chloroform/isoamylalcohol; and then reprecipitated with 2 vol of ice-cold absolute ethanol. Total cellular DNA was pelleted and resuspended in 400 μl of TE. Mitochondrial DNA was further purified from total cellular DNA by a previously described method of Garber et al (12) and as modified by Kim et al (100 μg of bisbenzimide per milliliter; Beckman Ti 70.1 rotor at 45,000 rpm for 48 h) (21).

TABLE 1. Isolates of *Fusarium oxysporum* f. sp. *niveum* used for determining mtDNA restriction fragment length polymorphism (RFLP)

Working number	Isolate	Pathotype	Origin	RFLP group
1-0225	FL-60-3A	Race 0	Florida	I
2-1172	FL-7IV-2(88)	Race 0	Florida	II
3-1033	OK-238(87)	Race 0	Oklahoma	I
4-0610	TX-J-3B-L(82)	Race 0	Texas	I
5-1256	FL-FG85-1(NM)	Race 0	Florida	I
6-1036	GA-591(88)	Race 1	Georgia	II
7-1049	OK-254(87)	Race 1	Oklahoma	I
8-1161	FL-LD8(87)	Race 1	Florida	I
9-1039	GA-568A(88)	Race 1	Georgia	I
10-1191	TX-J-Nav(88)	Race 1	Texas	I
11-1046	GA-557-I(88)	Race 1	Georgia	II
12-1065	NC-EE2-A(87)	Race 1	N. Carolina	IV
13-1167	FL-LD9(87)	Race 1	Florida	I
14-0041	SC-ATCC18467(78)	Race 1	S. Carolina	I
15-1076	SC-C(87)	Race 1	S. Carolina	I
16-0351	CA-Col(85)	Race 1	California	I
17-0348	CA-139(85)	Race 1	California	I
18-1195	TX-TS-U(88)	Race 1	Texas	I
19-0649	IN-8717(87)	Race 1	Indiana	I
20-1057	IN-8576(82)	Race 1	Indiana	I
21-1072	NC-EE-3(87)	Race 1	N. Carolina	I
22-0925	TWN-PSU-0974	Race 1	Taiwan	I
23-0228	FL-64-2	Race 1	Florida	II
24-1075	SC-B(87)	Race 1	S. Carolina	I
25-0172	TX-J-1-L(79)	Race 2	Texas	I
26-0291	ISL-59(73)	Race 2	Israel	III
27-1030	OK-270(87)	Race 2	Oklahoma	I
28-1174	FL-7III-1(88)	Race 2	Florida	II
29-0416	TX-CG133-L(86)	Race 2	Texas	I
30-0320	TX-LRGV-RS2(86)	Race 2	Texas	I
31-0314	TX-LRGV-RS1(86)	Race 2	Texas	I
32-0614	TX-CART-CG-1A(87)	Race 2	Texas	V
33-1264	FL-CS85-4(NM)	Race 2	Florida	I
34-1157	FL-LD10(87)	Race 2	Florida	I
35-1262	FL-FG85-2(NM)	Race 2	Florida	II
36-1254	OK-297(87)	Race 2	Oklahoma	II
37-0034	TX-HC3-13B(84)	Race 2	Texas	I
38-1051	TX-X1D(79)	Race 2	Texas	I
39-1147	TX-LRGV-V(88)	Race 2	Texas	I
40-0296	TX-J1-S(85)	Race 2	Texas	I
41-1143	TX-LRGV-M(88)	Race 2	Texas	I
42-1152	TX-LRGV-L(88)	Race 2	Texas	I
43-1228	OK-256(87)	N.P.[a]	Oklahoma	
44-1079	SC-A(87)	N.P.	S. Carolina	
45-1392	CHN(CI)-PRCF1(89)	Race 0	China	VI
46-1400	CHN(CI)-PRCF2(89)	Race 0	China	I
47-1396	CHN(CI)-PRCF3(89)	Race 1	China	VI
48-1384	CHN(CI)-PRCF4(89)	Race 1	China	VI
49-1380	CHN(CI)-PRCF5(89)	Race 1	China	VI
50-1388	CHN(CI)-PRCF6(89)	Race 1	China	VI
51-1376	CHN(CI)-PRCF7(89)	Race 1	China	I
52-1404	CHN(CI)-PRCF8(89)	Race 1	China	VI

[a] Nonpathogenic isolate of *F. oxysporum*.

Construction of a restriction map of mtDNA. One microgram of mtDNA from isolate FL-60-3A race 0 was digested with four restriction endonucleases (*Eco*RI, *Hin*dIII, *Pst*I, and *Hpa*I), either singly or in paired combinations (*Eco*RI/*Pst*I, *Eco*RI/*Hin*dIII, *Eco*RI/*Hpa*I, *Hin*dIII/*Pst*I, *Hpa*I/*Pst*I, and *Hpa*I/*Hin*dIII), according to the manufacturer's recommendation (Promega, Madison, WI, and BRL, Gaithersburg, MD). Restriction fragments were separated by gel electrophoresis in 0.8% agarose using TBE and a potential gradient of <5 V/cm for 12 h and transferred to nylon hybridization membranes (Genescreen Plus, DuPont, Wilmington, DE). Previously described *Pst*I clones of *F. o. niveum* mtDNA, pFON2a–pFON8b (21), ranging in size from 1.0 to 9.1 kb were labeled with [α-^{32}P] dATP using a random primed DNA labeling kit (USB, Cleveland, OH). Southern hybridizations with each [α-^{32}P] dATP labeled mtDNA clone were conducted as recommended for GeneScreen Plus by the manufacturer to determine the restriction sites of the four restriction enzymes and physical location of each clone.

RFLP analysis. Three restriction endonucleases, *Eco*RI, *Hin*dIII, and *Pst*I, producing 4, 21, and 10 fragments, respectively, were selected for use in the RFLP analysis of *F. o. niveum*. One microgram of total cellular DNA from each of 52 isolates was digested with the restriction enzymes. Restriction fragments were separated on 0.6–1.0% agarose gel by electrophoresis and immobilized onto Genescreen Plus membranes for Southern hybridization. Hybridization with each [α-^{32}P] dATP labeled mtDNA clone as well as a polyprobe (the mixture of equal molar ratio of all nine cloned fragments) was conducted. Hybridization bands were scored as binomial data: 1, if a given fragment was present for an isolate and 0, if it was absent. The hybridization banding patterns of three different enzymes were combined and analyzed by applying a cluster analysis with the unweighted paired group method using arithmetic means and Jaccard's similarity matrix with program NTSYS 1.50 (34).

Characterization of RFLP patterns based on the restriction map. A single isolate representative of each RFLP group detected was selected and digested with the restriction enzymes *Pst*I, *Hin*dIII, and *Eco*RI, and then transferred to nylon hybridization membranes. The mtDNA clones (pFON2a–pFON8b) were labeled with [α-^{32}P] dATP and hybridized with the representative blot. Possible sources of variation were determined by analysis of the mtDNA restriction map and comparison with the hybridization patterns obtained with each clone in the altered region. An autoradiogram of *Hin*dIII digestion probed with *Pst*I pFON5 was subjected to densitometry to quantify the molar ratio of hybridizing bands of RFLPG IV. Ultroscan XL Laser Densitometer (Pharmacia LKB, Uppsala, Sweden) with a line beam of 50 × 800 μm at 633 nm was used to detect the intensity of hybridizing bands.

Construction of a gene tree. A gene tree of mtDNA RFLP groups was constructed for the phylogeny of mtDNA RFLP groups (2). Characterization of mutations (i.e., base substitution or length mutation) and the number of mutations were determined by comparison of the mtDNA map from each RFLP group with the map of the reference isolate (FL-60-3A race 0).

RESULTS

RFLP of *F. o. niveum*. Six mtDNA RFLP groups were detected among the 50 pathogenic isolates of *F. o. niveum*; however, the restriction enzyme *Pst*I in combination with the polyprobe was the only restriction enzyme able to discern all the RFLP patterns. The patterns for the six RFLP groups detected by hybridization of the polyprobe to *Pst*I, *Hin*dIII, and *Eco*RI digests are shown in Figure 1. RFLPG I was the most common pattern and was present in a total of 34 isolates: 32 from the United States and

two from China. This group also contained isolates of all three races of *F. o. niveum*. Because RFLPG I was the most common pattern, and the mtDNA restriction map was constructed from isolate FL-60-3A race 0, which was in this group, every other polymorphism detected was compared to this pattern for establishing relationships. Isolates in RFLPG I all have 9.1-, 5.6-, 3.8-, 2.9-, 2.0-, 1.5-, and 1.0-kb *Pst*I fragments (Fig. 1). RFLPG II was the second most common pattern and included seven isolates, all from the United States. Again, all three races were included in this group. RFLPG II differed from RFLPG I in that the 1.5-kb fragment was absent in the *Pst*I digestion, but there were 0.9- and 0.6-kb fragments with homologous sequences to pFON7. RFLPG III contained only the single isolate ISL-59(73). This isolate originated from Israel and is the original strain first identified as race 2. It lacked the 1.0-, 1.5-, and 2.0-kb *Pst*I fragments typical of RFLPG I, but had 0.6-, 0.9-, and 3.0-kb fragments. RFLPG IV also contained a single isolate, race 1 from North Carolina (NC-EE2-A[87]). It differed from RFLPG I by having the 1.5-kb as well as the 0.9-kb and a 0.4-kb *Pst*I fragment and an additional 1.9-kb *Hin*dIII fragment. RFLPG V also contained a single isolate of race 2 from Texas. Compared to RFLPG II this isolate had lost the 0.6-, 2.9-, and 5.6-kb *Pst*I fragments, but gained 3.5- and 6.6-kb fragments, as well as gaining an extra 2.5-kb fragment in *Hin*dIII digestion. RFLPG VI contained six isolates from China, one race 0 and five race 1. This group was characterized by the lack of 1.0-, 1.5-, 2.9-, and 5.6-kb fragments after *Pst*I digestion and the loss of the 0.4-, 2.1-, both 2.7-kb fragments and the 0.8- and 0.9-kb fragments after *Hin*dIII digestion. It also lost a 23.3-kb fragment after *Eco*RI digestion. However, it contained a new 23.1-kb fragment in the *Pst*I digestion and a 15.3-kb fragment in the *Eco*RI digestion. Two nonpathogenic isolates, one from Oklahoma (OK-256[87]) and one from South Carolina (SC-A[87]) had very distinctive RFLP patterns with all restriction enzymes and were quite different from the pathogenic isolates. The hybridization patterns of 29 isolates including the two nonpathogens digested with *Pst*I and hybridized with the polyprobe are shown in Figure 2.

Cluster analysis. The relatedness among RFLP groups was analyzed by the unweighted paired group method using arithmetic means and Jaccard's similarity matrix based on the presence or absence of hybridizing bands. The dendrogram of the RFLP groups and the similarity between groups are presented in Figure 3. The cophenetic correlation coefficient representing goodness of fit of data was 0.997. Among the pathogenic groups, RFLPG IV was closest to RFLPG I (the most common pattern) with 90% similarity. RFLPG II and RFLPG III were clustered with a 90% similarity. These two main branches were connected with a 84% similarity. RFLPG V was quite distant (75%) from the other four groups, and RFLPG VI was the most distant from any other pathogenic RFLP group (65%). The matching distances of the two nonpathogenic RFLP groups were the furthest from any of the pathogenic groups (21 and 8%, respectively).

Restriction map of mtDNA. The mtDNA restriction map of isolate FL-60-3A race 0 is presented in Figure 4. The positions of the four, nine, 10, and 21 restriction sites of *Eco*RI, *Hpa*I, *Pst*I, and *Hin*dIII, respectively, in the mitochondrial genome were determined by hybridization with each clone and by double digestion of the clones. Hybridization showed the *Pst*I 1.5-kb clone (pFON7) had homology with the 2.0- (pFON6) and 9.1-kb clones (pFON2a), which resulted in two additional hybridizing bands in the *Pst*I digestion (Fig. 5).

Characterization of variation. When each RFLP group was analyzed with the map, the variations responsible for the different RFLP groups were identified. RFLPG II differed from RFLPG I by the presence of a *Pst*I site in the sequence homologous to pFON7, subsequently resulting in the loss of the 1.5-kb fragment

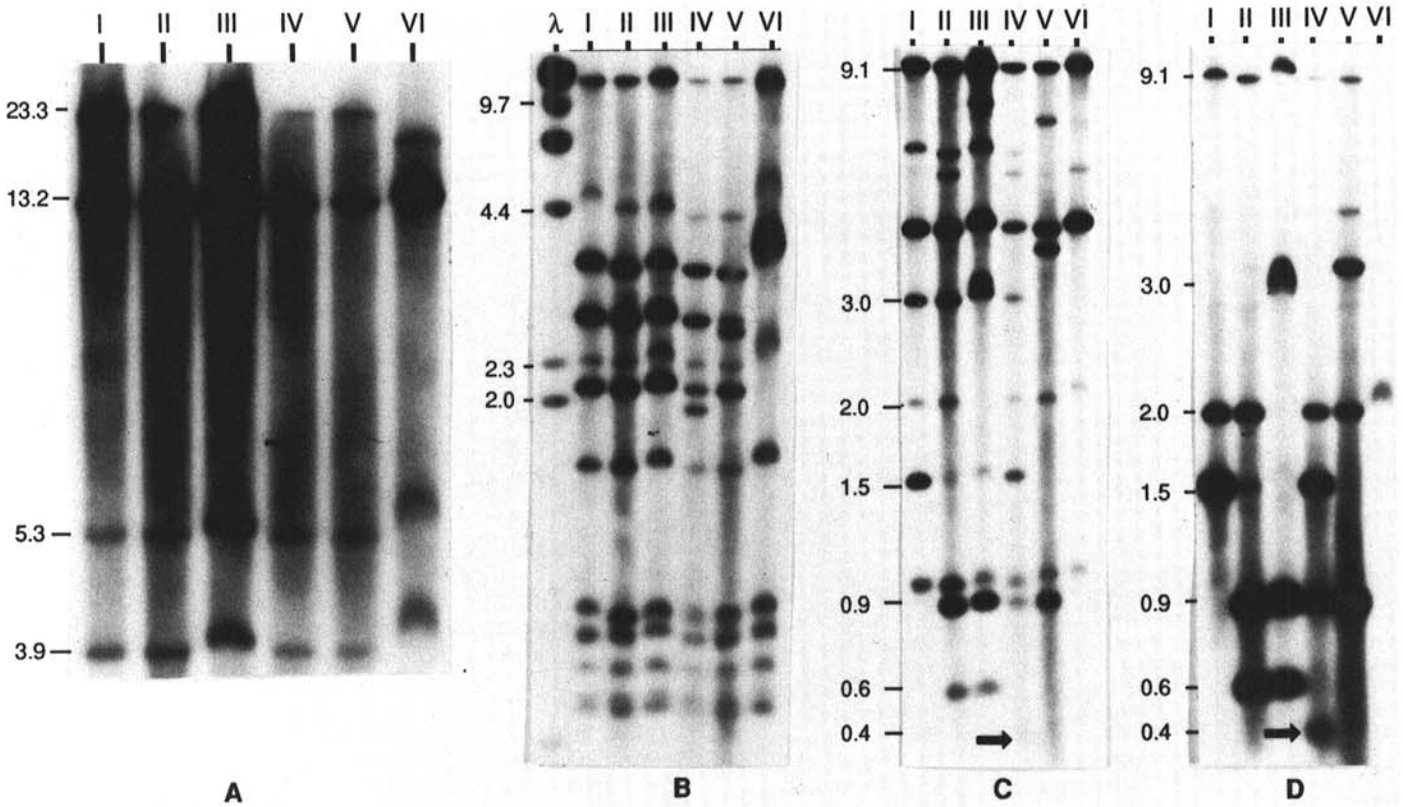

Fig. 1. Hybridization patterns of the six mtDNA restriction fragment length polymorphism groups (RFLPGs) detected in *Fusarium oxysporum* f. sp. *niveum*. **A,** *Eco*RI digests hybridized to polyprobe. Only two RFLPGs were detected (Lanes I–V and VI). **B,** *Hin*dIII digests hybridized to the polyprobe. Four RFLPGs were detected (Lanes I–III; IV; V; and VI). **C,** *Pst*I digests hybridized to the polyprobe. Six RFLPGs were detected. **D,** *Pst*I digests hybridized to the single pFON7 (1.5-kb) probe. The same six RFLPGs were detected with this single probe as with the polyprobe (Fig. 1C). Arrows represent the 0.4-kb fragment produced by *Pst*I digestion in RFLPG IV. Numbers refer to the sizes of fragments in kilobase (kb), and λ lane represents *Hin*dIII-digested λ DNA size marker.

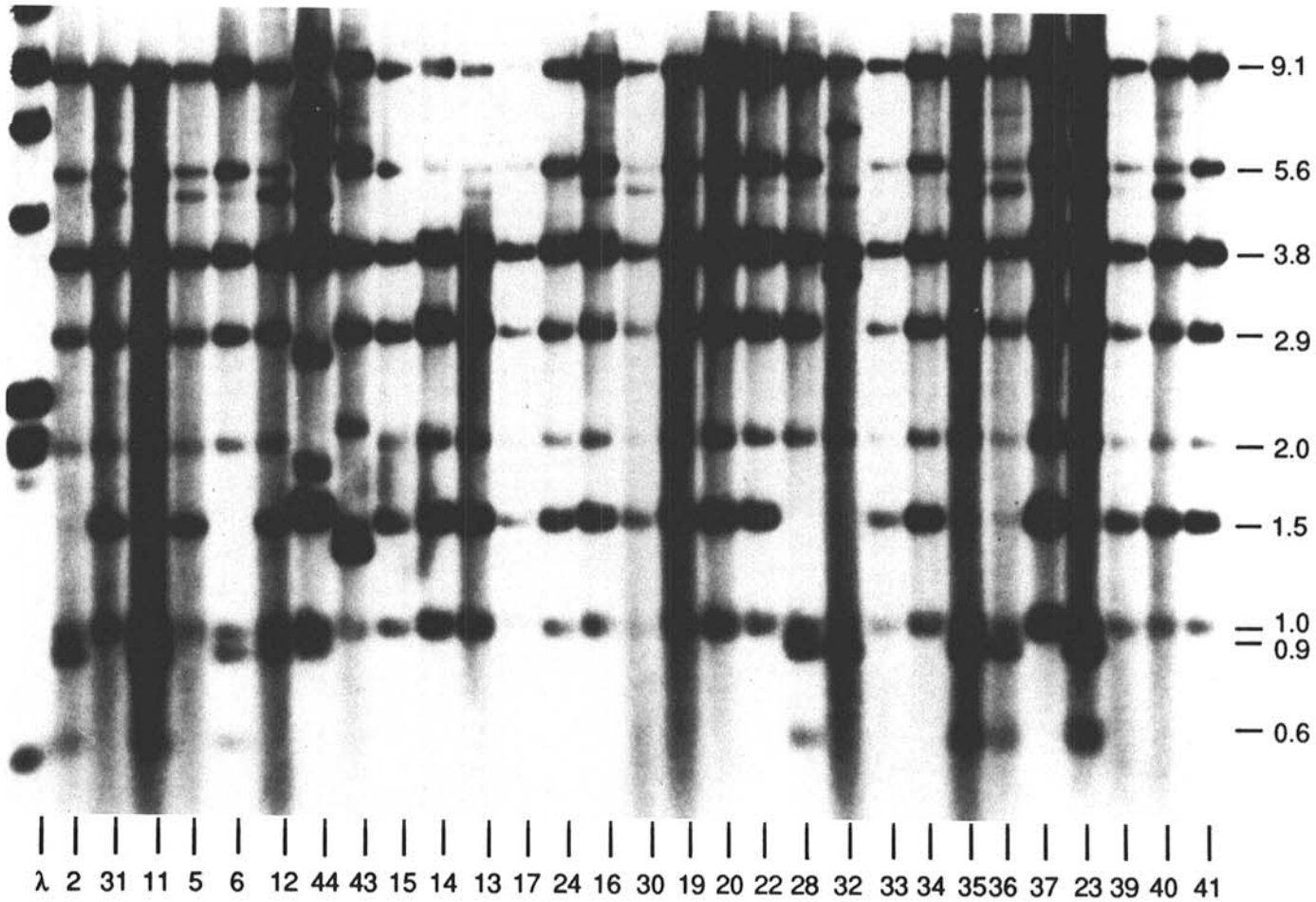

Fig. 2. Hybridization patterns of 27 isolates of *Fusarium oxysporum* f. sp. *niveum* and two nonpathogenic isolates of *F. oxysporum* (lanes 44 and 43) after *Pst*I digestion and hybridization to the polyprobe. Five of six *Pst*I restriction fragment length polymorphism groups are represented in this gel. Lane numbers at the bottom represent the working numbers of isolates in Table 1. Numbers on the right refer to fragment sizes (kb). λ Lane contains *Hin*dIII-digested λ DNA.

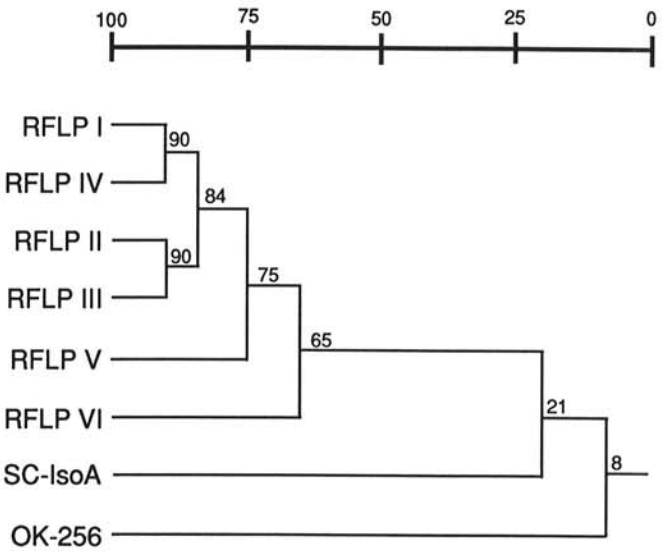

Fig. 3. Dendrogram of *Fusarium oxysporum* f. sp. *niveum* mtDNA restriction fragment length polymorphism (RFLP) groups generated by the unweighted paired group method using arithmetic means. Numbers refer to percentage of similarity between isolate clusters. The bottom branches (SC-IsoA and OK-256) are nonpathogenic isolates of *F. oxysporum* and are far removed from the pathogenic isolates.

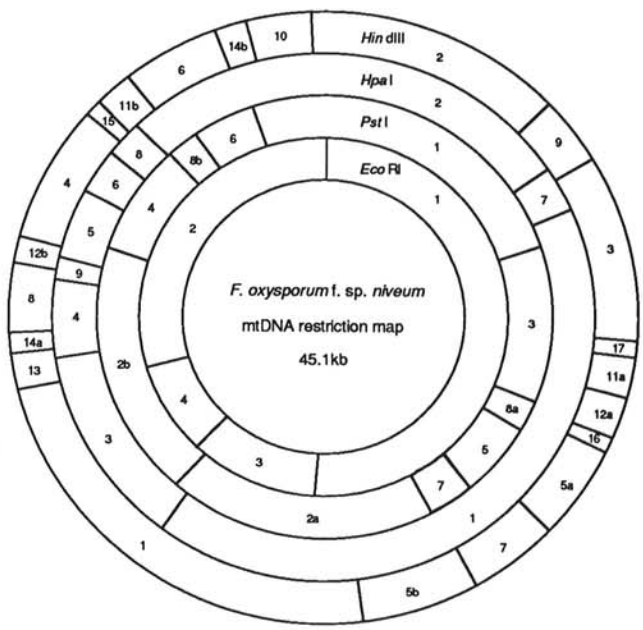

Fig. 4. Restriction map of mtDNA from *Fusarium oxysporum* f. sp. *niveum* race 0 (FL-60-3A). Each concentric circle represents the single enzyme digestion pattern produced by *Eco*RI, *Pst*I, *Hpa*I, and *Hin*dIII digestion, respectively. Numbers refer to the number of DNA fragments generated by each enzyme digestion from the largest to the smallest. The size of each fragment has been published elsewhere (21).

and two new fragments of 0.9 and 0.6 kb. RFLPG III had two additional *Pst*I sites in the sequence corresponding to pFON7 and lost the *Pst*I site that separated pFON6 and pFON8b. This resulted in 0.6-, 0.9-, and 3.0-kb fragments, respectively. RFLPG IV had the 1.5-kb fragment typical of RFLPG I, but it appeared also to have an extra altered copy of that 1.5-kb area that may have gained a new *Pst*I site, similar to that of RFLPG II, resulting in 0.9- and 0.6-kb fragments. However, there also appeared to be a deletion event of approximately 200 base pairs within the 0.6-kb fragment resulting in a 0.4-kb fragment instead and a 1.9-kb fragment rather than a 2.1-kb one in the *Hin*dIII digestion. However, other evidence seemed to indicate that the variation in RFLPG IV was due to reasons other than those for the other RFLP groups. First, the *Pst*I digestion pattern shown in Figure 1A contained each of the fragments of RFLPG I as well as the two additional bands of 0.9 and 0.4 kb. There were no changes in alignment of any of the other *Pst*I fragments and, therefore, did not appear to be a simple site mutation of RFLPG I. Second, there were no changes in fragment lengths of RFLPG I with any of the other enzyme digests except for one additional *Hin*dIII band at 1.9 kb in RFLPG IV (Fig. 1B), thus, ruling out a simple length mutation as the cause. Another possibility, although highly unlikely, would be a length mutation occurring exactly in the *Pst*I site; however, the presence of all identical fragments of RFLPG I in the other digests rule this out. It was known, however, that the two fragments (0.9 and 0.4 kb in *Pst*I digestion) originated from the 1.5-kb fragment based on their hybridization to the pFON7 clone shown in Figure 1D. Therefore, the other possibility considered was heteroplasmy, a case of two different mtDNA species in the same cell. The restriction map of the mtDNA (Fig. 5) showed that the *Pst*I clone pFON5 (2.9 kb) also hybridized to the *Hin*dIII 2.7-kb fragment (#5a) and to the *Hin*dIII 2.1-kb fragment (#7). This *Hin*dIII 2.1-kb fragment in turn hybridized with *Pst*I clone pFON7 (1.5 kb). The hybridization patterns of RFLPG I and IV digested with *Hin*dIII and probed with the *Pst*I pFON5 clone showed that it hybridized to the 2.7- and 2.1-kb fragments typical of RFLPG I, but also hybridized to an additional 1.9-kb fragment in RFLPG IV, indicating the possible presence of another mtDNA species. This extra fragment contained an additional *Pst*I site as well as a deletion of about 200 bases within clone pFON7 resulting in a 0.4-kb instead of 0.6-kb fragment in the *Pst*I digestion (Fig. 6A). Last, it was possible to visualize a density difference of the hybridizing bands of RFLPG IV after *Hin*dIII digestion in the autoradiogram (Fig. 6A). The densities of the 2.7-, 2.1- and 1.9-kb *Hin*dIII fragments were determined on a Ultroscan XL Laser Densitometer (Pharmacia LKB, Uppsala, Sweden). The density ratio (2.7:2.1:1.9 kb), as determined by the area under the curves (AUC), was 2:0.9:0.8, which was close to the expected 2:1:1 molar ratio reflected by size differences (Fig. 6B). Furthermore, the other potential parental type was found in another closely related formae speciales, *F. o. melonis*, as evidenced by the presence of the 1.9-

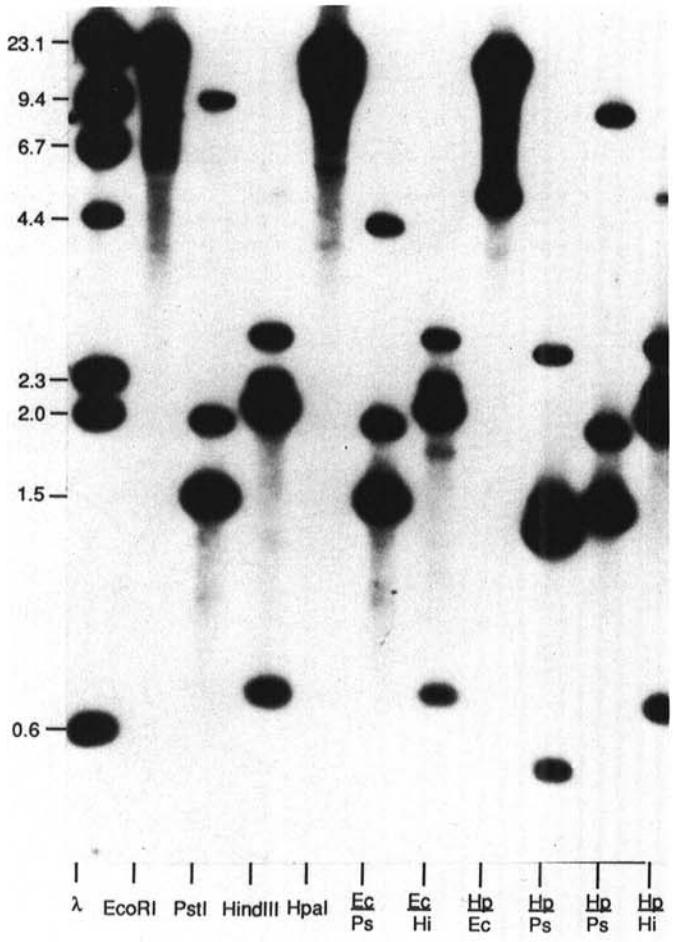

Fig. 5. Hybridization patterns of single and double enzyme-digested mtDNA with pFON7. Each lane is labeled with the enzyme used to digest the mtDNA: *Eco*RI (Ec), *Hin*dIII (Hi), *Hpa*I (Hp), and *Pst*I (Ps). In the double digestion lanes, the enzyme used in the first digestion is shown on the upper line, and the enzyme used second is below the line. Numbers refer to fragment sizes (kb), and λ represents the *Hin*dIII-digested λ DNA as a size marker.

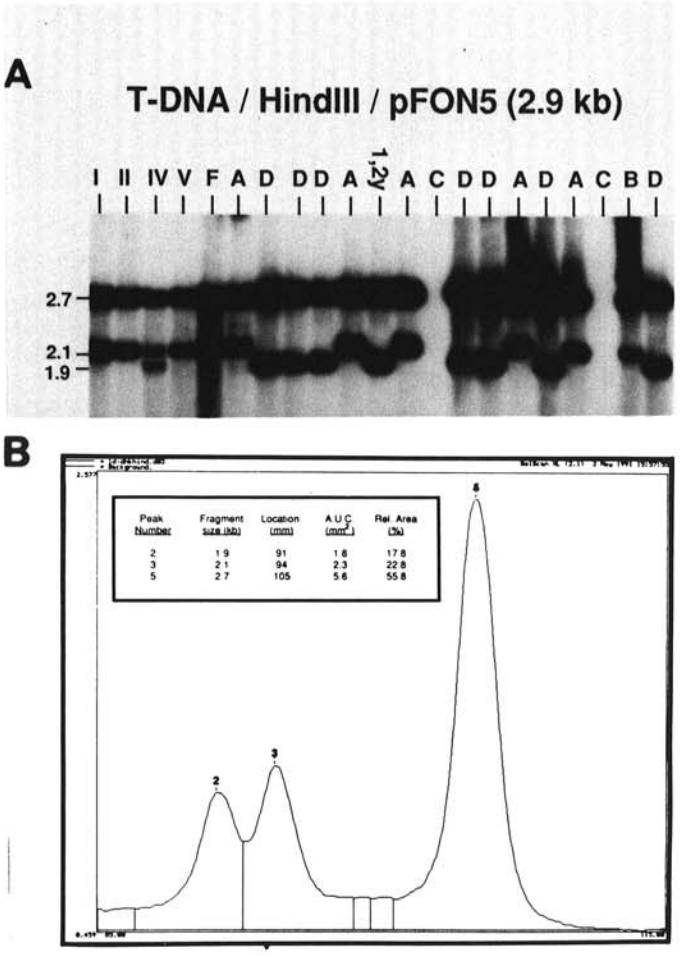

Fig. 6. Autoradiogram and densitometry of the hybridizing bands of restriction fragment length polymorphism group (RFLPG) IV. **A,** *Hin*dIII digests hybridized to mtDNA *Pst*I pFON5 probe. Three restriction patterns were detected depending on the presence of 1.9-kb and/or 2.1-kb fragments. Lanes I–V are *Fusarium oxysporum* f. sp. *niveum* and correspond to their RFLPG. Lanes identified by letters are mtDNA RFLPGs of *F. o. melonis* described by Jacobson and Gordon (18). Numbers on the left refer to fragment sizes (kb). **B,** optical density of the three hybridizing bands (2.7, 2.1, and 1.9 kb) of RFLPG IV (lane IV) with their size, location, area under the curve (AUC), and relative area.

kb fragment in *F. o. melonis* isolates of mtDNA RFLP D (Fig. 6A).

RFLPG V gained a new *Pst*I site in the 1.5-kb fragment but lost a site between the 1.5- and 2.9-kb fragments and another between a 1.0- and 5.6-kb segment resulting in 0.9-, 3.5-, and 6.6-kb fragments. In addition to these differences, RFLPG V also had a deletion in the 9.1-kb fragment around the area represented by *Hin*dIII digestion and close to pFON7 resulting in a 2.6-kb fragment rather than a 2.7-kb fragment in the *Hin*dIII digestion (Fig. 4). RFLPG VI resulted from a *Pst*I site mutation between 12.1 and 5.6 kb and a length mutation (deletion) from pFON3 to pFON2a.

It was concluded that all of the RFLP variants occurred within the 1.5-kb fragment (pFON7) or its homologous regions (pFON6 and pFON2a). Because of this, the *Pst*I digestion was hybridized with pFON7 by itself to determine whether it could discriminate the six RFLP groups detected by the polyprobe. Figure 1 compares the six RFLP groups detected by the polyprobe (Fig. 1C) and pFON7 (Fig. 1D). In all cases, pFON7 alone differentiated each RFLP group that the polyprobe did. Other single probe-enzyme combinations revealed some polymorphisms, but only the pFON7-*Pst*I detected all six RFLP groups, suggesting this probe is a unique variable area in the mitochondrial genome.

Construction of gene tree. Based on the characterization of the differences in RFLP groups, a gene tree defined as the phylogeny of a particular gene or stretch of DNA by Avise (2) was constructed with the fewest possible stepwise changes and is shown in Figure 7. RFLPG II is located at the center of the tree, and RFLPG I and RFLPG III are one step removed from RFLPG II. RFLPG VI is two steps away with both a site and length mutation. RFLPG IV, which represents the case of heteroplasmy, is connected to RFLPG I by a dotted line. RFLPG V was the most distant with four steps, three site mutations, and one length mutation from RFLPG II.

DISCUSSION

A close relationship between mtDNA RFLP haplotypes and vegetative compatibility groups (VCG) was observed in *F. o. melonis* (18). However, there was not a correlation between pathological races and mtDNA or VCG. Multiple races of *F. o. melonis* occurred within a single VCG, and a given race was associated with more than one VCG (18). In contrast, there was a close relationship between pathological race and VCG in *F. o. niveum* (24). In the present study, however, there was no corresponding relationship between mtDNA RFLP haplotypes, pathological race, or geographical origin among 50 isolates of *F. o. niveum*. The evolution of resistance and virulence in plants and pathogens is thought to occur in a stepwise fashion (10). Mutations conferring resistance in a host cause selection pressure for more virulent races of the pathogen. Likewise, a highly aggressive race of the pathogen can decimate its host unless new gene combinations or mutations conferring resistance occur (10). The observation that more than one pathological race may exist within each mtDNA RFLP group of *F. o. niveum* (Table 1) suggests that race differentiation occurred independently in each RFLP group under the selection pressure of resistance genes. Another explanation is that recombination through sexual reproduction (perhaps in the distant past) could have resulted in the gene(s) that influence host specificity being associated with different genetic backgrounds. Alternatively, derivatives of an originally clonal line may have accumulated the differences in mtDNA that are now observed.

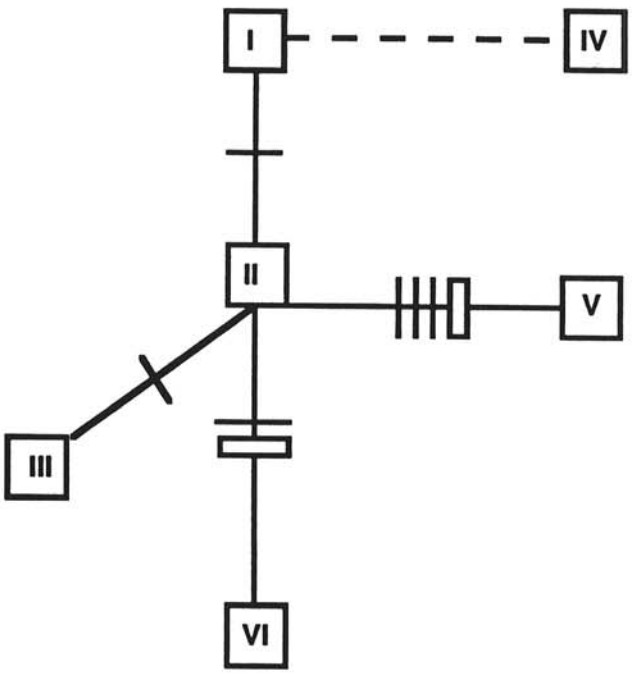

Fig. 7. Gene tree (see ref. 2) of six restriction fragment length polymorphism groups (RFLPGs) of *Fusarium oxysporum* generated by empirical analysis. Each bar represents the number of mutations needed to get to the next group. Solid bars represent site mutations, and open bars represent length mutations. Branch length is not proportional to the distance. The dotted line represents a branch showing heteroplasmy.

Based on size comparison of restriction fragments and the location of restriction recognition sites produced by *Pst*I and *Eco*RI, the restriction map of mtDNA from *F. o. niveum* race 0 isolate FL-60-3A is very similar to the mtDNA map of *F. o. melonis* (18), which is in general agreement with the map of *F. o. lycopersici* (25). A difference between *F. o. niveum* and *F. o. melonis*, however, was the presence of an additional 1.0-kb *Pst*I fragment (pFON8b) in *F. o. niveum*. However, an equivalent 1.0-kb *Pst*I fragment may also be present in *F. o. melonis*, but it was not cloned so its presence may have been masked by the other 1.0-kb fragment (18). Because the accuracy in measuring fragment sizes decreases with increasing fragment size, the differences in size of the two largest *Pst*I fragments (12.1 and 9.1 kb for *F. o. niveum* and 10.5 and 8.8 kb for *F. o. melonis*) needs to be further evaluated. It is possible that the two mtDNA maps are identical.

mtDNA RFLPs were used to estimate the genetic divergence among the collections of *F. o. niveum* and to determine the relationship among them. It is assumed that the degree of divergence reflects relative time since existant organisms diverged from a common ancestor (9,32). Both cladistic and phenetic methods have been used in similar studies with mtDNA to estimate the relationship among fungi (18,36). In this study, Jaccard's similarity coefficient (measuring the common present bands as a fraction of the total bands minus the common absent bands) was applied, and the dendrogram places all pathogenic groups into a tight cluster at a similarity level of 65% or greater and which are all well separated from the nonpathogenic isolates with less than 21% similarity. Although we were unable to distinguish races from *F. o. niveum* by mtDNA haplotype, mtDNA may be useful in distinguishing pathogenic isolates of *F. oxysporum* from nonpathogenic isolates. This has also recently been shown by Gordon and Okamoto (15) and Koch et al (23). Two nonpathogenic isolates displayed distinctive polymorphisms and did not group to any branches with pathogenic isolates. Similar results were obtained with nonpathogenic isolates in *F. o. melonis*, the causal agent of Fusarium wilt of muskmelon (T. R. Gordon, U. C. Berkeley, *personal communication*).

The gene tree was constructed empirically and is based on the fewest number of changes required to account for the haplotypes. It placed RFLPGs I, II, III, IV, and VI very close together (Fig. 7) and is similar to that produced by cluster analysis (Fig. 3), except that RFLPG VI is only two steps away from RFLPG II in the gene tree. This can be explained by the fact that a large deletion in the mtDNA from RFLPG VI is considered as a single step in the gene tree but is a multiple step event in the cluster analysis because a large deletion results in changes in fragment size from all restriction enzymes. In the gene tree, all

RFLP groups were close together and located within five steps from the closest to the most distant group. This close relationship of all RFLP groups may mean that *F. o. niveum* diverged fairly recently. Because RFLPG I had the most common pattern and was present throughout a wide geographical area, it may be the group most broadly adapted to the host plant. It could also indicate that this group is the oldest and has stabilized. Because only one isolate from Israel and Taiwan was used, the diversity of *F. o. niveum* in these areas cannot be evaluated.

Construction of a restriction map for mtDNA permits a molecular interpretation of how variations between RFLP groups occur. Length mutations, which were the major source of variations of mtDNA in *Neurospora crassa* (37) and were common in *F. o. melonis* (18), were rare in *F. o. niveum* and, instead, site mutations resulting from base substitution were more common. Only eight character changes separated all six *F. o. niveum* mtDNA RFLP groups. In contrast, 16 phylogenetically informative character changes were detected in the *F. o. melonis* mtDNA haplotypes. Therefore, *F. o. niveum* appears to be more homogeneous than *F. o. melonis*.

Heteroplasmy has been detected in a wide variety of organisms including animals (5), fish (14), insects (17), and plants (19). However, to our knowledge, this is the first report of natural heteroplasmy in a fungus. Although heterokaryosis within the same VCG was shown in *F. oxysporum* (24,33), the cytoplasmic exchanges were generally believed to occur under the forced situation such as complementation (33). However, in our study, the presence of two different mtDNA genomes in the same cell was detected from a natural isolate. Therefore, heterokaryosis and cytoplasmic fusion may be possible mechanisms for the exchange of genetic material in *F. o. niveum* in nature. The close relationship between *F. o. niveum* and *F. o. melonis* was suggested by the very similar or identical mtDNA restriction maps of reference strains. Additionally, the heteroplasmy evident in *F. o. niveum* consisted of the most common mtDNA from *F. o. niveum* (RFLPG I) and mtDNA from *F. o. melonis* (RFLPG D) (18). This might suggest that these two formae speciales are closely related and that RFLPG IV may represent a transitional group.

The 1.5-kb *Pst*I fragment (pFON7) in *F. o. niveum* is located between the cytochrome oxidase II gene and the 24s rRNA gene on the mtDNA map of *F. o. melonis* (18). However, a functional gene that covers this area has not been identified. pFON7 appears to be a unique fragment in that it has a high frequency of variation in which nonlethal deletions can occur. In addition, this sequence shows evidence for duplication in other areas of mtDNA by homologous hybridization. This fragment also revealed polymorphisms in other formae speciales (*F. o. betae* and *F. o. spinaciae*) (28). Therefore, it is possible that this could be an intron or insertion sequence in the mtDNA genome of *F. o. niveum*.

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