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Characterization of the Formae Speciales of *Fusarium oxysporum* Causing Wilts of Cucurbits by DNA Fingerprinting with Nuclear Repetitive DNA Sequences

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The genetic relatedness of five formae speciales of *Fusarium oxysporum* causing wilts of cucurbit plants was determined by DNA fingerprinting with the moderately repetitive DNA sequences FOLR1 to FOLR4. The four FOLR clones were chosen from a genomic library made from *F. oxysporum* f. sp. *lagenariae* 03-05118. Total DNAs from 50 strains representing five cucurbit-infecting formae speciales, *cucumerinum*, *melonis*, *lagenariae*, *niveum*, and *momordicae*, and 6 strains of formae speciales pathogenic to other plants were digested with *Eco*RV and hybridized with ^{32}P-labeled FOLR probes. The strains were clearly distinguishable at the formae specialis level on the basis of FOLR DNA fingerprints. Fifty-two fingerprint types were detected among the 56 strains by using all FOLR probes. These probes were used to infer phylogenetic relationships among the DNA fingerprint types by the unweighted pair group method using averages and parsimony analysis. The fingerprint types detected in each of the formae speciales *cucumerinum*, *lagenariae*, *niveum*, and *momordicae* were grouped into a single cluster. However, two different genetic groups occurred in the formae specialis *melonis*. The two groups also differed in pathogenicity: one group caused wilts of muskmelon and oriental melon, while the second was pathogenic only to muskmelon. The fingerprint types of different formae speciales pathogenic to plants other than cucurbits were distinguishable from one another and from the fingerprints of the cucurbit-infecting strains. These results suggest that the cucurbit-infecting formae speciales are intraspecific variants distinguishable at the DNA level and in their host range.

The phytopathogenic fungus *Fusarium oxysporum* Schlechtend.:Fr. causes destructive vascular wilts in a wide variety of crops. However, individual pathogenic strains within the species have a limited host range. Strains with similar or identical host ranges are assigned to intraspecific groups, called formae speciales (2, 46). The formae speciales are distinguished by the ability of their members to cause a wilt disease on a limited taxonomic range of host plants (2, 46). Some of the formae speciales are further divided into subgroups, named races, based on pathogenicity to a set of differential cultivars within the same plant species (2). *F. oxysporum* also includes nonpathogenic strains capable of persisting through asymptomatic colonization of plant roots and saprophytic growth on dead organic matter (8). Such pathogenic variation suggests an extreme genetic divergence within the species despite its highly conserved morphology (4).

In Japan, more than 10 cucurbit plant species and a number of their cultivars are grown for foods, cosmetics, and stocks for grafting (26). Grafting is in general use for growing cucurbit plants in Japan. Scions are grafted on the compatible stocks to escape attack from soilborne pathogens. For example, watermelon scions are grafted on bottle gourd stocks. Corresponding to these diverse plant species and cultivars, pathogenic variation of *F. oxysporum* on the cucurbit plants has been categorized into six formae speciales: formae specialis *cucumerinum* on *Cucumis sativus* L. (39), forma specialis *melonis* on *Cucumis melo* L. (20), forma specialis *lagenariae* on *Lagenaria siceraria* (Molina) Standley (27), forma specialis *niveum* on *Citrullus lanatus* (Thumb.) Matsum. et Nakai (2), forma specialis *luffae* on *Luffa cylindrica* Roem (14), and forma specialis *momordicae* on *Momordica charantia* L. (47).

The formae speciales pathogenic to cucurbit plants are basically host specific and distinguished by host species. However, exceptions to the concept of forma specialis have been reported in these formae speciales. McMillan (30) reported that isolates of *F. oxysporum* which were obtained from wilted cucumber plants in the Bahamas were also pathogenic to muskmelons and watermelons. Similar isolates were detected by Kim et al. (16). Martyn and McLaughlin (25) found that some isolates of the forma specialis *niveum* infected some summer squash cultivars. Nomura (36) showed that the forma specialis *lagenariae* was pathogenic not only to bottle gourds but also to pumpkins and malabar gourds. The forma specialis *momordicae*, the causal agent of balsam pear wilt, was found to cause disease also in bottle gourds, pumpkins, and malabar gourds (33). On the basis of the cross-infectivity of these formae speciales, the genetic relationships within and among these formae speciales have been questioned (15).

Knowledge of the amount and distribution of genetic variability within and among formae speciales is a prerequisite to study their genetic relationships (4, 29, 31). Over the past several years, genetic diversity in *F. oxysporum* has been examined by using various genetic markers, such as vegetative compatibility grouping (VCG) (3, 5, 6, 10, 12, 13, 19, 21, 24, 40, 41), isozyme profiles (3), and restriction fragment length polymorphisms (RFLPs) in mitochondrial and nuclear DNA (3, 11, 15–19, 23, 24). Of these procedures, RFLP analysis has the advantage of potentially detecting numerous polymorphisms at the DNA level. Recently, DNA fingerprinting with nuclear repetitive DNA sequences has been used to distinguish

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strains of *F. oxysporum* belonging to different pathogenic variants or from different geographic localities (4, 18).

In this study, our objectives were (i) to develop a sensitive DNA fingerprinting method for identifying individuals within single formae speciales of *F. oxysporum* causing wilts of cucurbit plants and (ii) to examine genetic diversity within the cucurbit-infecting formae speciales and to determine genetic relatedness among the formae speciales. The DNA fingerprinting method that we developed also was useful for identifying pathogenic variants of *F. oxysporum.*

MATERIALS AND METHODS

Fungal strains. Strains of *F. oxysporum* used in this study are listed in Table 1. These strains were collected from various locations in Japan (Table 1 and Fig. 1). All strains were obtained from single spores and maintained on potato dextrose agar. They were assayed for host specificity to confirm their previous definition at the forma specialis level.

Pathogenicity tests. Each strain was grown in 100-ml portions of potato dextrose broth in 300-ml Erlenmeyer flasks at 28°C for 5 days on an orbital shaker (125 rpm). The resulting culture was passed through four layers of cheesecloth, and the filtrate was centrifuged at 2,000 × *g* for 10 min. The conidial pellet was resuspended in distilled water to give a concentration of 10^7 conidia per ml. The pathogenicity of the *F. oxysporum* strains used was assayed by a root dip method (50). Plant seedlings were grown in soil sterilized with chloropicrin. We used seedlings that had fully expanded leaves for pathogenicity tests. If seeds were infested with the *Fusarium* pathogens, symptoms should appear on the seedlings before use for the tests. The healthy seedlings were removed from the soil, and their roots were washed gently with water. The roots were dipped in a spore suspension (10^7 conidia per ml) for 15 s. The inoculated seedlings were transplanted to plastic pots (9-cm diameter) filled with sterilized soil and placed in a greenhouse (25 to 32°C). External symptoms and vascular discoloration were scored 28 days after inoculation, and the pathogen was reisolated from vascular bundles of inoculated plants.

The cucurbit plant species and the cultivars used for pathogenicity tests were as follows: cucumber (*C. sativus* L. cv. Suhyou and *C. sativus* L. cv. Shimoshirazu jihai), muskmelon (*C. melo* L. cv. Amus and *C. melo* L. cv. Homerun star), oriental melon (*C. melo* L. var. *makuwa* Makino cv. Ogon 9 and *C. melo* L. var. *makuwa* Makino cv. New melon), hybrid melon (*C. melo* × *C. melo* var. *makuwa* cv. Prince), bottle gourd [*Lagenaria siceraria* (Molina) Standley cv. Sakigake and *Lagenaria siceraria* (Molina) Standley cv. Friend], pumpkin (*Cucurbita maxima* Duch. ex Lam. cv. Ebisu), malabar gourd (*Cucurbita ficifolia* Bouche cv. Kurodane kabocha), watermelon [*Citrullus lanatus* (Thumb.) Matsum. et Nakai cv. Asahi yamato and *Citrullus lanatus* (Thumb.) Matsum. et Nakai cv. Kodama], loofah (*Luffa cylindrica* Roem. cv. Futo hechima), balsam pear (*M. charantia* L. cv. Futo reishi), and wax gourd (*Benincasa hispida* Cogn. cv. Daimaru tougan).

DNA extraction. Fungal strains were grown in 200-ml portions of potato dextrose broth in 1-liter Roux bottles at 28°C for 4 days. Total DNA of each strain was prepared from the resulting mycelia by the method of Adachi et al. (1). Nuclear DNA was isolated by the method of Orbach et al. (38).

Recombinant λ phage DNA was isolated by the plate lysate method (44).

Cloning of repetitive DNA. Nuclear DNA from *F. oxysporum* f. sp. *lagenariae* 03-05118 was partially digested with *Sau*3AI to generate fragments of 15 to 20 kb. The *Sau*3AI fragments were cloned into λ phage vector λFix II (Stratagene) according to the manufacturer's recommendation. Genomic clones containing repetitive DNA sequences were selected from the resulting genomic library as described for *Magnaporthe grisea* by Hamer et al. (9). The library was screened by plaque hybridization with ^{32}P-labeled total genomic DNA of strain 03-05118 and nuclear rRNA gene (rDNA) clone Alt1 of *Alternaria alternata* (49). Alt1 was isolated as a nuclear rDNA clone containing two copies of the entire rDNA unit of *A. alternata* (49). We identified four clones, named FOLR1 to FOLR4, that carried moderately repetitive DNA sequences and were dispersed in the fungal chromosomes (unpublished data).

Hybridization. Fungal DNA was digested to completion with restriction endonucleases (Toyobo) and electrophoresed in 0.8% agarose gels by standard methods (44). The fractionated DNA was transferred to Hybond N+ nylon membranes (Amersham) by the alkaline transfer method (42).

DNA probes were produced by labeling with [α-^{32}P]dCTP by the random-primer method (7). Hybridization was carried out in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.7], and 1 mM EDTA) containing 5× Denhardt's solution (44), 0.5% sodium dodecyl sulfate, 100 μg of sonicated salmon sperm DNA per ml, and 50% formamide at 42°C. Hybridized blots were washed at 65°C; the final wash was done with 1× SSPE–0.1% sodium dodecyl sulfate.

Statistical analysis. The FOLR DNA fingerprint profiles were used to measure the genetic diversity within the formae speciales of *F. oxysporum*. Each band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 on the basis of the presence or absence of the band, respectively, for this position. Frequencies of bands at individual positions were tabulated for each forma specialis and for the total strains. Nei's (34) measure of genetic diversity (*H*) is given by the formula

$$H = 1 - \sum_{j=1}^{k} X_j^2$$

where *Xj* is the frequency of the *j*th allele.

The DNA fingerprints were analyzed separately to calculate the phenograms and parsimony trees by a binomial system based on the presence or absence of a given fragment. We estimated the similarity coefficient (*F*) for all possible pairs of strains from their fingerprints by the method of Nei and Li (35). A dendrogram was constructed from the similarity coefficient data by the unweighted pair group method with arithmetic average clustering (UPGMA) (45). The relationship among isolates was also analyzed by parsimony analysis using PAUP 3.1.1 (48). Trees were rooted by using a data set containing the value 0 for all numbers.

RESULTS

DNA typing of *F. oxysporum* strains. Total DNA of strain 03-05118 was digested with 10 restriction endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I, and *Xho*I) separately and subjected to Southern analysis with the FOLR clones as probes to determine the appropriate probe-enzyme combinations for DNA fingerprinting. Digestion of total DNA with various restriction enzymes produced multiple fragments hybridizable to the probes. *Eco*RV provided the most desirable resolution of fragment distribution in all probes. FOLR1 to FOLR4 probes hybridized to 22, 19, 23, and 13 *Eco*RV fragments (ranging in size from 0.5 to 20 kb), respectively, in strain 03-05118.

Total DNA of 50 strains representing five cucurbit-infecting

TABLE 1. Strains of *F. oxysporum* used in this study

Working no.	Strain[a]	Forma specialis	Origin		Source[b]
			Prefecture	Plant	
1	03-05116	*cucumerinum*	Unknown	Cucumber	NIAR
2	07-27508	*cucumerinum*	Unknown	Cucumber	NIAR
3	03-05117	*cucumerinum*	Unknown	Cucumber	NIAR
4	Cu: 2-1	*cucumerinum*	Unknown	Cucumber	NARC
5	Cu: 1-1	*cucumerinum*	Fukuoka	Cucumber	TCRS
6	SUF359	*cucumerinum*	Unknown	Cucumber	SU
7	Cu: 8-1 (JCM9284)	*cucumerinum*	Toyama	Cucumber	TCRS
8	Cu: 8-2	*cucumerinum*	Toyama	Cucumber	TCRS
9	Cucu01-011	*cucumerinum*	Ohita	Cucumber	This study
10	F. cucu	*cucumerinum*	Unknown	Cucumber	NARC
11	F. o. 5 No. 3	*cucumerinum*	Ibaraki	Cucumber	IAES
12	Cucu01-015 (JCM9285)	*cucumerinum*	Toyama	Cucumber	This study
13	Cucu01-016	*cucumerinum*	Toyama	Cucumber	This study
14	Cucu01-017	*cucumerinum*	Toyama	Cucumber	This study
15	Mel: 1-0	*melonis*	Unknown	Muskmelon	NRIV
16	Mel: 5-0	*melonis*	Shizuoka	Muskmelon	TCRS
17	Mel02005 (JCM9286)	*melonis*	Kumamoto	Muskmelon	KARC
18	Mel02008 (JCM9287)	*melonis*	Kumamoto	Muskmelon	KARC
19	Mel: 2-0	*melonis*	Fukuoka	Muskmelon	TCRS
20	Mel02010 (JCM9288)	*melonis*	Nagasaki	Muskmelon	This study
21	Mel02011	*melonis*	Nagasaki	Muskmelon	This study
22	Mel02013	*melonis*	Kumamoto	Muskmelon	KARC
23	90T1SV-1	*melonis*	Kagoshima	Muskmelon	KAES
24	90T2SV-2	*melonis*	Kagoshima	Muskmelon	KAES
25	B-1	*melonis*	Kanagawa	Muskmelon	ARIK
26	B-2	*melonis*	Kanagawa	Muskmelon	ARIK
27	C-1	*melonis*	Kanagawa	Muskmelon	ARIK
28	C-2	*melonis*	Kanagawa	Muskmelon	ARIK
29	Mel02025 (JCM9289)	*melonis*	Kumamoto	Muskmelon	KARC
30	Mel02026	*melonis*	Ibaraki	Muskmelon	IAES
31	01-03008	*lagenariae*	Mie	Bottle gourd	NIAR
32	03-05118	*lagenariae*	Unknown	Bottle gourd	NIAR
33	07-27503	*lagenariae*	Unknown	Bottle gourd	NIAR
34	Lag: 3-1 (JCM9293)	*lagenariae*	Kumamoto	Bottle gourd	TCRS
35	Lag: 1-1	*lagenariae*	Tochigi	Bottle gourd	TCRS
36	Lag: 7-1	*lagenariae*	Kumamoto	Bottle gourd	TCRS
37	No. 87	*lagenariae*	Tochigi	Bottle gourd	TAES
38	No. 134	*lagenariae*	Tochigi	Bottle gourd	TAES
39	03-05608	*niveum*	Unknown	Watermelon	NIAR
40	03-05543	*niveum*	Shizuoka	Watermelon	NIAR
41	Niv: 1-0	*niveum*	Fukuoka	Watermelon	TCRS
42	Niv: 2-0	*niveum*	Unknown	Watermelon	TCRS
43	SUF366	*niveum*	Unknown	Watermelon	SU
44	80WF-2	*niveum*	Kagoshima	Watermelon	KAES
45	80WF-3 (JCM9291)	*niveum*	Kagoshima	Watermelon	KAES
46	A-1	*niveum*	Kanagawa	Watermelon	ARIK
47	Niv04010	*niveum*	Unknown	Watermelon	NARC
48	90NF1-2	*momordicae*	Kagoshima	Balsam pear	KAES
49	90NF2-1 (JCM9292)	*momordicae*	Kagoshima	Balsam pear	KAES
50	90NF2-2	*momordicae*	Kagoshima	Balsam pear	KAES
51	03-05121	*lycopersici* (race 1)	Unknown	Tomato	NIAR
52	Ral08003	*radicis-lycopersici*	Miyazaki	Tomato	This study
53	SUF126	*melongenae*	Unknown	Eggplant	SU
54	03-05123	*raphani*	Tochigi	Radish	NIAR
55	07-27516	*conglutinans*	Unknown	Cabbage	NIAR
56	03-05557	*fragariae*	Shizuoka	Strawberry	NIAR

[a] Representative strains of the cucurbit-infecting formae speciales have been deposited in an international culture collection, Japan Collection of Microorganisms (JCM), and the accession numbers are shown in parentheses.

[b] Abbreviations: NIAR, National Institute of Agrobiological Resources; NARC, National Agricultural Research Center; TCRS, Toyama Vegetable and Ornamental Crops Research Station; SU, Sinshu University; IAES, Ibaraki Agricultural Experiment Station; TAES, Tochigi Agricultural Experiment Station; NRIV, National Research Institute of Vegetables, Ornamental Plants and Tea; KARC, Kumamoto Agricultural Research Center; ARIK, Agricultural Research Institute of Kanagawa Prefecture; KAES, Kagoshima Agricultural Experiment Station.

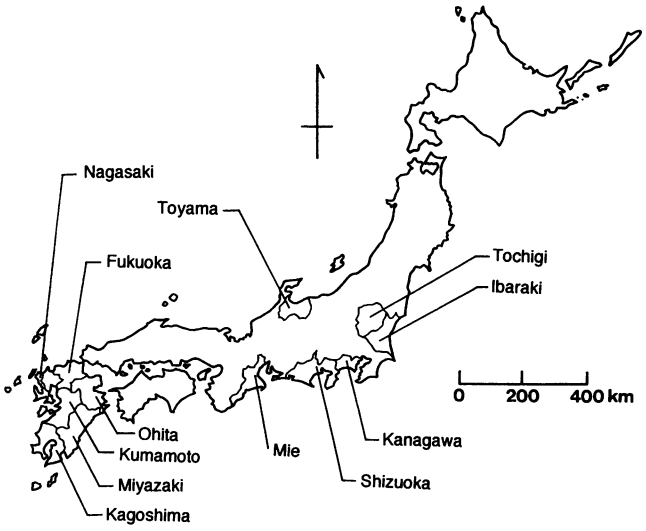

FIG. 1. Locations of the prefectures in Japan where *F. oxysporum* strains were collected. The arrow indicates north.

formae speciales and 6 strains pathogenic to plants other than cucurbits was digested with *Eco*RV and hybridized with ^{32}P-labeled FOLR probes. Figure 2 shows DNA fingerprints of the 56 strains with the FOLR4 probe. FOLR1 to FOLR4 probes hybridized to 5 to 26, 6 to 19, 6 to 25, and 1 to 17 *Eco*RV fragments, respectively, in each of the 56 strains, and several polymorphic bands were identified in DNA from these strains. Each probe produced different DNA fingerprints within a single forma specialis population. Major bands in the fingerprints were shared by strains within a single forma specialis, but minor bands tended to be more polymorphic among the strains. In general, however, strains within each of the formae speciales had similar banding patterns and were distinguished from strains of other formae speciales. Extensive polymorphisms in the fingerprints were observed not only in minor bands but also in major bands, when the fingerprints were compared between strains of different formae speciales. Strains of the forma specialis *melonis* had diverse banding patterns compared with those of other formae speciales (Fig. 2).

Genetic diversity within the cucurbit-infecting formae speciales. The polymorphisms detected by FOLR DNA fingerprinting were used to measure genetic diversity within each of the cucurbit-infecting formae speciales. FOLR1 to FOLR4 probes produced 53, 48, 43, and 39 different resolvable fragments, respectively, in the 56 strains tested. Nei's genetic diversity index in the total population was derived separately for the four FOLR DNA fingerprints (Table 2).

The genetic diversity within single formae speciales was significantly lower than that of the total population in every FOLR probe. However, there were differences in the levels of diversity among the formae speciales. In particular, forma specialis *melonis* carried a relatively high level of variation compared with that in the other formae speciales. This forma specialis contained two groups which were easily distinguishable from one another via FOLR DNA fingerprinting. Within each of these groups, diversity levels were similar to those seen in the other formae speciales. FOLR3 produced DNA fingerprints that could be used to clearly distinguish the two groups (Fig. 3). DNA fingerprint groups I and II of this forma specialis consisted of 10 strains (working numbers 15, 16, 19, 20, 21, 25,

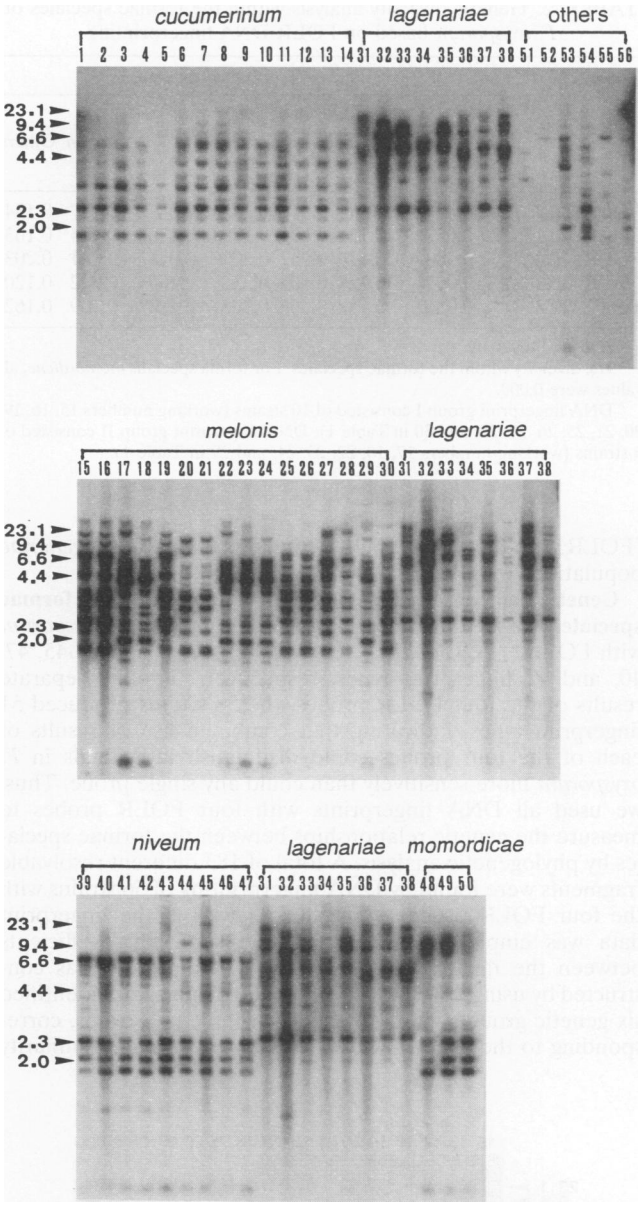

FIG. 2. FOLR4 DNA fingerprints of 56 strains from different formae speciales of *F. oxysporum*. Total DNA from each strain was digested with *Eco*RV and fractionated on a 0.8% agarose gel. The Southern blots were hybridized with ^{32}P-labeled FOLR DNA. Each blot contained DNA from eight strains of the forma specialis *lagenariae* for comparing DNA banding patterns between the formae speciales. The numbers above each lane indicate the strain working numbers shown in Table 1. The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated on the left in kilobases.

26, 27, 28, and 30) and 6 strains (working numbers 17, 18, 22, 23, 24, and 29), respectively (Tables 1 and 2). In contrast, we observed no genetic variation within forma specialis *momordicae*. This might be due to our restricted sample, only three strains, all from the same field in Kagoshima Prefecture.

The amount of diversity detected within a forma specialis was dependent upon the FOLR probe used. For example, with different probes, the genetic diversity ranged from 0.076

TABLE 2. Genetic diversity analysis within the formae speciales of *F. oxysporum* based on FOLR DNA fingerprinting

Probe	H_T[a]	H_S[b] within forma specialis:						
		cucumerinum	*melonis*[c] Group I	*melonis*[c] Group II	*melonis*[c] Total	*lagenariae*	*niveum*	Others
FOLR1	0.337	0.034	0.141	0.043	0.242	0.085	0.157	0.154
FOLR2	0.249	0.057	0.131	0.081	0.170	0.170	0.114	0.163
FOLR3	0.367	0.083	0.126	0.047	0.201	0.076	0.081	0.203
FOLR4	0.308	0.053	0.128	0.103	0.214	0.167	0.072	0.120
Total	0.318	0.056	0.132	0.066	0.208	0.120	0.109	0.162

[a] H_T, total diversity.
[b] H_S, diversity within the formae speciales. For forma specialis *momordicae*, all values were 0.000.
[c] DNA fingerprint group I consisted of 10 strains (working numbers 15, 16, 19, 20, 21, 25, 26, 27, 28, and 30 in Table 1); DNA fingerprint group II consisted of 6 strains (working numbers 17, 18, 22, 23, 24, and 29 in Table 1).

(FOLR3) to 0.167 (FOLR4) in the forma specialis *lagenariae* population.

Genetic relatedness between the cucurbit-infecting formae speciales. DNA fingerprinting of 56 strains of *F. oxysporum* with FOLR1, FOLR2, FOLR3, and FOLR4 produced 45, 47, 40, and 42 fingerprint types, respectively. Pooling separate results of the four FOLR probes, the 56 strains produced 51 fingerprint types, indicating that combined use of results of each of the four probes could distinguish individuals in *F. oxysporum* more sensitively than could any single probe. Thus, we used all DNA fingerprints with four FOLR probes to measure the genetic relationships between the formae speciales by phylogenetic analysis. A total of 183 different resolvable fragments were found by DNA fingerprinting of 56 strains with the four FOLR probes. A cluster analysis of the fingerprint data was employed to calculate the similarity coefficients between the fingerprint types, and a dendrogram was constructed by using UPGMA (Fig. 4). The dendrogram identified six genetic groups within the cucurbit-infecting strains, corresponding to the forma specialis classification, at the similarity level of more than 75% (Fig. 4). Each population of the formae speciales *lagenariae*, *cucumerinum*, *niveum*, and *momordicae* clustered in a single group. However, strains of the forma specialis *melonis* were divided into two groups. These two groups were completely correlated with the two DNA fingerprint groups identified in Table 2. *F. oxysporum* strains pathogenic to plants other than cucurbits were distinguished from one another and also from the strains of the cucurbit-infecting formae speciales on the dendrogram.

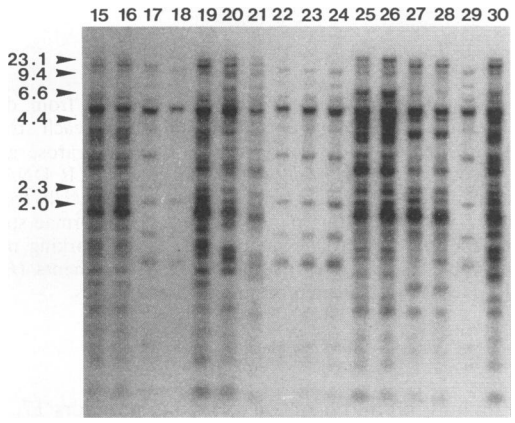

FIG. 3. FOLR3 DNA fingerprints of 16 strains of *F. oxysporum* f. sp. *melonis*. Total DNA was digested with *Eco*RV and fractionated on a 0.8% agarose gel. The Southern blots were hybridized with ^{32}P-labeled FOLR3 DNA. The numbers above each lane indicate the strain working numbers shown in Table 1. The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated on the left in kilobases.

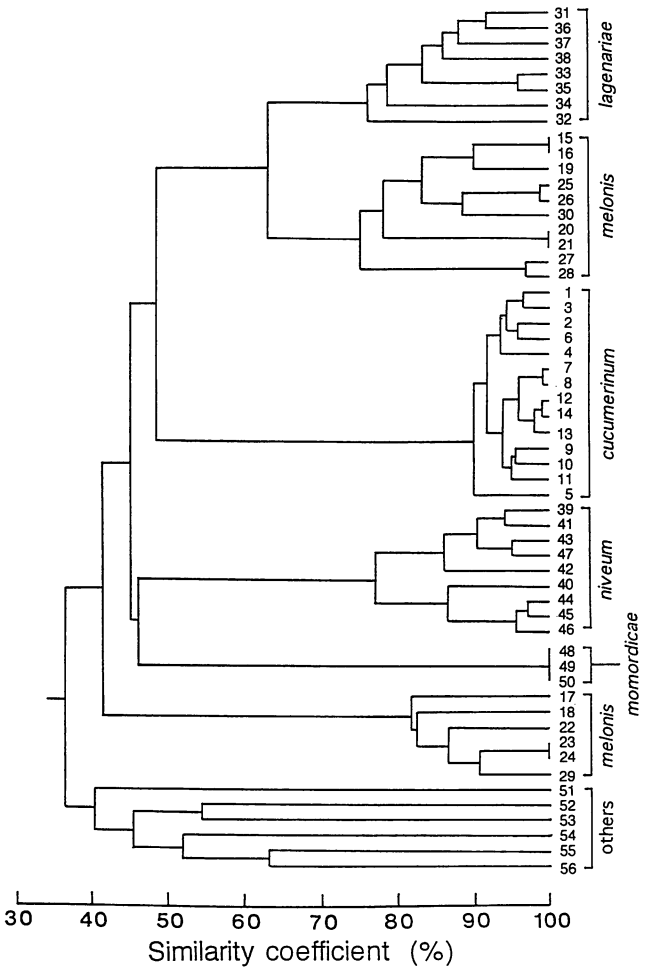

FIG. 4. Dendrogram showing the levels of genetic relatedness of 56 strains from different formae speciales of *F. oxysporum*. The 56 strains were divided into 51 fingerprint types on the basis of the fingerprints probed with FOLR1 to FOLR4. Similarity coefficients were calculated from the fingerprints by the method of Nei and Li (35). A dendrogram was constructed from the similarity coefficients by using UPGMA (45). The numbers on the right indicate the strain working numbers shown in Table 1.

The PAUP program was also used for phylogenetic analysis among the 51 fingerprint types. A data set containing null values for all loci was used as a designated ancestor for rooting trees. A total of 24 highly parsimonious trees were found by this analysis, and all had the same general form. Forma specialis-dependent grouping was evident also on the parsimony tree, and the branching patterns of the trees were identical to that of UPGMA (data not shown).

The forma specialis *lagenariae* contains pathogenic variants that infect pumpkins and malabar gourds in addition to the

TABLE 3. Pathogenicity of strains of *F. oxysporum* f. sp. *melonis* on four cultivars of muskmelon (*C. melo*) and oriental melon (*C. melo* var. *makuwa*)

Strain (working no.)	No. of diseased plants[a] of:			
	Cucumis melo cv.		*C. melo* var. *makuwa* cv.	
	Amus	Homerun star	Ogon 9	New melon
15	10	8	10	10
16	10	8	10	10
17	6	10	0	0
18	6	9	0	0
19	10	10	10	10
20	10	10	10	10
21	10	10	10	10
22	4	10	0	0
23	6	8	0	0
24	7	10	0	0
25	10	10	10	10
26	10	10	10	10
27	10	6	9	9
28	6	7	6	10
29	9	10	0	0
30	10	10	10	10

[a] Of 10 plants inoculated.

original host, bottle gourds (36). We used three pathogenic types in this study: (i) three strains (working numbers 31, 37, and 38) were pathogenic only to bottle gourds, (ii) three strains (working numbers 32, 35, and 36) were highly virulent to bottle gourds and weakly virulent to pumpkins and malabar gourds, and (iii) two strains (working numbers 33 and 34) were highly virulent to all three hosts. FOLR DNA fingerprinting could not differentiate these pathogenic variants.

The formae speciales *lagenariae* and *momordicae* have common host plants, i.e., bottle gourds, pumpkins, and malabar gourds. However, the strains were easily distinguished, corresponding to their forma specialis classification, by FOLR DNA fingerprinting: each population of these formae speciales was grouped into a distinct genetic cluster by phylogenetic analyses (Fig. 4).

Strains of the forma specialis *cucumerinum*, which were collected from different geographical locations in Japan, were more genetically similar than were those of the other formae speciales.

The forma specialis *melonis* population carried two genetic groups that were easily distinguishable from one another on the basis of DNA fingerprint profiles (Fig. 2 and 3) and phylogenetic analyses (Fig. 4). We have reported that the Japanese strains of the forma specialis *melonis* differ in pathogenicity, as indicated by differences in the infection spectra on Japanese cultivars of muskmelon and oriental melon (33). We assayed the 16 strains of the forma specialis *melonis* in this study by the root dip method using two cultivars each of muskmelon and oriental melon (Table 3). The assay divided these strains into two groups that corresponded to the groups determined by FOLR DNA fingerprinting (Table 2). One group pathogenic to both muskmelons and oriental melons corresponded to DNA fingerprint group I, and the other group, pathogenic only to muskmelons, corresponded to DNA fingerprint group II.

DISCUSSION

The forma specialis concept proposed by Snyder and Hansen (46) for *F. oxysporum* was based on strict host specificity of the strains. However, notable exceptions to this rule have been reported in the cucurbit-infecting formae speciales of *F. oxysporum* (16, 25, 30, 33, 36). The infection spectra of the cucurbit-infecting formae speciales are known to be complex: in some cases, a single forma specialis infects multiple genera or species of the family Cucurbitaceae and contains pathogenic variation (16, 25, 30, 33, 36); in other cases, different formae speciales share the same host plants (33, 36). These phenomena suggest that genetic variation exists within each forma specialis and that the formae speciales that attack related host plants may be closely related genetically and share characteristics necessary for pathogenicity (16).

Several studies have been done to determine genetic variation in the formae speciales of *F. oxysporum* on cucurbits by using VCGs (3, 10, 19, 40) and RFLPs in mitochondrial DNA (11, 15, 16). A VCG is defined as a group of isolates that are able to anastomose and form heterokaryons with each other but not with isolates outside the group (41). Larkin et al. (19) demonstrated a good correlation among strains of the forma specialis *niveum* between pathogenicity and VCG. Since *F. oxysporum* lacks a sexual stage, genetic interaction is thought to be restricted primarily to within a VCG. Thus, VCGs may represent genetically isolated populations that are correlated with specific traits, such as pathogenicity. However, VCG markers alone cannot be used to distinguish strains of *F. oxysporum* and to determine genetic relationship among the formae speciales, because of the lack of adequate polymorphisms to detect individuality (4, 21, 29, 31).

Kim et al. (16) determined the genetic divergence and the relatedness of five formae speciales within the Cucurbitaceae based on RFLPs of mitochondrial DNA. They identified a close relationship between the different formae speciales and presented evidence for genetic similarity. Both cluster and parsimony analyses of the mitochondrial DNA RFLPs indicated that all of the *F. oxysporum* formae speciales in cucurbits are closely related and that in some cases, isolates of different formae speciales were genetically more similar than isolates of the same forma specialis (16). On the basis of the complexity of infection spectra of the formae speciales on cucurbits and their RFLP analysis of mitochondrial DNA, Kim et al. (16) hypothesized that the genetic differences between the formae speciales were relatively small and that determinants for host specificity could be combined or lost in individual strains. Our results, based on FOLR DNA fingerprinting of nuclear DNA, are not consistent with this hypothesis. Cluster and parsimony analyses based on FOLR DNA fingerprinting readily distinguished the formae speciales. Our results suggest that the cucurbit-infecting formae speciales of *F. oxysporum* are intraspecies variants differing not only in pathogenicity but also in nuclear DNA content and organization. Differences in DNA fingerprints may reflect recent activity of mobile genetic elements. Thus, apparently substantial differences may occur between strains that are recent derivatives of a clonal ancestor. We are now characterizing FOLR sequences.

The forma specialis *lagenariae* contained three variants that differed in pathogenicity to pumpkins, malabar gourds, and bottle gourds. FOLR DNA fingerprint data demonstrated a close relationship among the pathogenic variants of the forma specialis *lagenariae*, despite differences in host specificity. Such genetic similarity suggests that pathogenic specialization could be caused by a genetically simple process within the forma specialis, such as mutation in a gene necessary for pathogenic-

ity to certain host plants. On the other hand, FOLR DNA fingerprinting divided strains of the forma specialis *melonis* into two different genetic groups according to host range: strains of DNA fingerprint group I cause wilt of both muskmelons and oriental melons; those of group II are pathogenic only to muskmelons. Phylogenetic analyses demonstrated that the two groups differed as much from each other as they did from other formae speciales. These differences indicate a possibility that similarities in pathogenicity between these two groups are a result of convergent evolution. Since these groups also differ in pathogenicity to oriental melons, we hypothesized that this difference might be related to the geographic origin of the host plants. Muskmelons and oriental melons have different geographic origins (26). Thus, genetic differences between the two groups could be due to geographic isolation of the pathogen populations during their establishment and prior to their dispersal throughout the world.

Risser et al. (43) reported that a French population of the forma specialis *melonis* contained four races. The races were distinguished on the basis of pathogenicity to a set of differential cultivars of muskmelon. In this study, the Japanese strains that we used were distinguished on the basis of pathogenicity to muskmelons and oriental melons. Pathogenicity of the foreign races of forma specialis *melonis* to oriental melons is unknown, and race descriptions of the Japanese strains are lacking. We are now identifying the races of the Japanese strains and testing pathogenicity of the foreign strains to oriental melons. In addition to pathogenicity tests, FOLR DNA fingerprinting of the foreign strains will provide a basis for evaluating the presented potential evolution of pathogenic specialization in *F. oxysporum*, taking into account the impact of geographic isolation and coevolution with host plants.

Strains of the forma specialis *cucumerinum* shared a higher level of genetic similarity than did the other forma specialis populations, although they were collected from different locations in Japan. McMillan (30) reported that strains of this forma specialis infected cucumbers, muskmelons, and watermelons. However, these pathogenic variants or races have never been reported in Japan. Uniformity in pathogenicity might reflect the close relationship among the Japanese strains.

Although Kim et al. (16) demonstrated a close relationship among the different formae speciales on cucurbits by RFLP analysis of mitochondrial DNA, DNA fingerprinting of nuclear DNA with moderately repetitive DNA sequences (FOLR clones) detected clear genetic differences among the formae speciales. These two studies used independent sets of strains. Kim et al. (16) used strains collected primarily from the United States and a few other countries, but we used only Japanese strains. We hope to measure the genetic diversity and the relationship among strains of *F. oxysporum* on the Cucurbitaceae collected from other countries by means of FOLR DNA fingerprinting and to analyze RFLPs of mitochondrial DNA from our Japanese strains. Such studies will provide useful information for unraveling the genetic basis of pathogenic specialization in the cucurbit-infecting strains of *F. oxysporum*.

Recently it has been demonstrated that hypervariable repetitive DNA in fungal chromosomes may be useful for differentiation of strains belonging to a particular pathotype or from a particular locale (4, 29, 31). Kistler et al. (18) used nuclear repetitive DNA to infer the genetic relationship among strains representing three crucifer-infecting formae speciales of *F. oxysporum*. The analysis indicated that a close relationship existed among members of the same forma specialis (18). This result is similar to our results with FOLR probes to determine genetic relationships among the cucurbit-infecting formae speciales. DNA fingerprinting with dispersed repetitive sequences also has been successfully applied to resolve variation in *Magnaporthe grisea* (9, 22), *Mycosphaerella graminicola* (28), *Cryphonectria parasitica* (32), and *Erysiphe graminis* (37).

Molecular characterization of the FOLR sequences remains to be done to provide more information. The fact that FOLR DNA fingerprinting can distinguish individuals of *F. oxysporum* indicates that FOLR markers will facilitate the studies of population dynamics of *F. oxysporum* over time and space and of evolutionary dynamics correlated to host plants.

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REFERENCES

1. **Adachi,Y., H. Watanabe, K. Tanabe, N. Doke, S. Nishimura, and T. Tsuge.** 1993. Nuclear ribosomal DNA as a probe for genetic variability in the Japanese pear pathotype of *Alternaria alternata*. Appl. Environ. Microbiol. **59**:3197–3205.
2. **Armstrong, G. M., and J. K. Armstrong.** 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt disease, p. 391–399. *In* P. E. Nelson, T. A. Toussoun, and R. J. Cook (ed.), *Fusarium*: disease, biology, and taxonomy. The Pennsylvania State University Press, University Park.
3. **Bosland, P. W., and P. H. Williams.** 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. **65**:2067–2073.
4. **Bruns, T. D., T. J. White, and J. W. Taylor.** 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. **22**:525–564.
5. **Correll, J. C., J. E. Puhalla, and R. W. Schneider.** 1986. Identification of *Fusarium oxysporum* f. sp. *apii* on the basis of colony size, virulence, and vegetative compatibility. Phytopathology **76**:396–400.
6. **Elmer, W. H., and C. T. Stephens.** 1989. Classification of *Fusarium oxysporum* f. sp. *asparagi* into vegetatively compatible groups. Phytopathology **79**:88–93.
7. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
8. **Gordon, T. R., and D. Okamoto.** 1992. Population structure and the relationship between pathogenic and non-pathogenic strains of *Fusarium oxysporum*. Phytopathology **82**:73–77.
9. **Hamer, J. E., L. Farral, M. J. Orbach, B. Valent, and F. G. Chumley.** 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proc. Natl. Acad. Sci. USA **86**:9981–9985.
10. **Jacobson, D. J., and T. R. Gordon.** 1988. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f. sp. *melonis*. Phytopathology **78**:668–672.
11. **Jacobson, D. J., and T. R. Gordon.** 1990. Variability of mitochondrial DNA as an indicator of relationships between populations of *Fusarium oxysporum* f. sp. *melonis*. Mycol. Res. **94**:734–744.
12. **Katan, T., and J. Katan.** 1988. Vegetative-compatibility grouping of *Fusarium oxysporum* f. sp. *vasinfectum* from tissue and the

rhizosphere of cotton plants. Phytopathology **78**:852–855.
13. **Katan, T., D. Zamir, M. Sarfatti, and J. Katan.** 1991. Vegetative compatibility groups and subgroups in *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology **81**:255–262.
14. **Kawai, I., H. Suzuki, and K. Kawai.** 1958. On the pathogenicity of the wilt *Fusarium* of the cucurbitaceous plants and their forms. Shizuoka Agric. Exp. Stn. Bull. **3**:49–68.
15. **Kim, D. H., R. D. Martyn, and C. W. Magill.** 1992. Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporum* f. sp. *niveum*. Phytopathology **82**:346–353.
16. **Kim, D. H., R. D. Martyn, and C. W. Magill.** 1993. Mitochondrial DNA (mtDNA)-relatedness among formae speciales of *Fusarium oxysporum* in the Cucurbitaceae. Phytopathology **83**:91–97.
17. **Kistler, H. C., P. W. Bosland, U. Benny, S. Leong, and P. H. Williams.** 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. Phytopathology **77**:1289–1293.
18. **Kistler, H. C., E. A. Momol, and U. Benny.** 1991. Repetitive genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. Phytopathology **81**:331–336.
19. **Larkin, R. P., D. L. Hopkins, and F. N. Martin.** 1990. Vegetative compatibility within *Fusarium oxysporum* f. sp. *niveum* and its relationship to virulence, aggressiveness, and race. Can. J. Microbiol. **36**:352–358.
20. **Leach, J. G., and T. M. Currence.** 1938. Fusarium wilt of muskmelon in Minnesota. Minn. Agric. Exp. Stn. Tech. Bull. **129**:32.
21. **Leslie, J. F.** 1993. Fungal vegetative compatibility. Annu. Rev. Phytopathol. **31**:127–150.
22. **Levy, M., J. Romao, M. A. Marchetti, and J. H. Hamer.** 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell **3**:95–102.
23. **Manicom, B. Q., M. Bar-Joseph, J. M. Kotze, and M. M. Becker.** 1990. A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*. Phytopathology **80**:336–339.
24. **Manicom, B. Q., M. Bar-Joseph, A. Rosner, H. Vigodsky-Haas, and J. M. Kotze.** 1987. Potential applications of random DNA probes and restriction fragment length polymorphisms in the taxonomy of the fusaria. Phytopathology **77**:669–672.
25. **Martyn, R. D., and R. J. McLaughlin.** 1983. Susceptibility of summer squash to the watermelon wilt pathogen (*Fusarium oxysporum* f. sp. *niveum*). Plant Dis. **67**:263–266.
26. **Matsuo, T. (ed.).** 1989. Collected data of plant genetic resources, vol. 2. Kodansya Scientific, Tokyo, Japan.
27. **Matsuo, T., and I. Yamamoto.** 1967. On *Fusarium oxysporum* f. sp. *lagenariae* n. f. causing wilt of *Lagenaria vulgaris* var. *hispida*. Trans. Mycol. Soc. Jpn. **2**:61–63.
28. **McDonald, B. A., and J. P. Martinez.** 1990. DNA restriction fragment polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. Phytopathology **80**:1368–1373.
29. **McDonald, B. A., J. M. McDermott, S. B. Goodwin, and R. W. Allard.** 1989. The population biology of host-pathogen interactions. Annu. Rev. Phytopathol. **27**:77–94.
30. **McMillan, R. T.** 1986. Cross pathogenicity studies with isolates of *Fusarium oxysporum* from either cucumber or watermelon pathogenic to both crop species. Ann. Appl. Biol. **109**:101–105.
31. **Michelmore, R. W., and S. H. Hulbert.** 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. **25**:383–404.
32. **Milgroom, M. G., S. E. Lipari, and W. A. Powell.** 1992. DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica*. Genetics **131**:297–306.
33. **Namiki, F., S. Izumi, T. Kayamura, and T. Shiomi.** 1992. Specialization of pathogenic strains of *Fusarium oxysporum* in the Cucurbitaceae. Ann. Phytopathol. Soc. Jpn. **58**:540–541. (In Japanese.)
34. **Nei, M.** 1975. Molecular population genetics and evolution. Elsevier, New York.
35. **Nei, M., and W. H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**:5269–5273.
36. **Nomura, Y.** 1992. Pathogenicity to pumpkins (*Cucurbita* spp.) of bottle gourd fusarium wilt organism (*Fusarium oxysporum* f. sp. *lagenariae*) isolated from acutely wilted cucurbitaceous plants. Ann. Phytopathol. Soc. Jpn. **58**:373–379.
37. **O'Dell, M., M. S. Wolfe, R. B. Flavell, C. G. Simpson, and R. W. Summers.** 1989. Molecular variation in populations of *Erysiphe graminis* on barley, oats and rye. Plant Pathol. **38**:340–351.
38. **Orbach, M. J., E. B. Porro, and C. Yanofsky.** 1986. Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol. Cell. Biol. **6**:2452–2461.
39. **Owen, J. H.** 1956. Cucumber wilt, caused by *Fusarium oxysporum* f. *cucumerinum* n. f. Phytopathology **46**:153–157.
40. **Ploetz, R. C., and J. C. Correll.** 1988. Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. Plant Dis. **72**:325–328.
41. **Puhalla, J. E.** 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. **63**:179–183.
42. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207–7221.
43. **Risser, G., Z. Banihashemi, and D. W. Davis.** 1976. A proposed nomenclature of *Fusarium oxysporum* f. sp. *melonis* races and resistance genes in *Cucumis melo*. Phytopathology **66**:1105–1106.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman & Co., San Francisco.
46. **Snyder, W. C., and H. N. Hansen.** 1940. The species concept in *Fusarium*. Am. J. Bot. **27**:64–67.
47. **Sun, S. K., and J. W. Huang.** 1983. A new Fusarium wilt of bitter gourd in Taiwan. Plant Dis. **67**:226–227.
48. **Swofford, D. L.** 1993. PAUP: phylogenetic analysis using parsimony, version 3.1.1 (computer program). Illinois Natural History Survey, Champaign.
49. **Tsuge, T., H. Kobayashi, and S. Nishimura.** 1989. Organization of ribosomal RNA genes in *Alternaria alternata* Japanese pear pathotype, a host-selective AK-toxin-producing fungus. Curr. Genet. **16**:267–272.
50. **Wellmann, F. L.** 1939. A technique for studying host resistance and pathogenicity in tomato Fusarium wilt. Phytopathology **29**:945–956.