Relatedness of Strains of *Fusarium oxysporum* from Crucifers Measured by Examination of Mitochondrial and Ribosomal DNA

H. Corby Kistler, Paul W. Bosland, Ulla Benny,
Sally Leong, and Paul H. Williams

The address of the first and third authors: Plant Pathology Department, University of Florida, Gainesville 32611. The address of the second, fourth, and fifth authors: Department of Plant Pathology, University of Wisconsin, Madison 53706. Present address of the second author: Department of Horticulture, New Mexico State University, Las Cruces 88003-3530.

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ABSTRACT

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DNA was examined from 25 isolates representing three formae speciales of *Fusarium oxysporum* from crucifers. The restriction enzyme fragment patterns of mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) were compared among isolates to examine phylogenetic relationships among pathotypes and to determine whether any restriction fragment-length polymorphisms (RFLPs) were associated with geographic origin. No differences in rDNA restriction fragment patterns were seen among isolates regardless of pathotype or geographic origin. RFLPs were detected in mtDNA, and these corresponded directly with forma specialis. A unique mtDNA restriction enzyme fragment pattern was detected in one isolate from Japan, indicating that geographical divergence is present in the pathogen population. All isolates examined contained plasmid-like DNA (plDNA). Three different plDNAs were detected and were also correlated with forma specialis.

The phytopathogenic fungus, *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans., causes the wilt or yellows disease of crucifers worldwide. Pathotypic variation of *F. oxysporum* on crucifers has been categorized into the formae speciales *conglutinans, raphani,* and *matthioli* based on the host species *Brassica oleracea* L., *Raphanus sativus* L., and *Matthiola incana* (L.) R. Br., respectively (2). Further differentiation into races is based on differential pathogenicity within a forma specialis. A correspondence of forma specialis with isozyme polymorphism and vegetative compatibility exists even among isolates from diverse geographic regions (2). In addition, two unique linear mitochondrial plasmid-like DNAs (plDNAs) are found in *F. oxysporum* strains that infect *Brassica* and *Raphanus*, and the presence of these plDNAs is correlated to the host range of the fungus (8).

Biological variation can be studied at the level of DNA sequences by comparing restriction endonuclease fragments. Studies of mitochondrial DNA (mtDNA) from *Aspergillus, Claviceps, Cochliobolus, Neurospora,* and *Penicillium* (4,7,9, 14,16) have demonstrated variation as determined by restriction enzyme fragment-length polymorphisms (RFLPs). RFLPs have also been detected in the tandemly repeated nuclear genes for ribosomal RNA (rDNA) in *Cochliobolus* and *Neurospora* (7,13). The small size of mtDNA and rDNA makes them suitable for restriction enzyme analysis. Thus, restriction site polymorphism provides yet another way to investigate molecular evolution of conspecific populations. Because of the rapid rate of evolution of mtDNA, which is estimated in animals to be 10 times faster than that of nuclear DNA (3), analysis of mtDNA may provide a more sensitive measure of divergence in populations than other methods.

MATERIALS AND METHODS

Fungal strains. Twenty-five virulent isolates of the three crucifer formae speciales representing diverse geographic origins and all known pathotypes were included (Table 1). Isolates were maintained in sterile soil at 4 C until studied. (In previous publications [e.g., 8,12], *F. o.* f. sp. *conglutinans* races 1 and 2, *F. o.* f. sp. *raphani*, and *F. o.* f. sp. *matthioli* races 1 and 2 were referred to as *F. o.* f. sp. *conglutinans* races 1, 5, 2, 3, and 4, respectively.)

A strain of *F. o.* f. sp. *lycopersici* race 1 and a homothallic strain of *Nectria haematococca* Berk & Br. (from F. Cervone and D. Parisot, respectively) were maintained in 50% glycerol at −80 C.

Isolation of DNA. Mycelium was grown in 1 L of potato-dextrose broth (Difco, Detroit, MI) that was gyrated at 125 rpm for 5–7 days at 24 C. The mycelium was filtered through a double layer of cheesecloth, frozen, and lyophilized.

Fungal DNA extractions were carried out by a modified method of Specht et al (15) that was described previously (8). Plasmid DNA grown in *Escherichia coli* strain HB101 was obtained by the alkali lysis method (10). DNA from *Magnaporthe grisea* Cavara was provided by Masatoki Taga (Sakata Seed Co.).

Restriction endonuclease analysis. Approximately 0.25 μg of DNA was digested with restriction enzymes as recommended by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, MD, or Promega Biotec, Madison, WI). Restriction fragments were separated by horizontal agarose gel electrophoresis (0.7% agarose), using Tris/borate buffer (10) and a potential gradient of 1–5 V cm^{-1}. DNA was detected by staining gels in a solution of ethidium bromide (0.5 $\mu g\ ml^{-1}$) followed by UV trans-illumination. Lambda phage DNA (Promega Biotec) cut by Hind III or Hind III and Bgl II restriction endonuclease was used for size markers. DNA was denatured, transferred to nitrocellulose, and hybridized to ^{32}P-labelled DNA probes as previously described (10). Nick translations of DNA (0.1 μg) used as probes were performed by standard methods (10) to give specific activities of $>5 \times 10^7$ dpm μg^{-1}.

DNA manipulations. DNA from mitochondrial fractions was digested with restriction enzyme Eco R1 and ligated with *E. coli* plasmid pUC19 (18), which had been linearized with the same endonuclease. Ligations were used to transform *E. coli* strain TB1, a r_k^- M_k^+ derivative of JM83 (18). Plasmid DNA of Amp^r, Lac^-

TABLE 1. Isolates of *Fusarium oxysporum* from crucifers used in this study

PHW#[a]	Pathotype[b]	Origin	Host species
2	Foc1	Florida	*Brassica oleracea*
81	Foc1	Wisconsin	*B. oleracea*
684	Foc2	California	*B. oleracea*
699	For	Wisconsin	*Raphanus sativus*
719	Foc1	Hungary	*B. oleracea*
722	Foc1	ATCC 9990[c]	*B. oleracea*
723	Foc1	ATCC 16600	*B. oleracea*
724	For	ATCC 16601	*R. sativus*
725	Fom1	ATCC 16602	*Matthiola incana*
726	Fom2	ATCC 16603	*M. incana*
760	For	Germany	*R. sativus*
768	Foc1	Wisconsin	*B. oleracea*
777	Foc1	Japan	*B. oleracea*
781	Fom1	Japan	*M. incana*
793	Fom1	CBS 247.61[d]	*M. incana*
795	For	CBS 262.50	*R. sativus*
796	For	CBS 488.67	?
806	Fom1	Italy	*M. incana*
808	Foc2	California	*B. oleracea*
809	Foc2	California	*B. oleracea*
811	Foc2	California	*B. oleracea*
815	For	France	*R. sativus*
821	For	Taiwan	*R. sativus*
1088	For	Japan	*R. sativus*
1094	Fom1	Japan	*M. incana*

[a] PHW = Laboratory designations of isolates maintained by Paul H. Williams, Department of Plant Pathology, University of Wisconsin-Madison 53706.
[b] Foc1 = *F. o.* f. sp. *conglutinans* race 1; Foc2 = F. o. f. sp. *conglutinans* race 2; For = *F. o.* f. sp. *raphani;* Fom1 = *F. o.* f. sp. *matthioli* race 1; Fom2 = *F. o.* f. sp. *matthioli* race 2.
[c] ATCC = American Type Culture Collection, Rockville, MD.
[d] CBS = Centraalbureau voor Schemmelcultures, Baarn, Netherlands.

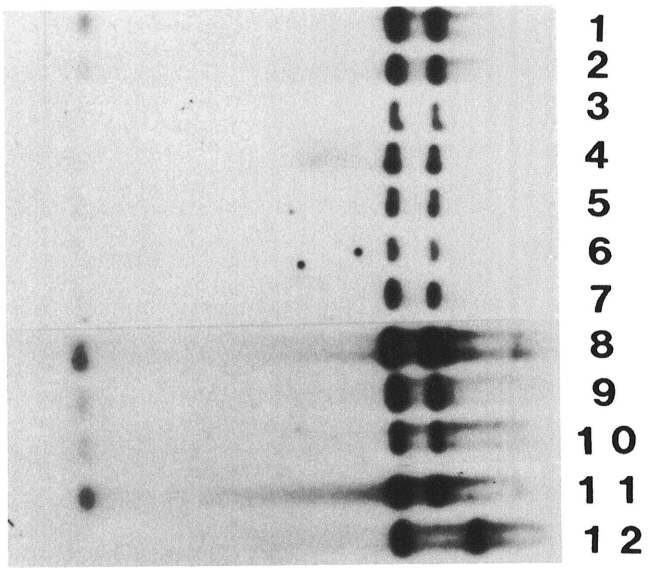

Fig. 1. Autoradiogram showing ribosomal DNA digested with Eco R1. Total nuclear DNA was digested with endonuclease Eco R1 and fractionated on a 0.7% agarose gel. A Southern blot of that gel was hybridized with the ^{32}P-labelled cloned rDNA from *Neurospora*, pMF2. Lanes contain DNA from strains 1) PHW 2, 2) PHW 699, 3) PHW 722, 4) PHW 724, 5) PHW 725, 6) PHW 726, 7) PHW 768, 8) PHW 806, 9) PHW 808, 10) PHW 811, 11) 1088, and 12) *Nectria haematococca* homothallic.

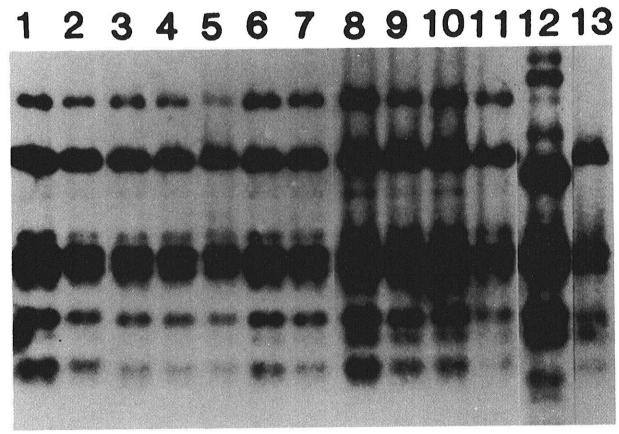

Fig. 2. Autoradiogram showing ribosomal DNA digested with Sau 3A. Total nuclear DNA was digested to completion with endonuclease Sau 3A and fractionated on a 1.5% agarose gel. Southern blot of that gel was hybridized with ^{32}P-labelled pMF2, the cloned *Neurospora* rDNA. Lanes contain DNA from 1) PHW 722, 2) PHW 723, 3) PHW 724, 4) PHW 725, 5) PHW 760, 6) PHW 768, 7) PHW 781, 8) PHW 793, 9) PHW 796, 10) PHW 806, 11) *F. o.* f. sp. *lycopersici*, 12) *Magnaporthe grisea*, and 13) *Nectria haematococca*.

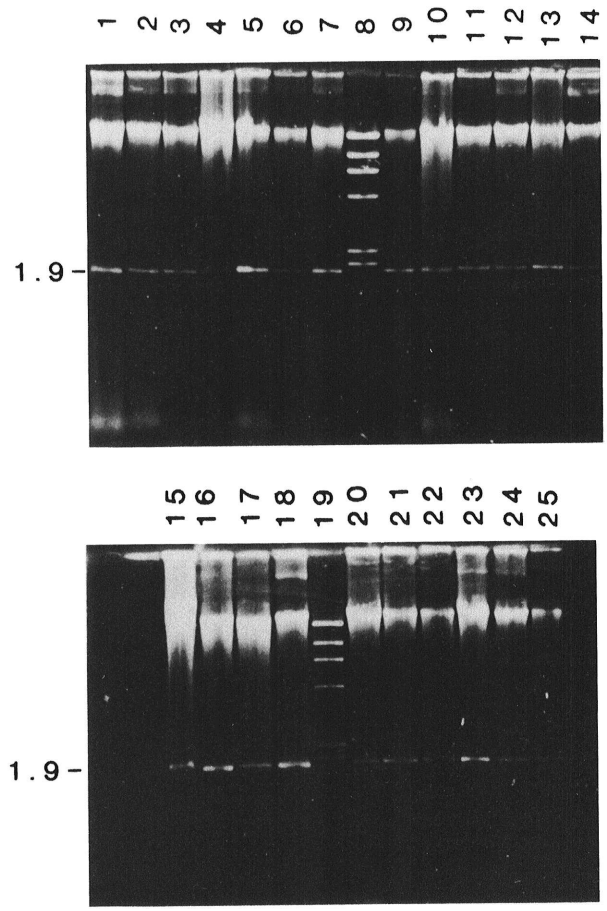

Fig. 3. Mitochondrial DNA fractions from 23 strains of *Fusarium oxysporum* from crucifers showing presence of 1.9 kb elements (noted at left). DNA was fractionated on a 0.7% agarose gel and stained with ethidium bromide. Lanes contain DNA from 1) PHW 2, 2) PHW 722, 3) PHW 723, 4) PHW 768, 5) PHW 777, 6) PHW 684, 7) PHW 808, 8) λ DNA digested with Hind III, 9) PHW 699, 10) PHW 724, 11) PHW 760, 12) PHW 781, 13) PHW 795, 14) PHW 796, 15) PHW 809, 16) PHW 811, 17) PHW 821, 18) PHW 1088, 19) λ Hind III, 20) PHW 719, 21) PHW 725, 22) PHW 793, 23) PHW 806, 24) PHW 1094, and 25) PHW 726.

transformants were screened by the alkali lysis mini-preparation method (10) for inserts of appropriate size.

Clones of plDNA from radish-infecting *F. oxysporum* (pCK1) (8) and nuclear rDNA from *Neurospora crassa* (pMF2) (6) have been described previously. The plasmid pMF2 was supplied by Robert Metzenberg (University of Wisconsin).

RESULTS

Southern hybridization analysis of nuclear DNA-containing fractions indicated that considerable homology exists between sequences in *F. oxysporum* and cloned genes for ribosomal RNA from *N. crassa* (Figs. 1 and 2). The hybridization probe pMF2 contains the entire coding regions for the highly conserved 17 S, 5.8 S, and 25 S ribosomal RNAs plus a small amount of noncoding spacer DNA (6). Heavily staining bands corresponding to those hybridizing with pMF2 were seen in ethidium bromide-stained gels of endonuclease-digested *F. oxysporum* DNA and were presumably repetitive sequences (data not shown).

DNA from *F. oxysporum* hybridizing to pMF2 (henceforth referred to as *Fusarium* rDNA) corresponds to a unit of 8.0 kb. No RFLPs were detected in rDNA from *F. oxysporum* when DNAs from different strains were digested with restriction enzymes Eco R1 (Fig. 1), Bgl II, Pst I, Sal I (not shown), or Sau 3A (Fig. 2). The restriction enzyme fragment pattern for rDNA was also identical for an isolate of *F. o.* f. sp. *lycopersici* (Fig. 2). Increasing differences were noted in RFLPs of rDNA from *N. haematococca* (imperfect form = *Fusarium solani*) and *M. grisea* (imperfect form = *Pyricularia oryzae*).

Linear plDNA associated with mitochondria have been previously described in cabbage- and radish-infecting strains of *F. oxysporum* (8). When mitochondrial DNA-containing fractions were examined, all crucifer pathotypes were found to contain plDNAs with an estimated size of 1.9 kb (Fig. 3). Ratios of high molecular weight mtDNA to plDNA varied among strains. Based on hybridization and restriction endonuclease mapping analysis among isolates of the three formae speciales, three distinct types of plDNAs were found. These are designated pFOXC1, pFOXC2, and pFOXC3, corresponding to plDNAs of formae speciales *conglutinans, raphani,* and *matthioli*, respectively. Although no homology can be detected between pFOXC1 and pFOXC2 based on hybridization analysis (8), pFOXC2 and pFOXC3 show distinct homology (Fig. 4B). However, the plDNAs pFOXC2 and pFOXC3 can be differentiated based on restriction endonuclease mapping. For example, the single Eco R1 site in pFOXC2 (8) is not found in pFOXC3. Thus, when probed with pCK1 (cloned 1.8 kb Bgl II pFOXC2 fragment) the Eco R1 digested mtDNA fraction shows a band homologous to the intact 1.9 kb pFOXC3 in *F. o.* f. sp. *matthioli* strains but exhibits two bands that correspond to the cleaved pFOXC2 in *F. o.* f. sp. *raphani* strains (Fig. 4B). No homology was found between pCK1 and *F. oxysporum* nuclear DNA nor between pCK1 and DNA of the mitochondrial chromosome from any tested crucifer-infecting *F. oxysporum* (8; Fig. 4B, *unpublished*).

Restriction enzyme digestion of mtDNA also revealed three distinct patterns with Eco R1 (Fig. 4A), Bgl II (Fig. 5), or Hind III (not shown). The mtDNA cleavage patterns within each forma specialis examined showed no variation, with the exception of isolate PHW 1088 from Japan, which exhibited a single-band heterogeneity when Bgl II was employed (Fig. 5, lane 12). Several restriction fragments in digests of mtDNA fractions appear to differ in stoichiometry among strains (cf, 0.7-kb band in lanes 1–7, Fig. 5). These bands were found to be fragments of plDNA in

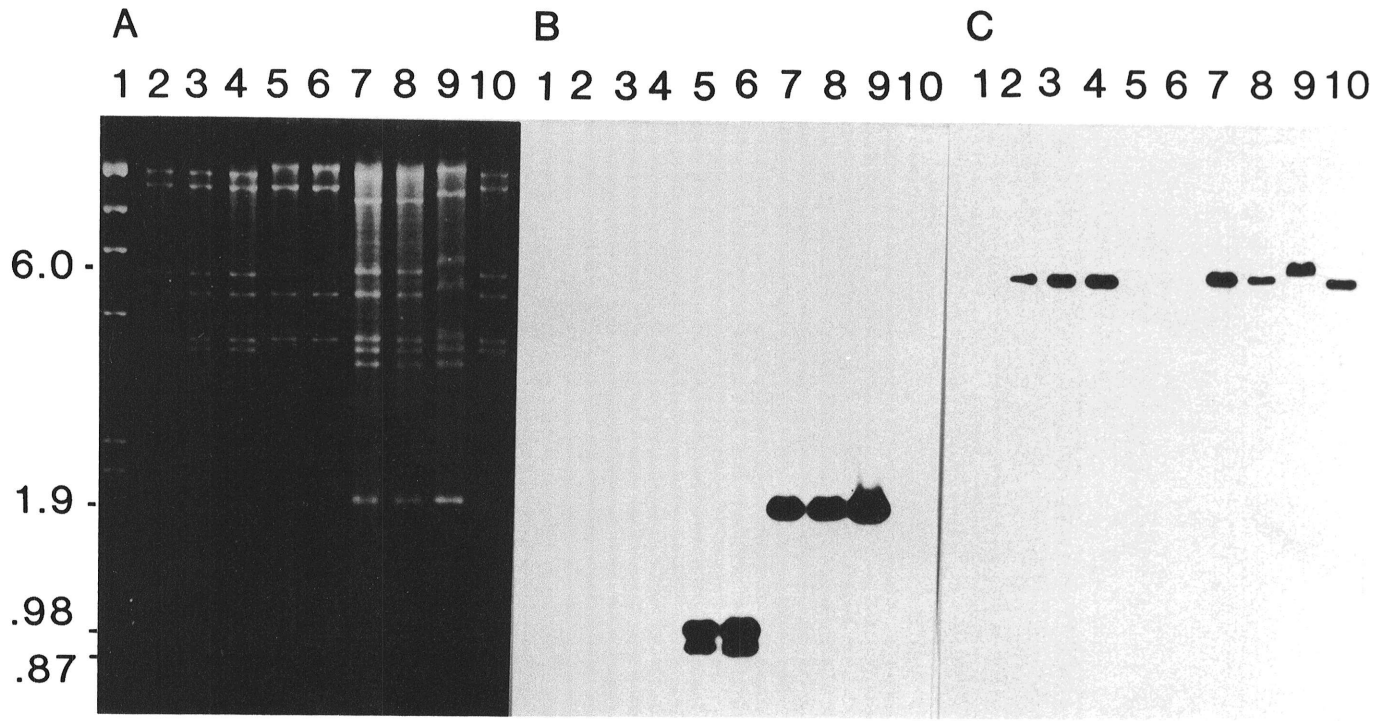

Fig. 4. Southern hybridization analysis of mitochondrial DNA fractions from nine strains digested with Eco R1. **A,** DNA fractionated on a 0.7% agarose gel and stained with ethidium bromide; **B,** Autoradiogram of Southern blot of gel hybridized with ^{32}P-labelled pCK1, containing 1.8 kb of DNA from the radish pathotype plDNA, pFOXC2; and **C,** Autoradiogram of Southern blot of gel hybridized with ^{32}P-labelled pUF1-11, a clone containing the 6.0 kb Eco R1 fragment of mtDNA from strain PHW 777. From left to right DNA are from (2–4) *Fusarium oxysporum* f. sp. *conglutinans* race 1 strains PHW 2, PHW 768, and PHW 777; (5 and 6) *F. o.* f. sp. *raphani* strains PHW 724 and PHW 796; (7 and 8) *F. o.* f. sp. *matthioli* race 1 strains PHW 781 and PHW 806; (9) *F. o.* f. sp. *matthioli* race 2 strain PHW 726; and (10) *F. o.* f. sp. *conglutinans* race 2 strain PHW 808. Lane 1 contains λ DNA digested with Hind III included as a size standard. Numbers at left denote sizes of hybridizing DNA fragments in kilobase pairs.

mitochondrial fractions as determined by molecular hybridization (e.g., Fig. 4B). No correlation was seen between the geographical source of the isolates and RFLPs. Thus, digestion patterns show conservation among isolates of a forma specialis regardless of their geographical origin.

When the size in kilobase pairs of mtDNA from each forma specialis was estimated by summation of the sizes of the restriction fragments, the mean size was 49.6 ± 6.3 for *F. o.* f. sp. *conglutinans*, 48.5 ± 7.6 for *F. o.* f. sp. *raphani*, and 52.3 ± 5.4 for *F. o.* f. sp. *matthioli*. This is similar to the size of mtDNA estimated in other studies of *F. oxysporum* (11). The smaller size of mtDNA in *F. o.* f. sp. *raphani* can in part be attributed to the lack of mitochondrial sequences found in the other pathotypes. When the 6.0-kb mtDNA Eco R1 fragment of *F. o.* f. sp. *conglutinans* strain PHW 777 was cloned in pUC19 and used to probe mtDNA of other pathotypes, sequences homologous to this fragment were totally absent in strains of *F. o.* f. sp. *raphani* (Fig. 4C).

DISCUSSION

The subspecific classification, forma specialis, of *F. oxysporum* found on crucifers is based on the host species from which the isolates are found. The intraspecific classification, race, is determined by ability to cause disease on particular cultivars of these host species. Studies on vegetative compatibility and isozyme polymorphisms have shown that *F. oxysporum* isolates from crucifers exist as at least three subpopulations (2). The detection of three distinct types of mitochondrial and plDNAs correlated with these subpopulations provide further evidence for the divergence of these pathotypes into three subspecific taxa and substantiate the notion that a barrier to genetic exchange between the formae speciales has occurred.

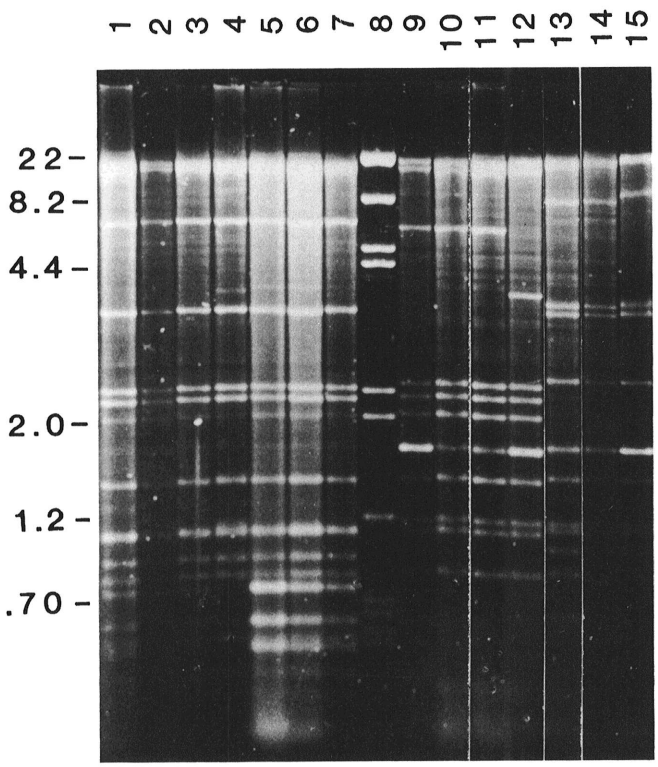

Fig. 5. Electrophoretic analysis of mitochondrial DNA fractions from 14 strains digested with Bgl II. DNA was fractionated on a 0.7% agarose gel and stained with ethidium bromide. Lanes contain DNA from *Fusarium oxysporum* f. sp. *conglutinans* race 1 strains PHW 2 (1), PHW 722 (2), PHW 723 (3), PHW 768 (4), PHW 777 (5), *F. o.* f. sp. *conglutinans* race 2 strains PHW 684 (6), PHW 808 (7); Bacteriophage λ digested with Hind III and Bgl II (8), *F. o.* f. sp. *raphani* strains PHW 699 (9), PHW 724 (10), PHW 796 (11), PHW 1088 (12), *F. o.* f. sp. *matthioli* race 1 strains PHW 781 (13), PHW 806 (14), and *F. o.* f. sp. *matthioli* race 2 strain PHW 726 (15). Note divergence in strain PHW 1088.

Conservation of restriction sites in *F. oxysporum* rDNA is unusually high when compared with the level of polymorphism found in species of *Neurospora*. When wild-type strains of *N. crassa, N. tetrasperma, N. sitophila, N. intermedia,* and *N. discreta* were examined, numerous RFLPs were observed, often showing differences among strains of the same species (4). Similar polymorphism was found in *Cochliobolus* rDNA (7). In addition, no heterogeneity in rDNA repeating units could be detected in *F. oxysporum* between individual strains, unlike that which has been reported in the yeast *Yarrowia lipolytica* (17). Conservation of restriction enzyme fragmentation patterns for rDNA of all *F. oxysporum* isolates (including a tomato pathogen) indicates that these sequences may be less likely to diverge and are characteristic of higher order taxa, perhaps species, in the genus *Fusarium*.

The level of polymorphism of mtDNA also was examined among formae speciales of *F. oxysporum* found on crucifers. Previous studies have shown that the level of polymorphism in mtDNA varies with the fungal genus and species under consideration. Within species of the genus *Neurospora* (4) and among strains of the species *Claviceps purpurea* (16) the level of polymorphism is high, with RFLPs corresponding to strain differences rather than differences in species. However, work with members of the genus *Aspergillus* suggests that even different species may have highly conserved or identical mtDNA restriction enzyme fragmentation patterns (9). Between these extreme cases is *Cochliobolus heterostrophus*, in which analysis of mtDNA RFLPs suggests two distinct subpopulations in the species that are related neither to geographic origin nor to other studied genetic characteristics (7). The mtDNA RFLPs in crucifer-infecting *F. oxysporum* suggest a level of polymorphism similar to that of *Cochliobolus*. Like *Cochliobolus*, distinct subpopulations in *F. oxysporum* are observed as defined by mtDNA RFLPs and these subpopulations are unrelated to the geographic origin of the isolates. Unlike *Cochliobolus*, however, polymorphisms usually are correlated with the pathotypically defined phenotype, forma specialis.

MtDNA patterns have also been used to detect genetic exchange between populations (1). The constancy of mtDNA RFLPs among isolates within a forma specialis provides further evidence that vegetative incompatibility may be operating to genetically isolate formae speciales of *F. oxysporum*, thus preventing a mixing of mitochondria among isolates.

Variation in the total mitochondrial genome among the pathotypes may be attributed, at least in the case of the *F. o.* f. sp. *raphani*, to a deletion or deletions in the mitochondrial chromosome. Most size variation in *Neurospora* and *Aspergillus* mtDNA has been attributed to small insertions and/or deletions (4,5).

The presence of a unique plDNA in each crucifer forma specialis is interesting in that these plDNAs may carry information necessary for pathogenic specialization (8). Although our observations are not inconsistent with that hypothesis, further work is required to substantiate or refute this possibility.

The finding that pathotypes have specific plDNAs and mtDNA RFLPs may help to clarify further relationships among the formae speciales of *F. oxysporum* and to provide other characteristics that may be useful in understanding the speciation and the evolution of this important group of pathogens.

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Genetics

Type of Gene Action in the Resistance to Maize Chlorotic Dwarf Virus in Corn

Eugen Rosenkranz and Gene E. Scott

First author: plant pathologist, Agricultural Research Service, U.S. Department of Agriculture, and professor, Department of Plant Pathology and Weed Science, Mississippi State University, Mississippi State 39762. Second author: supervisory agronomist, ARS, USDA, and professor, Department of Agronomy, Mississippi State University, Mississippi State 39762.
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ABSTRACT

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A six-parent diallel cross, comprised of three maize chlorotic dwarf virus (MCDV)-resistant (Ky122, Mp444, and Tx29A) and three MCDV-susceptible (AR234, Ky21, and T131) corn inbred lines, was chosen to estimate the genetic variance of host response to MCDV, and thus gain information on the type of gene action involved in the resistance to MCDV in corn. The 15 possible crosses (without reciprocals) were grown during 2 yr in a screenhouse into which leafhoppers from viruliferous colonies of *Graminella nigrifrons* were released at the rate of six and 12 insects per plant in 1984 and 1985, respectively. Each year, about 125 plants of each cross were evaluated for the presence of tertiary vein clearing, the diagnostic symptom of maize chlorotic dwarf. The results were consistent under the two levels of inoculum pressure. In both years, the mean disease incidence of the nine resistant (R) × susceptible (S) crosses was equal or similar in magnitude to the mean disease incidence for the combined three R × R and the three S × S crosses. The diallel analysis of the disease incidence data showed a relatively large and highly significant mean square for general combining ability and a relatively small and statistically nonsignificant mean square for specific combining ability. Thus, our results indicate that the total genetic variance in host response to MCDV among the 15 crosses was contained in the general combining ability, suggesting additive gene action, and that nonadditive gene action (dominance variance) was absent because the variance for specific combining ability was insignificant.

Additional key words: disease resistance, vector inoculation, *Zea mays*.

The etiology of the "corn stunting disease" in the United States of the 1960s was complicated by the presence of at least three causal agents. In the lower Midwest, maize dwarf mosaic virus and maize chlorotic dwarf virus (MCDV), the latter not identified as a distinct pathogen until 1968 (7,8), were causing severe losses in corn (*Zea mays* L.). In the South, especially in Mississippi and Louisiana, corn stunt spiroplasma (thought to be a virus at the time) occurred in the same corn fields with the above two viruses. Before the etiology of the corn stunting disease complex was clarified, a few field studies, conducted under natural infection, were carried out in an attempt to elucidate the inheritance of resistance to what was believed to have been either corn stunt (3) or maize dwarf mosaic (2,4). In all these cases, however, there are now strong indications that these researchers were working primarily with maize chlorotic