Chromosomal Polymorphism in *Fusarium oxysporum* f. sp. *niveum*

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ABSTRACT

Kim, D. H., Martyn, R. D., and Magill, C. W. 1993. Chromosomal polymorphism in *Fusarium oxysporum* f. sp. *niveum*. Phytopathology 83:1209-1216.

Genome size and chromosome number in seven geographically separated isolates representing six mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) groups (RFLPG I–VI) and the three known pathological races of *Fusarium oxysporum* f. sp. *niveum*, causal agent of Fusarium wilt of watermelon, were examined by transverse alternating field electrophoresis (TAFE). Chromosome-size DNA was separated on 0.8% agarose gel at 50 or 80 V with five switch intervals for 168 h. Six karyotypes were detected among the seven isolates. Two representative isolates from mtDNA RFLPG I had two karyotypes, whereas the representative isolates from mtDNA RFLPGs IV and V had the same karyotype. Putative chromosome number varied from five to 10 among the isolates and ranged in size from approximately 900 to 4,400 kb. Either one or both of the two largest putative chromosomes (3.6 and 4.4 Mb) were present in all isolates; however, smaller chromosomes varied both in number and size among isolates. Minimum genome size among the six isolates of *F. o. niveum* ranged from 15.8 to 26.0 Mb. When individual bands were extracted, labeled, and used as probes, 30 of the 40 randomly selected genomic cosmid clones from a reference strain hybridized to more than one chromosome band. When the procedure was reversed, three of 10 labeled clones hybridized to all chromosomes from the reference strain and also to several chromosomes from each of the other isolates, indicating the presence of common repeated sequences dispersed throughout the genomes.

Additional keywords: electrophoretic karyotype.

The small size of fungal chromosomes and the presence of an intact nuclear membrane during cell division make it difficult to apply cytogenetic analysis to fungal chromosomes. In addition, the lack of a sexual stage in many important plant-pathogenic fungi make them untenable for traditional genetic analyses. However, chromosome analyses of fungi have been enhanced by the development of pulsed field gel electrophoresis (PFGE) (36) for separation of large DNA fragments. This technique makes it possible to separate whole fungal chromosomes.

PFGE was first used to separate yeast chromosome-size DNA (36). It also has been used to resolve megabase-size DNA for physical mapping and chromosome-size DNA for electrophoretic karyotyping. With PFGE, fundamental information about the size of chromosomes and, therefore, the total number of bases in the genome can be determined. In addition, PFGE provides new methods for characterizing the number of chromosomes (30), analyzing genetic organization of the genomes (7), and allocating genes to linkage groups (19). The electrophoretic karyotypes of plant-pathogenic fungi representing more than 10 genera (27) have been described. The ability to separate individual chromosomes has many potential applications, including the use of DNA hybridization to locate and clone genes such as those affecting pathogenicity and virulence (40). In addition, the presence of dispensable mini- or B chromosomes and their possible role in pathogenicity have been examined by PFGE analysis (25).

Fusarium oxysporum Schlechtend.:Fr. f. sp. *niveum* (E.F. Sm.) W.C. Snyder & H.N. Hans., the causal agent of Fusarium wilt of watermelon, is known generally for its host specificity; however, exceptions have been reported (12,22,25). In addition, several pathological races have been described (20,21). Evidence for variation also is seen by the occurrence of different vegetative compatibility groups (VCGs; 16), isozyme polymorphisms (2), and mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) (10,11). However, no formal genetics or linkage relationships have been defined due to the absence of sexual reproduction and a lack of genetic markers in this fungus. Based on light and electron microscopy, the presence of at least three or four chromosomes per genome in *F. oxysporum* has been suggested (31). However, Momol et al (29) recently reported eight and 11 chromosomes, respectively, in *F. o. conglutinans* and *F. o. raphani*.

Three VCGs have been described in *F. o. niveum*, and there is a good correlation between VCG and the three known races (16). Results in our laboratory support a VCG/race correlation (D. Netzer and R. D. Martyn, *unpublished data*); however, we also have shown no correlation between race, mtDNA haplotype, isozyme polymorphism, or geographic origin of isolates (2,10,11). A similar lack of correlation between races of *F. o. melonis* (17), causal agent of Fusarium wilt of muskmelon, and either VCG, mtDNA, or origin was demonstrated by Jacobson and Gordon (8). In contrast, we have shown a close phylogenetic relationship between *F. o. niveum* and the other formae speciales causing wilt in cucurbit crops (12) based on mtDNA RFLPs, as well as a probable case of mtDNA heteroplasmy between *F. o. niveum* and *F. o. melonis* (11).

As part of an ongoing interest in the evolution of *F. oxysporum* formae speciales that cause vascular wilt in the Cucurbitaceae, chromosomal polymorphism (number and size) was examined in *F. o. niveum* by transverse alternating field gel electrophoresis (TAFE). Additionally, an initial study of chromosomal organization was examined by hybridization with randomly selected genomic clones of a reference strain of *F. o. niveum*.

MATERIALS AND METHODS

Fungal isolates. Seven isolates of *F. o. niveum*, representing six mtDNA RFLP groups (RFLPG) (11) and the three known pathological races from widely separated geographic origins, were examined (Table 1).

Protoplast formation. Protoplasts of *F. o. niveum* were prepared by a modified procedure of Kistler and Benny (14). Active cultures were obtained from stock soil tubes (24) by sprinkling soil onto potato-dextrose agar (PDA) and incubating at 25 C for 4 days. Microconidia were produced by seeding 50 ml of a Fusarium liquid-culture medium (5) in a 125-ml Erlenmeyer flask with a 5-mm plug from a PDA culture. The culture was incubated on a rotary shaker at 100 rpm for 3–4 days, filtered

through eight layers of cheesecloth, and pelleted by low-speed centrifugation. The enriched microconidia were resuspended in 50 ml of potato-dextrose broth (PDB) at a concentration of 2 $\times 10^7$ microconidia per milliliter. Germinating microconidia were precipitated by centrifugation at 1,500 *g* for 10 min, washed twice with 10 ml of washing solution (1.2 M $MgSO_4$, 10 mM Na_2PO_4, pH 5.8), resuspended in 2 ml of washing solution containing 2% (w/v) Novozym 234 (Novo Laboratories, Danbury, CT), and incubated at 30 C for 30–120 min until approximately all the cells were released as protoplasts. Protoplast suspensions were passed through glass wool, gently overlaid with an equal volume of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 50 mM $CaCl_2$), and centrifuged at 2,000 *g* for 10 min at 4 C in a swinging bucket rotor of a centrifuge. Protoplasts were recovered from the interface between the STC and the enzyme solution, washed in an equal volume of STC, precipitated by centrifugation at 2,500 *g* for 10 min at 4 C, and resuspended in STC at a concentration of 2 $\times 10^8$–8×10^8 protoplasts per milliliter. The protoplast suspension was mixed with an equal volume of 1.2% low melting point (LMP) agarose and cast in a precooled plug mold. Protoplast-containing agar plugs were incubated in lysis buffer (1% sodium lauryl sarcosinate, 500 mM EDTA, 10 mM Tris pH 8.0, 1 mg/ml of proteinase K) at 50 C for 48 h with one buffer change, and subsequently rinsed in 500 mM EDTA and stored in storage buffer (50 mM EDTA, 1 mM Tris, pH 8.0) at 4 C until use.

TABLE 1. Representative isolates of *Fusarium oxysporum* f. sp. *niveum* mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) groups used for chromosome analysis

Lane[a]	Isolate code	Geographic origin	Race[b]	mtDNA RFLP group[b]
1	FL-60-3A	Florida	0	I
2	TX-XID(79)	Texas	2	I
3	GA-557-1(88)	Georgia	1	II
4	ISL-59(73)	Israel	2	III
5	NC-EE2-A(87)	North Carolina	1	IV
6	TX-CART-CG-1A(87)	Texas	2	V
7	CHN(CI)-PRCF6(89)	China	1	VI

[a] Numbers refer to the lanes in the autoradiograms in Figures 1, 2, and 3.
[b] Kim et al (11).

TAFE electrophoresis. TAFE agarose gel electrophoresis with the Gene Line system (Beckman Instruments, Palo Alto, CA) was conducted at 10 C in TAE running buffer (10 mM Tris, pH 7.6, 4 mM glacial acetic acid, 0.5 mM EDTA free acid). Protoplast plugs containing a total of 1×10^7–4×10^7 protoplasts were loaded in each lane of a 0.8% agarose gel along with two standard chromosome-size markers: *Saccharomyces cerevisiae* strain YNN295 and *Schizosaccharomyces pombe* (ATCC 2411). In initial separations, chromosome-size DNA fragments were subjected to electrophoresis consecutively on the same gel at a constant 80 V with five switch intervals: 1) 5 min for 24 h, 2) 8 min for 24 h, 3) 10 min for 24 h, 4) 15 min for 48 h, and 5) 20 min for 48 h. To better separate the large chromosomes, additional switch intervals, times, and voltages were used: 1) 5 min for 24 h, 2) 10 min for 24 h, 3) 20 min for 24 h, and 4) 30 min for 48 h at a constant 80 V, and 5) 60 min for 48 h at 50 V. The gels were then stained with ethidium bromide (0.5 μg/ml) for 30 min and destained in distilled water for 30 min. The size of each fragment was estimated by comparison with the standard chromosomal bands of *S. cerevisiae* and *Schizosaccharomyces pombe*, assuming there was a linear relationship between mobility of DNA and molecular size (23). Negative photographs of gels stained with ethidium bromide were scanned with a densitometer (Ultroscan XL laser densitometer, Pharmacia LKB, Uppsala, Sweden) to measure relative intensity of the stain in each band.

Southern analysis. DNA hybridization was used to screen a series of cosmids to identify those that might serve as chromosome-specific probes. Forty recombinant clones with an average insert size of 40 kb of DNA from *F. o. niveum* isolate FL-60-3A were selected randomly from a genomic library. The library was constructed by ligating size-selected fragments after partial digestion with *Mbo*I in cosmid vector CosHyg1 (O. C. Yoder, Cornell University, Ithaca, NY, and Ely Lilly & Co., Indianapolis, IN).

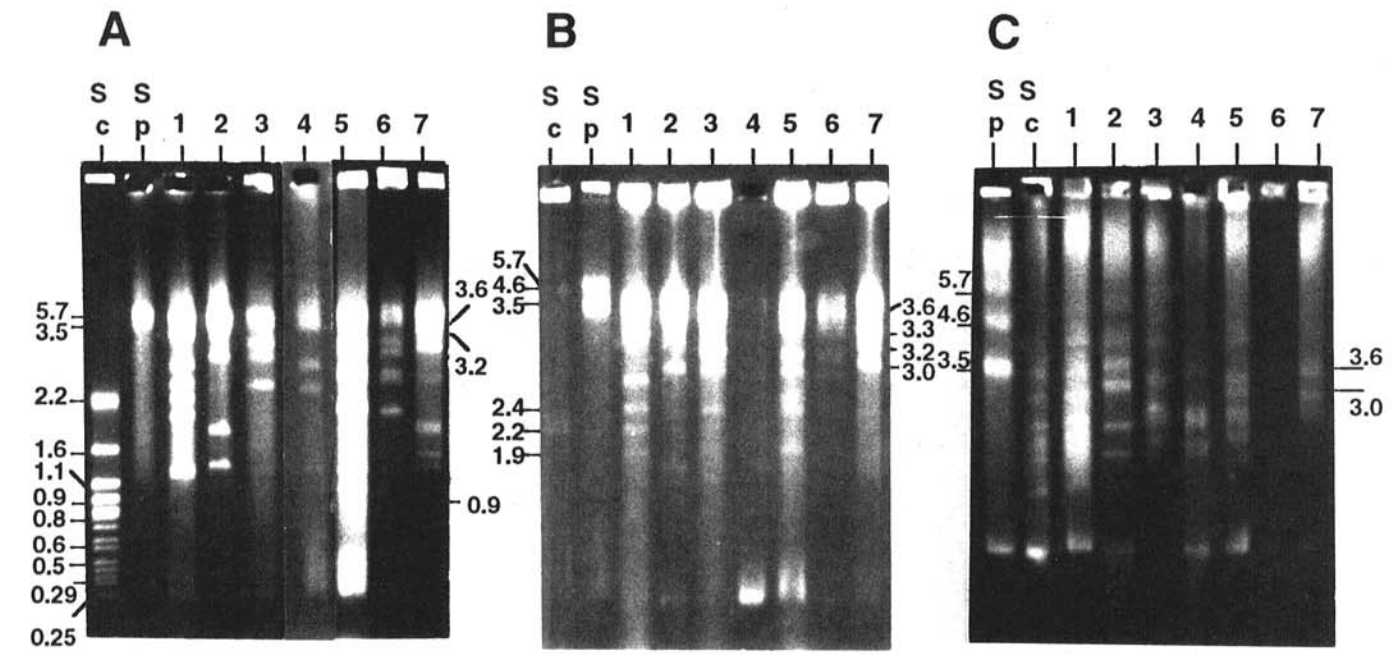

Fig. 1. Effect of switching time intervals on transverse alternating field electrophoresis (TAFE) separation of *Fusarium oxysporum* f. sp. *niveum* chromosomes. **A,** Small chromosome-size bands (<2,700 kb) were separated with five switch intervals (5 min/24 h, 8 min/24 h, 10 min/24 h, 15 min/48 h, and 20 min/48 h at 80 V). Due to the poor yield of protoplasts in one isolate, lane 4 was cut from a separate run conducted under the same conditions; **B,** larger chromosomes were further separated with an additional switch interval of 30 min/48 h at 80 V; **C,** the largest chromosomes (>3,100 kb) were separated with switch intervals of 5 min/24 h, 10 min/24 h, 20 min/24 h, 30 min/48 h all at 80 V, and 60 min/48 h at 50 V. Lane numbers (top) refer to isolates in Table 1. Sc and Sp represent *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively, which were used for standard size markers. Size markers are in megabases.

The cosmid clones were extracted from *Escherichia coli* host strain DH5αMCR by an alkaline lysis minipreparation method (34), and 0.1 μg of DNA from each clone was dot blotted onto hybridizing membrane (Genescreen Plus, Du Pont De Nemours and Co., Inc., Wilmington, DE). The blots were hybridized to individual chromosome-size DNA bands of *F. o. niveum* race 0 (FL-60-3A) excised from 1.0% LMP agarose TAFE gels, labeled with [α-^{32}P]dATP, using the random primed DNA labeling procedure of Feinberg and Vogelstein (6) with the aid of a kit from United States Biochemicals (Cleveland, OH), and used as probes.

Based on their hybridization pattern with the different chromosomal probes, several recombinant clones were selected for Southern hybridization analysis of TAFE gels. Chromosomal DNA in the TAFE gel containing the seven representative isolates was UV-irradiated for 2 min to nick the DNA, depurinated with 0.25 N HCl for 25 min, and transferred to hybridizing membrane by capillary action with 0.4 N NaOH. Each cosmid clone was labeled with [α-^{32}P]dATP by a nick translation kit from Bethesda Research Laboratories (Gaithersburg, MD) and hybridized to the TAFE blots. To determine whether clones that had hybridized to a single chromosome-size band contained single-copy unique sequences and if clones that hybridized to multiple chromosome-size bands contained repetitive sequences, representative clones were hybridized to blots containing *Pst*I-digested DNA from each of the seven isolates of *F. o. niveum*. The hybridization patterns as well as the size of hybridizing bands were compared with the *Pst*I digestion patterns of the clones themselves.

RESULTS

Protoplast preparation. For all isolates except ISL-59(73), a protoplast yield of 99% was reached between 30 and 120 min of digestion of young (<12 h old), germinating microconidia in 2% Novozym 234 solution. However, protoplast yield of isolate ISL-59(73) was less than 70% of that of other isolates. In addition, cultures that acquired a dark pigmentation after approximately 12 h in PDB produced fewer protoplasts (≤30%) than did younger, unpigmented cultures.

Chromosomal polymorphisms. When the maximum switch interval was 20 min, small chromosome-size bands (<3.0 Mb) from *F. o. niveum* and all chromosomes from *S. cerevisiae* were resolved, whereas the larger bands (>3.0 Mb) from *F. o. niveum* and the three chromosomes from *Schizosaccharomyces pombe* remained unresolved (Fig. 1A). The number of ethidium bromide-stained bands in TAFE gels varied from four to eight among the *Fusarium* isolates (Fig. 1A). However, when the longest switch interval was increased to 30 min, several large bands further separated into two bands. For example, the second band (after 20-min switching intervals) from the top in lane 7 was resolved into two distinct bands containing two chromosome-size fragments (II and III) (Fig. 1B). In addition, there was better resolution of the *Schizosaccharomyces pombe* chromosomes because two bands, one at 3.5 Mb and one consisting of a mixture of chromosomes I and II, were now evident. Increasing the maximum switching interval to 60 min resolved three distinct *Schizosaccharomyces pombe* chromosomes, and each of the largest chromosomal bands in the *Fusarium* isolates was resolved to either single (lane 7), double (lanes 1, 3, 5, and 6), or triple (lane 2) bands (Fig. 1C). Scans of the original 75- × 95-mm negatives of stained gels showed that the densities of the double-band areas contained twice as much stained DNA as neighboring bands in the same isolate (data not shown). Chromosomal bands were numbered from I–X in order of decreasing size in accordance with conventions proposed by Magee et al (19), and consequently, chromosome bands with the same number may not be the same chromosome in different isolates.

Most isolates exhibited chromosome-size bands of either 4.4 or 3.6 Mb or both. Although most chromosome-size bands were resolved in the conditions stated for Figure 1A, there was no single set of conditions that distinguished all chromosome-size bands from all *F. o. niveum* isolates. Therefore, the number and size of chromosome-size bands were determined based on comparison of results obtained from several different running conditions. Based on this, the number of chromosome-size bands varied from five to ten among isolates and ranged from approximately 900 to 4,400 kb. In Figure 1C, the ethidium bromide-stained smear visible above the 5.7-Mb band in all lanes was considered an artifact due to the extended running conditions. Although we

TABLE 2. Chromosome number and sizes in *Fusarium oxysporum* f. sp. *niveum*

Chromosome number	Isolate number[a]						
	1	2	3	4	5	6	7
I	4,400[b]	4,400	4,400	3,600	4,400	4,400	3,600
II	3,500	3,600	3,400	3,100	3,400	3,400	3,300
III	3,200	3,400	3,100	2,800	3,300	3,300	3,200
IV	2,700	2,800	2,800	2,300	3,100	3,100	3,000
V	2,400	1,800	2,300	2,000	2,800	2,800	2,500
VI	2,200	1,300		1,100	2,600	2,600	1,700
VII	1,900			900	2,500	2,500	1,700
VIII	1,100				1,900	1,900	1,400
IX	1,000				1,100	1,100	1,200
X					900	900	
Total[c]	22.4	17.3	16.0	15.8	26.0	26.0	21.6

[a] Numbers refer to the isolates of *F. o. niveum* designated in Table 1.
[b] Numbers refer to the size of chromosomal bands in kilobases.
[c] Numbers refer to the size of chromosomal bands in megabases.

TABLE 3. Hybridization patterns of 40 clones to whole-chromosome probes

Clone number	1+2[a]	3	4	5	6	7	8+9
1A4				+[b]	+	+	+
1A5	+	+					
1B4		+	+	+	+	+	+
1B5	+		+	+	+		+
1C5	+					+	
1B12	+		+	+	+	+	
1G7	+	+	+	+	+	+	+
2D5		+					
4B8					+	+	+
4B10	+						
4B12	+		+	+	+		+
7C6	+	+	+	+	+	+	+
4H12	+	+	+	+	+	+	
6A1	+	+	+	+	+	+	+
6D4	+			+	+	+	+
6F7	+						
7B8	+				+		
7F3	+						
7G2	+				+	+	
7H3	+	+	+	+	+	+	+
7H7	+						
2E6	+						
3F9	+						
7B9	+	+	+	+	+	+	+
8E4	+		+	+	+	+	
4G6	+						
10C1	+						
1C7	+		+	+	+		+
1F3	+			+	+	+	+
2A2	+				+	+	
2B3			+	+	+	+	+
3E5	+		+	+	+		
3F2	+				+		
3G5	+		+	+			+
3H8	+		+	+			+
4B6	+						
5E1	+				+	+	+
5F12	+				+	+	
6A10	+		+	+	+	+	+
6B10	+		+	+	+		

[a] Data represent the probe set containing DNA from two chromosomes.
[b] + represents hybridization with the whole-chromosome probe.

cannot definitely rule out the possibility that additional chromosome-size bands larger than 5.7 Mb are present in *F. o. niveum*, we believe that there are not any bands larger than 5.7 Mb. This is based on the fact that no similar bands or smears larger than the 5.7 Mb *Schizosaccharomyces pombe* marker were visible in the other gels (Fig. 1A and B) and none of the multiple-hybridizing clones (described later) hybridized to this area.

The minimum genome size of *F. o. niveum*, calculated as the sum of each chromosome-size band, ranged from 15.8 to 26.0 Mb (Table 2). Based on the number and size of chromosomal bands in the TAFE gels, six karyotypes were detected among the seven isolates (Fig. 1). Two isolates from mtDNA RFLPG I (lanes 1 and 2) had different karyotypes, whereas isolates from mtDNA RFLPGs IV and V (lanes 5 and 6) had the same karyotype. The remaining three isolates (lanes 3, 4, and 7) were separate karyotypes. Lane 1 (FL-60-3A, race 0, RFLPG I) contained nine chromosomes (I–IX) ranging from 1,000 to 4,400 kb with a minimum genome size of 22.4 Mb, whereas lane 2 (TX-XID(79), race 2, RFLPG I) contained six chromosomes ranging from 1,300 to 4,400 kb with a minimum genome size of 17.3 Mb. Lane 3 (GA-557-1(88), race 1, RFLPG II) had the fewest number of chromosomes, five, ranging from 2,300 to 4,400 kb and a minimum genome size of 16.0 Mb. Lane 4 (ISL-59(73), race 2, RFLPG III) had seven chromosomes ranging from 900 to 3,600 kb, and its minimum genome size was the smallest at 15.8 Mb. Lanes 5 (NC-EE2-A(87), race 1, RFLPG IV) and 6 (TX-CART-CG-1A(87), race 2, RFLPG V) had the same banding patterns. Both isolates contained 10 chromosomes ranging from 900 to 4,400 kb and had the largest minimum genome size at 26.0 Mb. Lane 7 (CHN(Cl)-PRCF6(89), race 1, RFLPG VI) had nine chromosomes ranging from 1,200 to 3,600 kb with a minimum genome size of 21.6 Mb. The entire procedure was repeated with a separate set of protoplasts, and the putative chromosome banding pattern of each isolate was identical to the first set.

Preparation of chromosomal probes. Chromosome-size DNA from the reference strain FL-60-3A (race 0) was separated by TAFE on LMP agarose under the same conditions as those described for Figure 1A. Due to the increased concentration of the LMP agarose required to obtain stable gels and the very similar size of the two smallest chromosomes (1,000 and 1,100 kb), resolution of the two largest and two smallest bands was not sufficient to permit clean excision of each band. Therefore, chromosome bands I and II were combined as were those for chromosomes VIII and IX. In all, seven sets of "whole-chromosome" probes were made. Each whole-chromosome probe was hybridized to a dot blot containing DNA from 40 randomly selected recombinant cosmid clones with an average insert size of 40 kb of DNA from FL-60-3A; the resulting hybridization pattern is shown in Table 3. Thirty of 40 clones hybridized to more than one labeled whole-chromosome probe, and five hybridized to all seven whole-chromosome probes. Ten clones hybridized to a single whole-chromosome probe. However, nine of these hybridized with the whole-chromosome probe containing both bands I and II. Only cosmid 2D5 hybridized to a single chromosome-size probe (chromosome III).

Characterization of chromosomes. To characterize chromosomes from different isolates, 10 recombinant clones (1A5, 1G7, 2D5, 3F9, 4G6, 4H12, 6A1, 6A10, 7C6, and 7H7) were selected and hybridized to the TAFE blots of the seven *F. o. niveum* isolates. Three clones (3F9, 4G6, and 7H7) hybridized to the probe consisting of chromosomes I and II of isolate FL-60-3A, and one clone (2D5) was specific to chromosome III. The remaining six clones (1A5, 1G7, 4H12, 6A1, 6A10, and 7C6) hybridized to more than one of the chromosome-size probes from isolate FL-60-3A. All three chromosome I- or II-specific clones hybridized to the largest chromosome from all isolates. Clone 2D5 was specific to chromosome III of FL-60-3A; however, it also hybridized to chromosome I of TX-XID(79), GA-557-1(88), ISL-59(73), NC-EE2-A(87), and TX-CART-CG-1A(87) and to chromosomes I and III of CHN(Cl)-PRCF6(89) (data not shown). For the six multiple-hybridizing clones (1A5, 1G7, 4H12, 6A1, 6A10, and 7C6), clone 1A5 hybridized to chromosomes I and III of FL-60-3A but only to chromosome I from the other isolates. Three clones (1G7, 6A1, and 7C6) hybridized to all chromosomes from isolate FL-60-3A. However, the hybridization patterns of these three clones to chromosomes from other isolates varied, i.e., they hybridized to all chromosomes in some isolates but only to certain chromosomes in other isolates (Fig. 2). Clone 6A10

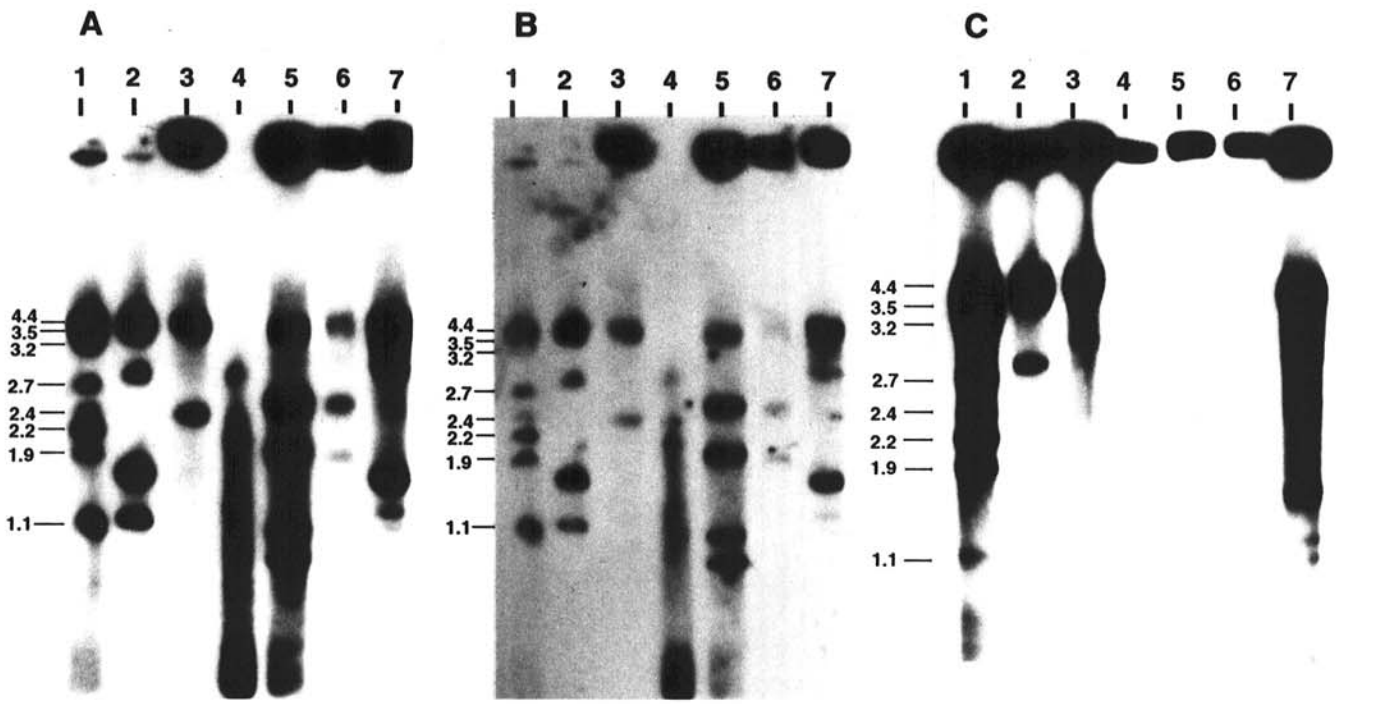

Fig. 2. Hybridization patterns of chromosome-size DNA to multiple chromosome-hybridizing clones. Chromosomes were separated based on the conditions in Figure 1A and probed with each clone. Sample 4 appears to be degraded in this preparation, but the hybridization pattern was deduced from several repeated trials. Hybridization pattern of clones **A**, 6A1; **B**, 6A10, which has a similar hybridizing pattern to that of 6A1 except it does not hybridize to the 3.2-Mb chromosome from FL-60-3A (lane 1); and **C**, 7C6. Lane numbers (top) refer to isolates in Table 1. Size markers (left) are in megabases.

showed a similar hybridization pattern to that of 6A1 except it did not hybridize to chromosome III from FL-60-3A (Fig. 2A and B). Clone 7C6 hybridized to all chromosomal bands from isolates FL-60-3A and CHN(Cl)-PRCF6(89); however, it hybridized only to the large chromosomes (≥2,800 kb) from other isolates. Hybridizing patterns of the multiple-hybridizing clones are summarized in Table 4 and are in agreement with the dot blot results (Table 3). Two isolates (NC-EE2-A(87) [lane 5] and TX-CART-CG-1A(87) [lane 6]) that displayed the same karyotype in ethidium bromide-stained gels also showed identical hybridization patterns with all clones examined. However, none of the 10 clones hybridized to chromosomes IV or V from either of these two isolates.

The hybridization patterns on Southern blots of genomic DNA from each isolate digested with *Pst*I and probed with chromosome-specific clone 4G6 and the multiple-hybridizing clone 6A1 were compared to the restriction enzyme-digestion pattern of the clones themselves (Fig. 3). Hybridization with labeled 4G6 DNA exhibited a pattern typical of a single-copy fragment and showed seven or nine discrete bands depending on the isolate (Fig. 3A). Size summation of bands that hybridized with probe 4G6 was approximately 50–60 kb, which was close to the size of the insert in the 4G6 cosmid. However, hybridization to labeled DNA from cosmid 6A1 produced numerous bands in all isolates, typical of a repetitive sequence (Fig. 3B).

DISCUSSION

Efficiency of protoplast production by *F. o. niveum* depended on the source of tissue as well as the physiological status of the fungus. Young microconidia germlings, assumed to have a thin wall structure with fewer constituents to inhibit digestion, were the best source of protoplasts, whereas old cultures and non-germinated microconidia were the least effective. Similar results were reported for protoplast production from *Trichoderma harzianum* (37).

Six karyotypes were repeatedly identified among seven isolates chosen as representative of *F. o. niveum*. Several studies have

TABLE 4. Chromosomal polymorphisms in *Fusarium oxysporum* f. sp. *niveum* as demonstrated by multiple-hybridizing clones

Clone	Isolate number[a]	Chromosome number[b]									
		I	II	III	IV	V	VI	VII	VIII	IX	X
1A5	1	+									
	2	+									
	3	+									
	4	+									
	5	+									
	6	+									
	7	+									
1G7	1	+	+	+	+	+	+	+	+	+	
	2	+	+	+	+						
	3	+	+	+	+	+					
	4			+							
	5	+	?	?							
	6	+	?	?							
	7	+	+		+		+	?	+	+	
4H12	1	+	+	+	+	+	+	+			
	2	+	+		+	+					
	3	+	+			+					
	4	+	+								
	5	+	?	?							
	6	+	?	?							
	7	+	+			+	+	?			
6A1	1	+	+	+	+	+	+	+	+	+	
	2	+	+	+	+	+	+				
	3	+	+			+					
	4	+	+	+	+	+	+				
	5	?	?				+	+	+	+	+
	6	?	?				+	+	+	+	+
	7	+	+	+	+	+	+	?	+	+	
6A10	1	+	+		+	+	+	+	+	+	
	2	+	+	+	+	+	+				
	3	+	+			+					
	4	+	+	+	+	+	+				
	5	?	?				+	+	+	+	+
	6	?	?				+	+	+	+	+
	7	+	+	+	+	+	+	?	+	+	
7C6	1	+	+	+	+	+	+	+	+	+	
	2	+	+	+	+						
	3	+	+	+							
	4	+	+	+							
	5	?	?								
	6	?	?								
	7	+	+	+	+	+	+	?	+	+	

[a] Isolate numbers of *F. o. niveum* are designated in Table 1.

[b] Putative chromosomes for each clone and isolate are listed from the largest (I) to the smallest (X). Identical chromosome numbers do not necessarily indicate identical chromsomes among isolates (See text and Table 2). + indicates hybridization with the labeled probe. ? indicates that hybridization to this chromosome was not confirmed due to the presence of a double band.

shown that the electrophoretic karyotype of a fungal isolate can spontaneously change during subculturing (34,39). However, no variation of chromosome-size DNA-band pattern within a single isolate of *F. o. niveum* was observed in our study, even after subculturing one isolate 10 times (data not shown). Thus, it appears that the electrophoretic karyotype in *F. o. niveum* is stable and can be applied as a genetic marker.

A recent study with *Magnaporthe grisea* (39) showed that karyotypes were variable enough to obscure the relatedness of isolates on the basis of pathogenic or genetic lineage. Similarly, variations in karyotypes also were observed between and within a group of *Septoria nodorum* strains adapted to wheat or to barley (3). In *F. o. niveum*, variation in virulence (races) occurs (21,22), and the variations correspond closely to vegetative compatibility (16); however, there does not appear to be a correlation between virulence (race phenotype), mtDNA RFLP haplotype, or geographic origin of the isolate (10,11). In addition, Kim et al (9) showed that virulence and VCG apparently are genetically separable in *F. o. niveum* because transformation of *F. o. niveum* race 2 with a cosmid clone of race 0 DNA resulted in a concomitant change in virulence (race phenotype) but not in vegetative compatibility. In the present study, the fact that each of the three isolates of race 1 had a different karyotype, as did each of the three race 2 isolates, and that there was an overlap in karyotypes between race 1 and race 2 isolates implies there is no association between karyotype and virulence (race). Additionally, even though the sample size was small, the fact that different electrophoretic karyotypes occurred in isolates with the same mtDNA haplotype and that the same karyotype occurred in isolates in different mtDNA haplotypes seems to suggest that no relationship exists between mtDNA haplotype and electrophoretic karyotype.

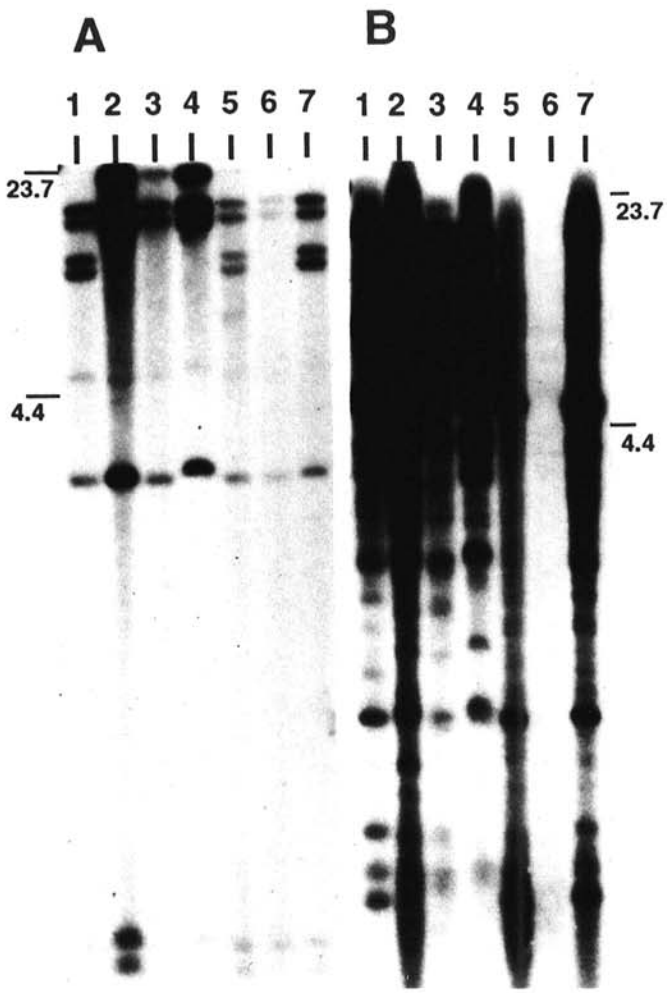

Fig. 3. Southern analysis of *Pst*I-digested genomic DNA hybridized with clones **A,** 4G6 and **B,** 6A1. Hybridization pattern of clones **A,** 4G6 showing a discrete band pattern typical of a single-copy sequence and **B,** 6A1 showing multiple-hybridizing bands typical of a repeated-sequence DNA probe. In this particular gel, lane 6 did not contain an adequate concentration of DNA, and the hybridization pattern is not well presented. Lane numbers (top) refer to isolates in Table 1. Size markers (left) are in kilobases, and the topmost bands were estimated to be 23.7 kb.

RFLP analysis and phylogeny studies based on mtDNA RFLPs (11) indicated that *F. o. niveum* isolates are closely related; however, a wide variation in the number and size of chromosomes occurred in the isolates examined. The number of chromosomes ranged from five to 10, which is comparable to that reported for other *F. oxysporum* formae speciales (28,29). Chromosomal number and size predict a total minimum genome size of 15.8 to 26.0 Mb for *F. o. niveum* depending on the isolate used. This is somewhat smaller than that of *F. o. conglutinans* (31.4–32.1 Mb) (28) and *Aspergillus niger* (35.5–38.5 Mb) (4), as well as another imperfect fungus, *Cladosporium fulvum* (44 Mb) (38). Comparison of chromosomal polymorphisms of several plant pathogenic fungi with various frequencies of meiosis in nature led Kistler and Miao (15) to suggest that the occurrence and extent of chromosomal polymorphisms is inversely correlated to the frequency of meiosis. In our study, six karyotypes from seven isolates were detected, and total genome DNA content, as determined by the sum of each chromosome-size DNA, was highly variable; the smallest minimum genome size in *F. o. niveum* (isolate GA-557-1(88)) was approximately 60% of the largest (isolates NC-EE2-A(87) and TX-CART-CG-1A(87)). These are minimal estimates because there still may be two chromosomes that cannot be separated. Thus, the difference in genome size within *F. o. niveum* could be greater or less. Due to a lack of chromosome-specific probes, it is not possible to analyze the specific content of each chromosome and identify identical chromosomes among the isolates. However, *F. o. niveum* is a strictly asexually reproducing fungus, and its considerable polymorphisms in chromosomal number and size is in accordance with Kistler and Miao's hypothesis (15).

Aneuploidy (1), deletion (23,32), duplication (3), translocation (13), and the presence of B chromosomes (26) all have been suggested as sources of chromosomal variation in fungi. It is difficult to identify the basis for variation in the chromosomes of *F. o. niveum*. Hybridization of clone 1A5 to chromosomes I and III in one isolate but only to chromosome I in others could result from translocation, transposition, multiple base changes, or deletions. The observation that clone 2D5 hybridized to chromosome I and/or III in different isolates also suggests translocations but could arise from transposition. It can be postulated that repeated sequences present on different chromosomes may serve as points for homologous pairing and mitotic exchange, leading to a variety of chromosomal rearrangements. Others have pointed out that the reproductive losses associated with aberrant chromosomes during meiosis are not a restraint to chromosome rearrangements in asexually reproducing species (33,34).

In an initial attempt to identify the chromosomal origin of a series of cosmids made from DNA of isolate FL-60-3A, the chromosome-size bands of the same isolate were extracted after TAFE separation in LMP agarose, labeled, and used to probe dot blots containing DNA from 40 cosmids. Most cosmids (35 of 40) hybridized to a probe made by combining the two largest chromosomes, and the majority showed homology to more than one chromosomal probe. An explanation based on cross-contamination of the probes by chromosomal fragments does not seem likely because some cosmids did hybridize to specific-chromosome probes. This was verified by reversing the procedure and using individual cosmids to probe Southern blots of the separated chromosomes. Three of the 10 cosmids tested hybridized to all nine chromosomes from the source isolate and to most chromosomes of the other isolates as well. Because the cosmids were made from genomic fragments selected via sucrose-density centrifugation to be near 40 kb, it is almost certain that each contains a contiguous DNA segment from a single chromosome. Even with the smallest estimate for the total size of the genome, an average cosmid would contain less than 0.25% of the sequences.

These observations imply that the chromosomes of *F. o. niveum* contain common repeated sequences that are dispersed throughout the genome. Repeated sequences should provide relatively strong signals compared to unique sequences after random labeling and, because the cosmids are fairly large, any common repeat included in the cosmid DNA is likely to be detected by hybridization. Under this model, chromosome VI is likely to contain a relatively high proportion of repeats common to other chromosomes because it hybridized to 23 of the cosmids. Each of the 18 clones found to contain sequences homologous to the probe made by labeling the combined bands for chromosomes VIII and IX also were homologous to at least three other chromosomes. The most logical explanation for this observation is that these chromosomes share repeated sequences that may not be common in the other chromosomes.

Considering the fact that all clones used showed sufficient sequence homology to hybridize to one or more chromosomes of each of the isolates and vice versa, the minimum genetic information required to be a same species seems to be present in each isolate. Electrophoretic karyotyping will make it possible to assign cloned genes into linkage groups and should eventually help resolve the basis for chromosomal variation in this group of fungi.

Numerous fungi have polymorphic chromosomes, and very few have been shown to be truly dispensible (15). However, the large variation among small chromosomes present in *F. o. niveum* isolates is consistent with the possibility that they might be dispensable B chromosomes. In the absence of a sexual cycle or mapped mutants, it is not possible to determine if they assort regularly as homologous or if they contain functional genes.

The size of the hybridizing bands and the hybridizing patterns of DNA from the seven isolates when probed with the chromosome I-specific clone 4G6 seem to suggest that it contains a sequence unique to chromosome I. In contrast, cosmid clone 6A1 hybridized to most or all the chromosomes and to many fragments on *PstI*-digested DNA, showing a pattern typical of repeated-sequence DNA probes. Moreover, the intensity of hybridization to certain chromosome-size bands was different from the others suggesting that the copy number of these repeated sequences may vary for some chromosomes. This clone may have promise for identification of specific subpopulations of *F. o. niveum* in the way the "MGR" (*Magnaporthe grisea* repeat) sequence has been used to identify pathotypes of the rice blast fungus *M. grisea* (18).

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