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Electrophoretic Karyotypes Distinguish the Biological Species of *Gibberella fujikuroi* (*Fusarium* Section *Liseola*)

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Using pulsed field gel electrophoresis, we resolved the chromosomes of strains belonging to six different mating populations (biological species) of the ascomycete fungus *Gibberella fujikuroi* (anamorph *Fusarium* section *Liseola*). In all 34 strains examined, gross karyotypes suggest a haploid number of 12 chromosomes in all of the mating populations, although in some strains chromosomes 4 and 5 appear as a doublet band with an obviously higher density. All strains have two chromosome bands that are as large or larger than the largest chromosome of *Schizosaccharomyces pombe* (5.7 Mbp), and one of these bands is larger than the largest chromosome of *Neurospora crassa* (10 Mbp). All strains from the same mating population have a similar electrophoretic karyotype, regardless of geographic or host origin, but each mating population has a distinctive karyotype. Comparison of karyotype profiles following Southern analysis using homologous and heterologous nuclear gene probes and single-copy restriction fragment length polymorphism probes revealed some differences in hybridization between, but not within, biological species. Estimated genome sizes are 45–50 Mbp for mating populations A, B, D, and F, and 50–55 Mbp for mating populations C and E. The smallest of the 12 chromosomes varies the most between mating populations and was present in all of the field strains examined, but it can be lost following meiosis. Thus, karyotype analysis is a useful tool for the study of taxonomy, genome organization, and evolution of this group of fungi.

Additional keywords: contour-clamped homogeneous electric field (CHEF) gels, fungal taxonomy, *Fusarium moniliforme, Fusarium subglutinans, Fusarium proliferatum*

Gibberella fujikuroi (Sawada) Ito in Ito & K. Kimura encompasses at least six distinct mating populations (biological species [Klittich and Leslie 1992; Leslie 1991]) that can be resolved into one or more morphological anamorphs, including *Fusarium moniliforme* J. Sheld., *F. subglutinans* (Wollenweb. & Reinking) P. E. Nelson, T. A. Toussoun, & Marasas, and *F. proliferatum* (T. Matsushima) Nirenberg, depending upon the taxonomist and the value assigned to different morphological characters (Nelson *et al.* 1983; Nirenberg 1989; Snyder and Hansen 1945). Collectively these fungi can pathogenize a variety of commercially important hosts, including asparagus (Elmer and Ferrandino 1992), figs (Caldis 1927), maize (Leslie *et al.* 1990), mango (Varma *et al.* 1974), pine (Correll *et al.* 1992), pineapple (Rohrbach and Pfeiffer 1976), rice (Sun and Snyder 1981), sorghum (Leslie *et al.* 1990), stone fruits (Subbarao and Michailides 1992), and sugarcane (Martin *et al.* 1989). Some strains also produce significant quantities of mycotoxins, such as fumonisins (Gelderblom *et al.* 1988), fusarin C (Wiebe and Bjeldanes 1981), fusaric acid (Marasas *et al.* 1984), and moniliformin (Marasas *et al.* 1984), which may adversely affect human and animal health. Members of the different mating populations may be found preferentially on different host plants (Leslie and Plattner 1991) and may also differ in the spectrum of mycotoxins that they produce (Leslie and Plattner 1991; Leslie *et al.* 1992) and in their sensitivity to various antifungal agents (Yan *et al.* 1993).

The sexual stage of *F. moniliforme* was first described by Wineland (1924) as *Gibberella moniliformis*. The species was synonymized with *G. fujikuroi* by Booth (1971). The fungus is heterothallic, with a one-locus, two-allele mating system (Gordon 1961). Perithecia of *G. fujikuroi* have been observed under field conditions in rice in Japan and Taiwan (Hsieh *et al.* 1977) and in maize (Roane 1950; Voorheese 1933).

Cytological studies of fungal chromosomes have usually been done with material from the sexual stage (e.g., Raju 1980; Carmi *et al.* 1978), and the lack of a sexual stage has often inhibited such studies. Most fungal chromosomes are quite small, and it is difficult to accurately count chromosomes and construct karyotypes for these organisms by using classical staining and light microscopy. Howson *et al.* (1963) examined developing *F. moniliforme* asci undergoing meiosis by staining with acetoorcein and acetocarmine and gave $n = 4$. Twenty years later, a limited genetic map containing four linkage groups was constructed by Puhalla and Spieth (1983, 1985) for strains from mating population A, but some markers remained unlinked, and they suggested that additional chromosomes remained to be identified.

Since the introduction of pulsed-field gel electrophoresis (PFGE) in 1982, rapid advances have been made in the reso-

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lution of large DNA molecules. Most fungal chromosomes are small enough to be resolved as individual bands in PFGE gels. Electrophoretic karyotyping permits such studies in organisms in which classical cytological analyses (which are normally conducted at pachytene) are not practical, e.g., those lacking a known sexual reproductive phase, as well as organisms in which comprehensive genetic data are not available. PFGE techniques have been used to construct karyotypes of various fungi, including several *Fusarium* spp. (Boehm *et al.* 1994; Fekete *et al.* 1993; Migheli *et al.* 1993; Nagy and Hornok 1994). In most of these cases, however, investigators have focused on only a single species and have not used this powerful tool to elucidate the relationships between related groups, as has been done in classic karyotype studies of cryptic species of *Drosophila* (Clayton and Guest 1986).

Variation in fungal electrophoretic karyotypes was recently reviewed by Kistler and Miao (1992). Two basic types of variants have been found. Chromosome length polymorphisms were first reported by Magee and Magee (1987) in *Candida albicans*. Each of the five isolates that they examined had a unique electrophoretic karyotype. Since then, numerous reports of chromosome length polymorphisms that are stably inherited and transmitted have been made (cf. Kistler and Miao 1992). It has been hypothesized that chromosome length polymorphisms in plant-pathogenic fungi could be related to a lack of meiosis under field conditions. Chromosome length polymorphisms could be derived from genome rearrangements such as translocations (Orbach *et al.* 1988), deletions, or insertions (Ono and Ishino-Arao 1988). Variability in the number of chromosomes on PFGE has also been reported for a variety of fungi, including *Bremia lactucae* (Francis and Michelmore 1993), *Colletotrichum gloeosporioides* (Masel *et al.* 1990), *Fusarium oxysporum* (Boehm *et al.* 1994), *Leptosphaeria maculans* (Plummer and Howlett 1993), *Magnaporthe grisea* (Valent and Chumley 1991; Talbot *et al.* 1993), *Phytophthora megasperma* (Howlett 1989), *Tilletia caries* (Mills and McCluskey 1990), and *Ustilago maydis* (Kinscherf and Leong 1988). The variability in the number of chromosomal bands could result from aneuploidy, as has been observed in some industrial strains of *Saccharomyces cerevisiae* (Bakalinsky and Snow 1990).

The apparent close relationship of the different mating populations within *G. fujikuroi* makes this group a logical candidate for examination for karyotypic diversity that could accompany speciation and host specialization. Our objectives in this study were to determine if karyotypes within a mating population (biological species) were consistent and stable, and to determine if the karyotypes of the different mating populations were the same or different.

RESULTS

Karyotype of *G. fujikuroi* mating population A.

Several running conditions for the contour-clamped homogeneous electric field (CHEF) gels were tested to resolve *G. fujikuroi* chromosomes. When chromosomal DNA from *F. moniliforme* strain A-00102 was resolved under conditions used to separate DNA pieces 2–10 Mbp in size, 10 bands were consistently observed (Fig. 1). These separations usually required 17–20 days. Variations on these basic conditions were used to resolve *S. cerevisiae* size standards and some specific size ranges, especially the smaller ones, which often could be completed in 1–3 days. Because of the relative intensity of UV light fluorescence following ethidium bromide staining, two of the 10 bands were judged to be doublets. We resolved the karyotypes of a total of eight strains from this mating population (Table 1 and Fig. 1). Five of these strains (A-00102, A-00149, A-00549, A-04643, and A-05113) all had the same karyotype, with chromosomes 4 and 5 and chromosomes 8 and 9 running as doublets. In isolates A-00488 and A-02949, chromosomes 8 and 9 can be separated, while in strain A-00498 all 12 of the chromosomes can be resolved. Since this organism has 12 genetic linkage groups based on restriction fragment length polymorphism (RFLP) mapping studies (Xu and Leslie, 1993), we concluded that the 12 bands seen in the A-00498 karyotype each represent one chromosome.

To examine karyotypic variation in more detail, more exhaustive studies of strain A-00102 were made. Isolates from different subcultures of this strain were purified by subculturing uninucleate microconidia. No alterations in either the number or the position of the bands were observed.

Mitochondrial DNA appears as a diffuse band at the leading edge of CHEF gels used to resolve smaller chromosomes; however, the mtDNA is much smaller than the smallest chromosomes, probably about 50 kbp, and is easy to distinguish from the nuclear chromosomal bands. The presence of mtDNA was confirmed by Southern hybridizations with cloned fragments of the mitochondrial genome from strain A-00102 (J.-R. Xu and J. F. Leslie, unpublished).

To confirm that all of the putative chromosomal DNA bands on the CHEF gels contained functional coding sequences, labeled cDNAs were constructed from total poly(A)$^+$ mRNA recovered from mycelia of strain A-00102 growing in liquid culture. Total labeled cDNAs hybridized to all of the bands on the gel, suggesting that all of the observed chromosome bands contained functional genes. Moreover, radiolabeled telomeric DNA from *F. oxysporum* (Powell and

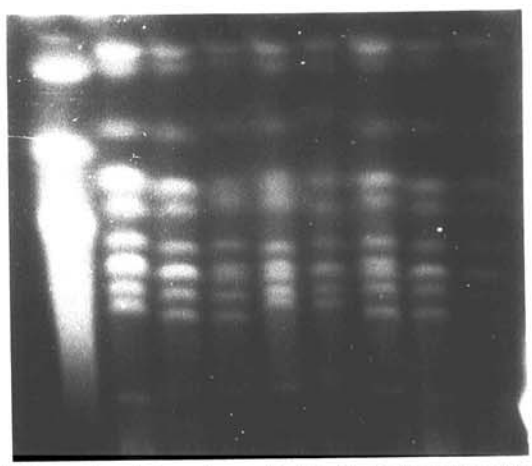

Fig. 1. Electrophoretic karyotypes of eight strains in mating population A of *Gibberella fujikuroi*. A 0.55% agarose gel was run in 1× Tris-borate-EDTA (Maniatis *et al.* 1982) at 40 V with a 1-hr switching interval for 3 days; the voltage was then changed to 50 V and run for 14 additional days. The lanes contain (from left to right) *Schizosaccharomyces pombe* and *G. fujikuroi* strains A-00102, A-00149, A-00488, A-00498, A-00549, A-02949, A-04643, and A-05113.

Kistler 1990) hybridized to all putative chromosomal bands (data not shown).

Representative RFLP probes from the 12 known genetic linkage groups of *G. fujikuroi* mating population A (Xu and Leslie, 1993) and heterologous probes representing known structural loci in other filamentous fungi were hybridized to CHEF gel blots (Table 2). All 12 chromosome bands can be tagged with probes belonging to specific linkage groups (Fig. 2), leading to the correlation of genetically defined linkage groups with CHEF-gel-defined chromosomes. Among strains in mating population A, even though there is some minor chromosome length polymorphism, the electrophoretic patterns are quite similar (Fig. 1). Some minor chromosome length polymorphisms can be observed in the strains belonging to mating population A, but the RFLP probes tested all hybridized to the same chromosome. These results indicate that all of these strains have a similar genome organization pattern, in spite of diverse host and geographic origins. The relative lack of chromosome aberrations or other significant chromosome length polymorphisms suggests that the karyotype of this biological species is relatively homogeneous.

Karyotypes of other *G. fujikuroi* mating populations.

In addition to karyotypes from strains in mating population A, we also karyotyped five strains from mating population B, two from mating population C, five from mating population D, seven from mating population E, and six from mating population F (Table 1). Strains belonging to the same mating population had similar electrophoretic karyotypes, which were unique for each mating population (Fig. 3). Minor chromosome length polymorphisms, similar to those observed in mating population A, were also observed between strains within the other five mating populations. All of the strains examined have 12 chromosomes; no chromosome

Table 1. *Gibberella fujikuroi* strains used in this study

Strain	MP and MT[a]	Host	Geographic origin	Other numbers[b]
A-00102	A^+	Sorghum	San Joaquin Co., CA	PTS-F80, PEN-M3120, FGSC-7598
A-00149	A^-	Maize	Visalia, CA	PTS-F237, PEN-M3125, FGSC-7600
A-00488	A^-	Maize	Transkei, South Africa	MRC-826, PEN-M1325
A-00498	A^-	Maize	Rossville, KS	PEN-M6459
A-00549	A^-	Maize	Silver Lake, KS	PEN-M6466
A-02949	A^-	Maize	Crowder, MS	PEN-M3797, FGSC-7605
A-04516	A^+	Maize	Katmandu, Nepal	PEN-M5500, FGSC-7606
A-04522	A^+	Maize	Katmandu, Nepal	PEN-M5538
A-04643	A^-	Progeny from A-00149 female × A-04522 male		
A-05113	A^-	Progeny from A-00149 female × A-04516 male		FGSC-7607
B-00278	B^+	Sugarcane	Hsingying, Taiwan	PTS-F1251, PEN-M3127, FGSC-7608
B-00281	B^-	Sugarcane	Hsingying, Taiwan	PTS-F1254, PEN-M3128, FGSC-7609
B-01722	B^-	Sorghum	Laguna, Philippines	PEN-M5476
B-01762	B^+	Unknown	South Africa	MRC-1240
B-03810	B^-	Unknown	Unknown	PEN-M846, WG-63320
C-01994	C^+	Rice	Taiwan	PEN-M1149, EGK-FSL290
C-01996	C^-	Rice	Taiwan	PEN-M1151, EGK-FSL294
D-00502	D^+	Maize	Rossville, KS	PEN-M6471, FGSC-7612
D-00666	D^-	Maize	Highland, KS	PEN-M5123
D-00720	D^-	Sorghum	Powhattan-1, KS	PEN-M6485
D-01591	D^+	Maize	Beijing-1, China	PEN-M5360
D-02945	D^-	Sorghum	Holcomb, MS	PEN-M3793, FGSC-7613
E-00434	E^-	Maize	Breakover, KS	PEN-M6496
E-00507	E^-	Maize	Rossville, KS	PEN-M5119
E-00551	E^-	Maize	Silver Lake, KS	PEN-M6501
E-00731	E^+	Maize	Powhattan, KS	PEN-M5126
E-00990	E^-	Maize	St. Elmo, IL	PEN-M3696, FGSC-7616
E-02192	E^+	Maize	St. Elmo, IL	PEN-M3693, FGSC-7617
E-03809	E^-	Maize	Iran	PEN-M845
F-00728	F^+	Sorghum	Powhattan, KS	PEN-M5598, MRC-5713
F-00965	F^+	Sorghum	Zeandale, KS	PEN-M5134, MRC-5707
F-01051	F^+	Sorghum	Alma, KS	PEN-M5594, MRC-5709
F-01377	F^+	Sorghum	WaKeeney, KS	PEN-M5555, MRC-5706, FGSC-7619
F-01540	F^-	Progeny from cross F-01377 female × F-00966 male		FGSC-7620
F-04091	F^-	Progeny from cross F-01377 female × F-01540 male		FGSC-7054

[a] MP = mating population (A–F); MT = mating type (+ or –).

[b] EGK = E. G. Kuhlman, USDA Forest Service, Athens, GA; FGSC = Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City; MRC = Walter F. O. Marasas, Program on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, Republic of South Africa; PEN = Paul E. Nelson, Fusarium Research Center, Department of Plant Pathology, Pennsylvania State University, University Park; PTS = Philip T. Spieth, Department of Plant Pathology, University of California, Berkeley; and WG = W. Gerlach, Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Mikrobiologie, Berlin.

number polymorphisms were observed between strains from the same or different mating populations.

The sizes of the *G. fujikuroi* chromosomes (Table 3) were estimated from comparisons with *S. cerevisiae, Schizosaccharomyces pombe,* and *Neurospora crassa* chromosomes (Vollrath and Davis 1987; Orbach 1992; Orbach *et al.* 1988); the range of chromosome sizes observed was 0.7–12 Mbp. To accurately estimate the size of the smallest chromosome, conditions for resolving DNA fragments of 0.2–2.0 Mbp were used. For mating populations A, B, D, and F, the total genome size is 45–50 Mbp, and for mating populations C and E it is 50–55 Mbp.

Comparative genome organization of mating populations A–F.

We wanted to determine if the genomes of the different mating populations were similar in physical organization. We found that the rDNA probes amplified from A-00102 all hybridized to chromosome 2 for all of the strains examined except F-00965, in which it hybridized to chromosome 3. Also, 17 of 19 RFLP probes from chromosomes 1–11 hybridized to the same chromosome in representative strains from the other mating populations if the chromosomes were arranged according to their size. These data support the hypothesis that these mating populations are closely related and have similar genome organization.

The RFLP hybridization patterns also indicate some significant differences in genome organization in the mating populations. Two probes in particular deserve further mention. One probe, 39E-L, hybridized to the chromosome 4–5 doublet and the chromosome 8–9 doublet of A-00102 and F-04091, to chromosome 6 of C-01996, and to the chromosome 4–5 doublet of D-00502 and E-00990, but it did not hybridize at all to B-00278. A second probe, 11P43, hybridized to chromosomes 1 and 11 of A-00102, E-00990, and F-04091 and to chromosomes 3 and 11 of B-00278 and D-00502, but it did not hybridize at all with C-01996. These data indicate that chromosome rearrangements and differences in genome organization do exist in the mating populations, although these differences do not appear to be extensive.

Chromosome 12.

Chromosome 12 deserves some special mention. This chromosome is the smallest and can vary significantly in size, ranging from 700 kbp in A-00102 to 900 kbp in B-00278 and E-00990; we also observed variability of this chromosome within a mating population. Compared with chromosomes 1–11, the smallest chromosome appears to have more length polymorphisms, both within and between populations; however, a 100-kbp change in this chromosome would be very readily detected, but a change of the same magnitude might be impossible to detect in some of the larger chromosomes. When RFLP probes derived from this chromosome in A-00102 were checked for cross-hybridization with representatives of the other mating populations, 5E32 was found to hybridize only to strains from mating populations A and F, while 6E88 hybridized solely to strains belonging to mating population A. Thus, this chromosome has a number of interesting characteristics that warrant further investigation.

DISCUSSION

The development of PFGE has led to significant advances in the study of fungal chromosomes. Limitations of studies to fungi with an identifiable sexual stage have been removed, since cells from vegetative tissue can be used as a DNA source. Similarly, difficulties associated with small chromosome size for light microscopy (e.g., Perkins 1979) and the complexities of serial synaptonemal complex studies (e.g., Carmi *et al.* 1978) are conveniently bypassed. Variation in chromosome number and size has been reported for a number of fungi (cf. Mills and McCluskey 1990; Kistler and Miao

Table 2. Chromosomal location of selected nuclear gene probes

Probe (gene)	Chromosomes	Source[a]	Reference
pKY (β-tubulin)	1	*Gibberella fujikuroi*	K. Yan and M. B. Dickman (unpublished)
pMF (rDNA)	2	*Neurospora crassa*	Free *et al.* (1979)
pA102-2 (*nnu* global nitroen regulator)	1	*G. fujikuroi*	Dickman and Leslie (1992)
pU5-11 (cutinase)	1	*Fusarium solani*	Soliday *et al.* (1989)
pNLA-7 (telomere)	All	*F. oxysporum*	Powell and Kistler (1990)
cDNA (poly(A)$^+$ RNA)	All	*G. fujikuroi*	
6E51 (anonymous)	1	*G. fujikuroi*	Xu and Leslie (1993)
11P10 (anonymous)	2, 8	*G. fujikuroi*	Xu and Leslie (1993)
6E14 (anonymous)	3	*G. fujikuroi*	Xu and Leslie (1993)
75-E (anonymous)	4	*G. fujikuroi*	Xu and Leslie (1993)
7E49 (anonymous)	5	*G. fujikuroi*	Xu and Leslie (1993)
73-E (anonymous)	6	*G. fujikuroi*	Xu and Leslie (1993)
7E77 (anonymous)	7	*G. fujikuroi*	Xu and Leslie (1993)
5E51 (anonymous)	8	*G. fujikuroi*	Xu and Leslie (1993)
P-39 (anonymous)	9	*G. fujikuroi*	Xu and Leslie (1993)
P-13 (anonymous)	10	*G. fujikuroi*	Xu and Leslie (1993)
P-15 (anonymous)	10	*G. fujikuroi*	Xu and Leslie (1993)
P-25 (anonymous)	10	*G. fujikuroi*	Xu and Leslie (1993)
6E74 (anonymous)	11	*G. fujikuroi*	Xu and Leslie (1993)
5E32 (anonymous)	12	*G. fujikuroi*	Xu and Leslie (1993)
6E88 (anonymous)	12	*G. fujikuroi*	Xu and Leslie (1993)
11P43 (anonymous)	Variable	*G. fujikuroi*	Xu and Leslie (1993)
39E-L (anonymous)	Variable	*G. fujikuroi*	Xu and Leslie (1993)
7E79 (anonymous)	2, 9	*G. fujikuroi*	Xu and Leslie (1993)

[a] All *G. fujikuroi* strains were from mating population A.

1992), and the hypothesis that such variability is the norm for plant-pathogenic fungi has been advanced. For most plant-pathogenic fungi, especially those with no known sexual stage, genetic linkage groups have not been identified, and estimates of chromosome number from PFGE studies may be the only data available on chromosome number and genome organization. Thus, it is particularly important to pay attention to procedural details in the development of electrophoretic karyotypes. Care must be exercised in using band density on CHEF gels to estimate chromosome size and copy number and to ensure that all of the chromosomal bands present have been identified, especially if some of the chromosomes are at or near the upper limit of size resolution (Vollrath and Davis 1987). For diploid or dikaryotic fungi, additional care needs to be exercised in the study of karyotype composition and variability, since length polymorphisms can lead to overestimation of the haploid chromosome number. For example, Magee and Magee (1987) identified 11 chromosome bands in *C. albicans,* but this pathogenic yeast is diploid ($n = 7$). Thus, four of the seven chromosomes must have length polymorphisms that were large enough to be detected through PFGE. Special care also needs to be exercised to be sure that cultures used in karyotype studies can be traced to uninucleate origins, since heterokaryons or mixed cultures could result in chromosome number variation due to chromosome length polymorphisms.

The development of electrophoretic karyotypes that contain chromosomes not previously envisioned in the fungal genome has provided a new area of inquiry for fungal geneticists (Kistler and Miao 1992; Miao *et al.* 1991). In many cases the number of chromosomes observed was larger than expected, and the variation in number and size within a single species was unanticipated (Mills and McCluskey 1990).

G. fujikuroi genome description.

We used CHEF-gel karyotyping to characterize the genomes of six distinct biological species in the *G. fujikuroi* species complex, to determine if genome composition was related to the differences detected by means of biological species–mating population criteria (Leslie 1991). In all six mating populations, the haploid chromosome number (n) was 12, which is quite different from the mere four chromosomes indicated by earlier observations (Howson *et al.* 1963). Based on hybridization with total genomic mRNA and to telomere probes, all of the bands that we observed contain transcribed sequences and structures appropriate for intact chromosomes. Since all 12 chromosomes were seen in all of the field-collected isolates that we examined, we presume that all of the chromosomes carry material that is required for survival under field conditions.

Electrophoretic karyotypes have been used to correlate genetic and physical maps and are especially useful for tying linkage group fragments together and for grouping loci that are too far apart for linkage to be observed by means of measures of recombination. For mating population A, 12 genetic linkage groups have been identified (Xu and Leslie 1993), and we have correlated these linkage groups with the chromosome bands resolved on CHEF gels. Such results are similar to those for other genetically well-characterized filamentous fungi, such as *N. crassa* (Orbach *et al.* 1988) and *Aspergillus nidulans* (Brody and Carbon 1989), in which the

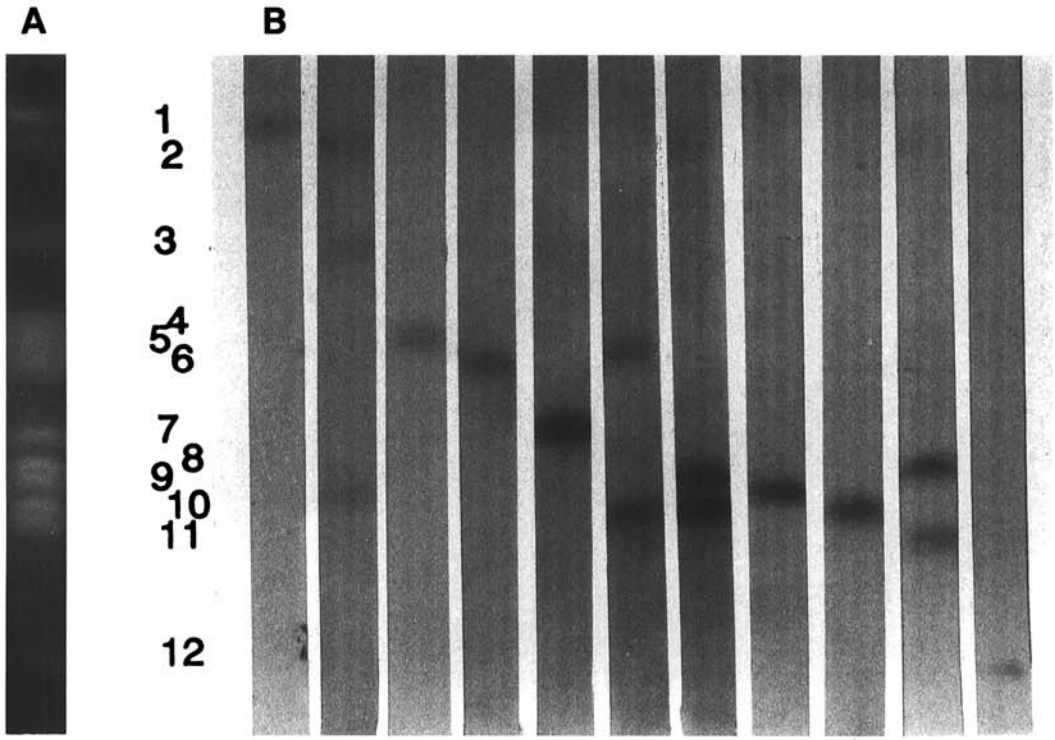

Fig. 2. Southern blotting and tagging of chromosomes from *Gibberella fujikuroi* strain A-00498 with restriction fragment length polymorphism (RFLP) probes. **A,** Contour-clamped homogeneous electric field gel with A-00498 chromosomes. **B,** Hybridization of different RFLP probes to the blotted chromosomes. The RFLP probes were (from left to right) 6E51; 6E14 and 7E79; 75-E; 73-E; 7E77; 7E49 and P-13; P-15 and 11P10; P-39; P-25; 6E74 and 5E51; and 5E32. The chromosomes are ordered and named according to size, the largest being designated chromosome 1.

number of bands on CHEF gels is conserved and equal to the number of genetic linkage groups. The correlation that we observed when Southern blots of CHEF gels were probed with mapped RFLP probes provides further evidence that the number of chromosomes in *G. fujikuroi* is 12. Earlier cytological studies may have been inaccurate, because many of the *G. fujikuroi* chromosomes are quite small and are difficult to count accurately. In *N. crassa* all of the chromosomes can be seen clearly under a light microscope and are 4 Mbp or larger (Orbach *et al.* 1988; Perkins 1979). In *G. fujikuroi,* however, it is difficult to count more than 10 chromosomes at meiosis (N. B. Raju, personal communication), and only five or six of the 12 chromosomes are as large as those of *N. crassa*. Thus electrophoretic karyotyping may be the only available technique for obtaining an accurate karyotype for these fungi.

We used the bands in the CHEF gels to estimate the total genome size (in megabase pairs) of each mating population. Total genome size ranges from approximately 45 to 54 Mbp, a 20% total spread in content, and in the same size range as the estimated 47 Mbp for *N. crassa* (Orbach *et al* 1988), but significantly larger than the 31 Mbp estimated for *A. nidulans* (Brody and Carbon 1989). Some size variation occurs in the smaller chromosomes, but most of the variability that we observed occurred in the largest two chromosomes. The sizes of these chromosomes are the most difficult to estimate, since they are near the upper limit of resolution in CHEF gel technology. Chromosome 2 appears to be especially variable, since it can range from approximately 10 Mbp in mating population E to as little as 5.7 Mbp in mating population D. This chromosome carries the rDNA region, and amplification of this region could account for some of the differences that we observed, but seems unlikely to account for all of the observed differences. If differences in the sizes of chromosomes 1 and 2 are excluded, then the total range of differences between the mating populations is less than 3 Mbp.

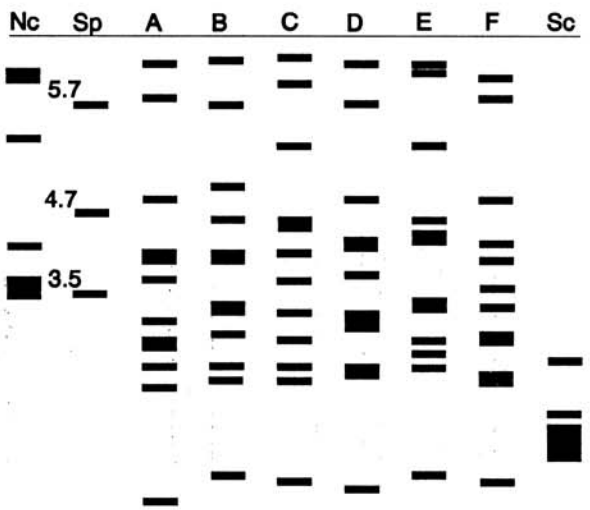

Fig. 3. Computer-generated cartoon electrophoretic karyotypes of representatives of the six mating populations of *Gibberella fujikuroi*. Doublet bands on agarose gels were drawn with double density. Strains used were (from left to right) *Neurospora crassa; Schizosaccharomyces pombe; G. fujikuroi* strains A-00102, B-00278, C-01996, D-00502, E-00990, and F-04091; and *Saccharomyces cerevisiae*.

In all cases one, and in some cases two, of the chromosomes are at or near the upper size limit for the resolution of DNA molecules by CHEF gel electrophoresis. We also ran some CHEF gels for longer periods and used lower agarose concentrations, but no additional chromosome bands were resolved from the largest chromosome. This result provides further evidence that we have identified all of the *G. fujikuroi* chromosomes in our karyotypes. As electrophoresis protocols are improved, it may be necessary for us to revise our estimates of the sizes of these chromosomes and, consequently, the total size of the genome.

Compared with some other fungi, the variability we observed within our strain sample was more similar to that of the sexually reproducing *A. nidulans* observed by Geiser *et al.* (1994) than that of the asexually reproducing population of *F. oxysporum* f. sp. *cubense* characterized by Boehm *et al.* (1994). Chromosome number was conserved across all six mating populations, and in the 11 largest chromosomes 17 of the 19 probes examined mapped to the same chromosome, irrespective of mating population. Genome rearrangements such as duplications, deletions, and translocations were detected in the mating populations by our RFLP probes. These kinds of genome rearrangements could be a part of the cause of the chromosome length polymorphisms between different mating populations, and they may be associated with the speciation process. Thus, within this cluster of biological species, basic genome organization has been preserved, even though there are significant differences in some areas.

Within the mating populations we observed relatively little variability except for chromosome 12. Minor chromosome length polymorphisms do occur within mating populations, however. We used the polymorphisms in strains of mating population A to resolve all 12 chromosomes in A-00498 and confirm our original diagnosis of doublet bands in A-00102. These minor chromosome size variations among strains of mating population A do not appear to affect genome organization, because all of the RFLP probes examined hybridized to the same chromosome in all mating population A strains.

The relative uniformity of karyotypes from strains within a mating population could be accounted for if these strains

Table 3. Estimated chromosome sizes (in Mbp) of representative strains of *Gibberella fujikuroi* mating populations A–F

	Chromosome												
Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Total**
A-00102	10	6.5	4.9	4.1	4.0	3.6	3.0	2.6	2.5	2.2	2.0	0.7	46.1
B-00278	11	6.0	4.9	4.5	4.1	4.0	3.0	3.0	2.6	2.2	2.1	0.9	48.3
C-01996	12	9.0	5.2	4.4	4.4	4.0	3.5	3.0	2.6	2.2	2.1	0.8	53.2
D-01591	12	5.7	4.9	4.1	4.1	3.6	2.8	2.8	2.8	2.2	2.2	0.8	48.0
E-00990	12	10	5.2	4.5	4.3	4.2	3.0	3.0	2.5	2.3	2.2	0.9	54.1
F-04091	10	6.0	4.9	4.0	4.0	3.5	3.0	2.5	2.5	2.2	2.1	0.8	45.5

were regularly participating in sexual exchange under field conditions. On the basis of available data, such an explanation could account for the uniformity observed in mating populations A, D, and E, but not mating population F; there is insufficient population data to test this hypothesis in mating populations B and C. In isolates of mating population F from the United States, female fertility is rare (Klittich and Leslie 1992), and variability with respect to the number of vegetative compatibility groups is quite low. If regular sexual reproduction is not required to maintain the population, then it is possible that the karyotypic homogeneity that we observed is due to a bottleneck effect. Such a bottleneck could occur if mating population F was introduced into the United States along with its sorghum host from Africa and has not had sufficient time to develop the karyotypic diversity observed in some other fungi in which sexual reproduction is apparently not a common part of the life cycle.

Chromosome 12 variability.

One unexpected result of studying fungal electrophoretic karyotypes has been the discovery of a supernumerary B-like chromosome or chromosomes (Jones and Rees 1982) in these organisms. Naturally occurring dispensable chromosomes have also been reported in *B. lactucae* (Francis and Michelmore 1993), *C. gloeosporioides* (Masel *et al.* 1990), *M. grisea* (Valent and Chumley 1991; Talbot *et al.* 1993), *F. oxysporum* (Boehm *et al.* 1994), and *Cochliobolus heterostrophus* (Tzeng *et al.* 1992). In all of these cases, dispensable chromosomes were found in some isolates but not in others. So far the best characterized B-like chromosome in plant-pathogenic fungi is the 1.6-Mbp chromosome of *Nectria haematococca* (Miao *et al.* 1991), which carries the pisatin demethylase (*pda*) gene. This chromosome is not stable in meiosis and is correlated with pathogenicity in pea.

Chromosome 12 behaves as a dispensable chromosome in meiosis and is subject to size variation within a mating population. It contains sequences that hybridize to telomeric repeats and hybridizes to our bulk cDNA probe, indicating that this band represents a true chromosome and that it carries transcribed genes. From other work (Xu and Leslie 1993) we know that this chromosome can be lost during meiosis and does not carry material that is essential for growth under our laboratory conditions. Migheli *et al.* (1993) described a strain from mating population A or F that does not have a band in this size range. Thus the function of this chromosome and the genetic material that it carries remain unknown.

In our survey we have never seen more than one chromosome in this size range per isolate and thus cannot say whether there are multiple dispensable chromosomes in *G. fujikuroi*. Certainly, however, there are differences between the chromosomes that we examined. When RFLP probes from the largest 11 chromosomes of strain A-00102 were hybridized with CHEF gel blots of representatives of the other mating populations, 17 of 19 probes detected homologous sequences in all five other mating populations, and the remaining two probes detected homologous sequences in four of the other five mating populations. In contrast, of the two probes that we used from chromosome 12, only one detected homologous sequences in any of the other mating populations, and the one which did detect homologous sequences detected them only in mating population F. Thus, the similarities between the chromosomes in position 12 in the karyotypes may be relatively small, and it is possible that DNA sequences from these chromosomes could be useful in developing diagnostics for the different mating populations.

Other *Fusarium* karyotypes.

Our description of an electrophoretic karyotype for *Fusarium* spp. is not the first. Previous work with *F. oxysporum* (Boehm *et al.* 1994) and with *F. solani* (sexual stage *Nectria haematococca*) (Kistler and Benny 1992; Miao *et al.* 1991) has shown that the karyotype in *N. haematococca* can vary significantly and may contain one to several "B" chromosomes that carry determinants for pathogenicity. Fekete *et al.* (1993) described electrophoretic karyotypes for eight different *Fusarium* species—*F. avenaceum, F. camptoceras, F. chlamydosporum, F. fusarioides, F. pallidoroseum, F. poae, F. sporotrichioides,* and *F. tricinctum.* Chromosomes were between 0.4 and 6.5 Mbp, and different species had six to nine chromosomes in their genome. Total genomic DNA content ranged from approximately 27 to 30 Mbp.

Migheli *et al.* (1993) examined strains of *F. moniliforme, F. proliferatum,* and *F. sacchari* that all belong to the *G. fujikuroi* species complex, which should have karyotypes similar to those that we have described. Within *G. fujikuroi,* strains can be placed in one of six mating populations, identified by the letters A–F. Strains of the same mating population are potentially sexually compatible with each other, but generally are not cross-fertile with strains from other mating populations (Leslie 1991). These mating populations correlate with different anamorphic *Fusarium* spp.; in particular, *F. moniliforme* contains mating populations A and F, *F. subglutinans* (also termed *F. sacchari*) contains mating populations B and E, and *F. proliferatum* (also termed *F. fujikuroi*) contains mating populations C and D (Leslie 1991). A seventh mating population, G, corresponding to *F. nygamai,* has recently been identified (Klaasen and Nelson 1993) but has not yet been formally described, and no karyotypic studies of representative strains have been conducted.

The karyotypes we resolved in this study differ significantly from those described by Migheli *et al.* (1993) for strains from the same morphological species. These differences are briefly summarized below, but it is important to remember that our conclusions are supported by genetic linkage analyses (Xu and Leslie 1993) and by molecular data indicating that each band has telomeres and transcribed sequences.

For *F. moniliforme,* Migheli *et al.* (1993) described karyotypes for two strains, one with 10 and the other with 11 chromosomes; mating population data were not given. The largest chromosome they resolved was 5.8 Mbp, so chromosomes 1 and 2 were probably not separated, and either chromosome 6 or chromosome 7 was missing, since they identified only one band smaller than 4 Mbp but larger than the 2.5- to 2.6-Mbp doublet containing chromosomes 7 and 8. Additionally, one of their strains appears to be missing chromosome 12.

For *F. subglutinans* (*F. sacchari*), they identified 11 chromosomes and estimated the genome size as 38.9 Mbp. The total genome size is significantly less than what we propose for either mating population B (48.3 Mbp) or mating popula-

tion E (54.1 Mbp). Differences in chromosome number here could be resolved if the single band they observed at 5.1 Mbp were actually a doublet band corresponding to chromosomes 3 and 4 or if their doublet band 3 was actually a triplet corresponding to chromosomes 4, 5, and 6. Their karyotype lacks the chromosome 7–8 doublet, but has two resolvable chromosomes instead.

For *F. proliferatum* (*F. fujikuroi*), they identified seven chromosomes and estimated that the total genome size was 18.1 Mbp. This is about one-third the size that we found for mating population C (53.2 Mbp) and less than one-half the size that we propose for mating population D (48 Mbp). They reported no doublets for any of the bands that they observed and included only one band larger than 4 Mbp in their karyotype of this strain, while we have identified either five (in mating population D) or six (in mating population C).

Electrophoretic karyotypes and taxonomic distinctions.

Variability in chromosome number and size has been reported in several fungal species (cf. Mills and McCluskey 1990; Kistler and Miao 1992). Explanations or mechanisms responsible for such genome rearrangements include insertions, duplications, deletions, and translocations. Differences in karyotypes that are correlated with genetic or biochemical traits or with pathogenicity have been described in several fungi, including *Tilletia caries* (Russell and Mills 1993), *Kluyveromyces marxianus* (Steensma *et al.* 1988), and *Candida* spp. (Kwon-Chung *et al.* 1988; Vazquez *et al.* 1991). The biological significance of these correlations is not understood, however. Putative aneuploids have been identified in some plant-pathogenic fungi, including *M. grisea* (Valent and Chumley 1991; Talbot *et al.* 1993), *U. maydis* (Kinscherf and Leong 1988), and *C. gloeosporioides* (Masel *et al.* 1990), but it is not clear what advantage or role this aneuploidy might play in the life cycle and natural history of these fungi. Some of the variability seen in pathogenic fungi might also be attributable to taxonomic difficulties with these organisms. For example, all of the mating populations in *G. fujikuroi* had but a single anamorph under the old classification system of Snyder and Hansen (1945). If this species concept were applied to these strains without knowledge of the mating populations, then we would have concluded that this species has extensive polymorphisms for chromosome length. Thus some of the polymorphisms presently being observed may be attributable to the inappropriate grouping of diverse isolates under a common name. Such problems will be especially difficult to resolve with groups that have no known sexual stage, since the identification of biological species as an independent measure of relatedness will be difficult, if not impossible.

The data from this study are consistent with the hypothesis that mating populations A–F of *G. fujikuroi* are distinct entities that warrant resolution at the species level. All of the mating populations are reproductively isolated from one another (Leslie 1991) except for mating populations C and D, which can occasionally produce a few perithecia containing a few (<0.01%) viable ascospores (A. E. Desjardins, personal communication). This reproductive isolation would permit differentiation from a progenitor karyotype without imposing a genetic load due to structural rearrangements in the genome and could serve to reinforce the isolating mechanisms that originally led to the speciation event. As expected under this hypothesis, there was more variability between mating populations than there was within a mating population. The differences in the ease with which spheroplasts could be released from strains belonging to the different mating populations is also consistent with this hypothesis, as are data from studies of isozymes (Huss and Leslie 1993). Although the karyotypes we observed were constant and diagnostic for a mating population, we do not think it a good idea to use these karyotypes as a routine identification procedure, since simple tests such as crosses with standard testers and isozyme profiles are also available and are faster and technically simpler. Electrophoretic karyotypes are a useful backup identification tool and are essential to an understanding of the genome organization and evolutionary differentiation of these fungi.

The similarities between the different mating populations of *G. fujikuroi* indicated by the cross-hybridization of the RFLP probes from A-00102 with members of the other five mating populations suggests that it should be possible to generate genetic maps for these mating populations by means of this common set of probes. Their genomes could also be compared to study the evolutionary events that have led to the differentiation of these groups. In plants, this approach has been used to compare tomato, potato, and pepper (Tanksley *et al.* 1988; Bonierbale *et al.* 1988) and to compare maize and sorghum (Bennetzen and Freeling 1993; Hulbert *et al.* 1990; Whitkus *et al.* 1992). The few viable progeny of crosses between strains belonging to mating populations C and D could provide some clues to genomic regions that must be retained for the progeny to be viable. It also is possible that the genomic similarity within *G. fujikuroi* could extend to other closely related fungi which will be more difficult to study, either because they are homothallic, like *Gibberella zeae,* or because they have no known sexual stage, like *F. oxysporum* and its numerous pathogenic forms.

MATERIALS AND METHODS

Isolates and culture methods.

All strains of *G. fujikuroi* used in this study are listed in Table 1. All isolates originated from single uninucleate microconidia. Strains were cultured on complete medium (Correll *et al.* 1987) or potato-dextrose agar (Difco) at 25° C and maintained for long-term storage in 15% glycerol at –70° C. Sexual crosses were made on carrot agar as described by Klittich and Leslie (1988).

Saccharomyces cerevisiae BWG 1-7a, *Schizosaccharomyces pombe* 24843, and *Neurospora crassa* OR23 *A* were used as sources of chromosomal DNA for size reference markers. The yeasts were cultured on YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose), and *N. crassa* on Vogel's minimal N (Vogel 1964).

Preparation of intact chromosomal DNA.

G. fujikuroi conidia were collected from 2- to 3-day-old liquid cultures by filtration and then washed twice in sterile distilled water. In a 250-ml Erlenmeyer flask, 50–100 ml of potato-dextrose broth (Difco) was inoculated with $1–5 \times 10^6$ conidia per milliliter; the flask was placed on a rotary shaker (150 rpm) and then incubated for 10–16 hr at room temperature (22–24° C). Germinated spores were collected and washed three times with either 1.2 M sorbitol or OM buffer

(1.2 M $MgSO_4$ and 10 mM Na phosphate, pH 5.8) (Carle and Olson 1985). Washed germlings were suspended in 5 ml of OM buffer containing Novozym 234 (10 mg/ml) (Novo Biolabs, Danbury, CT) and 50 µl of β-mercaptoethanol and incubated for 2 hr to overnight at 32° C while being shaken at 60–70 rpm. This suspension was filtered through two layers of cheesecloth and a 44-µm-mesh nylon screen. The filtrate, containing spheroplasts, was washed twice with STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.6) and then pelleted by centrifugation (3000 × *g* for 5 min) and resuspended at a concentration of 2×10^8 cells per milliliter. The suspension was placed at 37° C and mixed with an equal volume of molten 1.4% low-melting-point agarose (Fisher Scientific, Pittsburgh, PA) in 1.2 M sorbitol and 50 mM EDTA (pH 8.0). The agarose-spheroplast mixture was pipetted into a sample mold and solidified on ice for 15 min. The agarose plugs were incubated in NDS buffer (0.5 M EDTA, 10 mM Tris-HCL, and 1% Na *N*-lauroyl sarcosinate, pH 8.0) containing proteinase K (2 mg/ml) at 50° C for 48 hr with two buffer changes. Plugs were washed three times with 50 mM EDTA (pH 8.0) at 37° C and stored at 4° C in 50 mM EDTA. The chromosomal DNA was stable for up to six months.

Standard methods were used for preparing chromosome-sized DNAs of *S. cerevisiae* (Vollrath and Davis 1987), *S. pombe* (Gunderson and Chu 1991), and *N. crassa* (Orbach *et al.* 1988).

Spheroplast plug preparation.

Proper preparation of the spheroplast plugs is critical for good resolution of *G. fujikuroi* chromosomes on CHEF gels. A concentration of 2×10^8 spheroplasts per milliliter yielded the best DNA concentration for clear, sharp bands. Contamination of the plugs with conidia amounting to a few percent of the total did not affect the results, but damaged spheroplasts or DNase contamination resulted in smearing when the DNA was resolved on the CHEF gels. Although strains from the six *G. fujikuroi* mating populations have similar mycelial and conidial morphology, they differ substantially in the ease with which spheroplasts can be released. Spheroplasts were usually released relatively easily from strains belonging to mating population A; treatment with Novozym 234 for 2–4 hr normally sufficed. Spheroplasts were much more difficult to obtain from strains belonging to populations C and E, requiring increased levels of Novozym 234 (up to 20 mg/ml), the addition of 1% β-mercaptoethanol, and longer incubation.

CHEF gel electrophoresis conditions.

PFGE was performed with a CHEF system (CHEF DRII, Bio-Rad, Richmond, CA). Gels were run in a cold room (4° C); the buffer was 0.5× Tris-borate-EDTA (Maniatis *et al.* 1982), maintained at 9–12° C and kept in constant recirculation. Electrode buffer was changed every 6 or 7 days for gels running longer than 1 week. Regular DNA-grade agarose (Fisher Scientific) was used in gels for resolving DNA molecules 3 Mbp or smaller, and chromosome-grade agarose (Bio-Rad) for resolving DNA molecules larger than 3 Mbp.

Different electrophoretic conditions were used to resolve chromosomal DNAs of different sizes. To resolve DNA bands in the 0.1–1.5 Mbp range the conditions were a 1% agarose gel, 200 V, a 70-sec switch time for 14.5 hr, and a 2-min switch time for 10 hr. To resolve bands in the 1.5–3.5 Mbp range the conditions were a 0.6% agarose gel, 45 V, a 60-min switch time for 48 hr, a 55-min switch time for 72 hr, a 45-min switch time for 72 hr, and a 37-min switch time for 72 hr. To resolve bands in the 2–10 Mbp range the conditions were a 0.5% agarose gel, 40 V, and a 60-min switch time for 72 hr, and then 50 V and a 60-min switch time for an additional 9–10 days. After electrophoresis, the gel was stained in ethidium bromide (0.5 µg/ml) for 30 min, destained in water overnight, and photographed under illumination with ultraviolet light (254 nm).

Preparation of probes.

All plasmids used for probes were grown in *Escherichia coli* DH5α; plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly 1979). Random anonymous genomic clones used for constructing an RFLP map of *F. moniliforme* were used for tagging different chromosomes (Xu and Leslie 1993); the heterologous probes used are identified in Table 2. With the exception of the cDNA probe, all probes were radiolabeled with α-^{32}P to a specific activity of 5×10^8 cpm/µg of DNA by either nick translation (Rigby *et al.* 1977) or random hexamer priming (Feinberg and Vogelstein 1983). Free radioactive nucleotides were removed by gel filtration through Sephadex G-50.

The cDNA probe was prepared from total mRNA from *F. moniliforme* strain A-00102 that had been purified with the Fast Tract mRNA Isolation kit (Invitrogen Co., San Diego, CA). Poly(A)$^+$ RNA (1–4 µg) was denatured at 70° C for 10 min and then placed on ice. The sample was incubated at 37° C for 1 hr in a final reaction volume of 50 µl containing 5× reaction buffer; 10 mM dithiothreitol; 0.5 mM (each) dATP, dCTP, and dTTP; oligo-(dT) (40 µg/ml); 2.4 µM dGTP; 200 units of RNase H-reverse transcriptase; and 100 µCi (α-^{32}P)dGTP. After the incubation, 5 M NaOH was added to the reaction solution to a final concentration of 0.3 M, and the solution was cooled to room temperature. After it had cooled, 1.0 M Tris-HCl (pH 7.4) and 12 N HCl were added to make final concentrations of 0.25 M and 0.18 N, respectively. The mixture was extracted once with phenol $CHCl_3$, and the upper (aqueous) phase placed on an Elutip microcolumn (Schleicher & Schuell, Keene, NH) to separate the labeled cDNA from the unincorporated nucleotides.

The 18S and 28S rDNA probes were amplified by the polymerase chain reaction (PCR) using the NS and NL primers (White *et al.* 1990; Vilgalys and Hester 1990). PCR reactions contained 0.25 unit of *Taq* polymerase (Promega, Madison, WI); 1× *Taq* buffer (Promega); 0.1 mM (each) dATP, dGTP, dCTP, and dTTP (USBiochemicals, Cleveland, OH); 25 ng of genomic DNA; and 0.2 µM each primer. The amplification reactions were incubated in a Perkin-Elmer Cetus DNA Thermal Cycler for 45 cycles of 1 min at 94° C, 1 min at 55° C, and 2 min at 72° C.

Southern blotting and hybridization conditions.

CHEF gels were irradiated with UV light (254 nm) for 2 min to nick the chromosomal DNA. Gels containing nicked DNA were depurinated by immersing them in 250 mM HCl for two 15-min periods; the DNA was denatured with 250 ml of 0.5 M NaOH and 1.5 M NaCl for 30 min and then neutralized in 0.5 M Tris-HCl and 1.5 M NaCl (pH 7.5) for 10 min.

The DNA was transferred to nylon membranes (MSI, Westboro, MA, or Amersham, Arlington Heights, IL), with either 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) or 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.7), respectively, by capillary blotting overnight. The DNA was cross-linked by irradiating the membrane with UV light (254 nm) and was baked at 80° C for 1–2 hr. Prehybridization was done in a solution of 5× SSPE, 50% formamide, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA (20 µg/ml) for 2–4 hr at either 37 or 42° C, depending upon the source of the probe. Hybridizations were continued for 24 hr, with a probe concentration of 1×10^6 to 2×10^6 cpm/ml. Blots were washed under stringency conditions appropriate for the source of the probe. Filters were washed in 3× SSPE/SSC and 0.2% SDS at 42° C for 15 min and then washed twice, for 30 min each wash, in 0.5× SSPE/SSC and 0.1% SDS at 60° C. The membranes were then exposed to Kodak X-Omat film with intensifying screens (Dupont, Wilmington, DE). After autoradiography, the membranes were stripped of labeled probe by washing in boiling 0.5% SDS and then were reprobed. These blots could be stripped and reprobed at least 12 times and still yielded clear hybridization signals.

Routine molecular manipulations not otherwise specified followed Maniatis *et al.* (1982) and Sambrook *et al.* (1989).

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