

Electrophoretic Karyotypes of *Magnaporthe grisea* Pathogens of Diverse Grasses

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Received 19 September 1995. Accepted 16 January 1996.

We have analyzed the chromosomes of a wide variety of strains of *Magnaporthe grisea* using pulsed-field gel electrophoresis (PFGE), in combination with Southern hybridization and genetic crosses. Strains analyzed included rice pathogens, field pathogens of grasses other than rice, and fertile laboratory strains. All *M. grisea* strains examined contain chromosomes in the size range from 2,000 kilobases (kb) to greater than 10,000 kb. Some strains also contain chromosomes, termed "mini-chromosomes," that range in size from 500 to 2,000 kb. Variation in chromosome numbers seems to be mainly among the mini-chromosomes. Infertile field isolates, including most rice pathogens, have a relatively high number of chromosome length polymorphisms. In contrast, some *M. grisea* strains from diverse hosts around the world are interfertile and have a relatively uniform karyotype, although translocations are present even among these fertile strains. There appears to be a correlation between the presence of mini-chromosomes and low levels of sexual fertility, although it is not yet clear if mini-chromosomes are a result or a cause of the low fertility. A mini-chromosome from a Chinese rice pathogen failed to segregate in crosses and it contained at least one DNA sequence that was found in mini-chromosomes of diverse strains, but not found in the larger standard chromosomes from these strains. These mini-chromosomes appear to be nonessential for growth and pathogenicity. Thus, we conclude that many small *M. grisea* chromosomes are like B-chromosomes found in plants and animals. However, size alone does not predict whether a small chromosome has B-chromosome-like properties. This is demonstrated by the normal Mendelian segregation of a small chromosome present in one parental strain used in an *M. grisea* RFLP mapping project that was apparently formed through an unequal translocation between two larger chromosomes.

Additional keywords: B-chromosomes; chromosomal rearrangements; mini-chromosomes, rice blast disease; sexual fertility.

Magnaporthe grisea (Hebert) Barr (anamorph *Pyricularia grisea* Sacc.; Rossman et al. 1990) is a filamentous, heterothallic ascomycete that includes pathogens of rice (*Oryza sativa* L.) as well as pathogens of a broad range of other grasses (Ou 1985; Valent and Chumley 1991). Genetic studies of host range and mechanisms of pathogenesis have been hampered by the low fertility of most isolates of the fungus collected from the field. Many of the early studies have been conducted using strains of *M. grisea* that parasitize finger millet, *Eleusine coracana* (L.) Gaertn., or weeping lovegrass, *Eragrostis curvula* (Schr.) Nees, because some field isolates infecting these grasses are hermaphrodites that cross to produce viable ascospores (Yaegashi 1978). Crosses have generally not been possible between field isolates that are pathogenic to rice because they are female sterile. Efforts have gone into identifying field isolates of the rice pathogen that are able to function as sexually fertile males, and in using these field isolates to produce fertile laboratory strains for analysis of pathogenicity toward rice (Kolmer and Ellingboe 1988; Valent et al. 1991). A rare hermaphroditic field isolate of the rice pathogen from French Guiana (Leung et al. 1988; Silué et al. 1992) has facilitated genetic analysis of rice pathogens.

Strains of the rice blast fungus are reported to be quite variable in the field, with the frequent appearance of new races (Ou 1985) defined by the spectrum of rice cultivars infected. This variability has been reported to be correlated with constant changes in chromosome numbers due to non-disjunction, asynchronous division, and lagging chromosomes during nuclear division (Ou 1980a; Ou 1980b; Tolmsoff 1983). The chromosome number has been reported to range from 2 to 12 (Kameswar and Crill 1981) with most strains having 3 or 6. A more recent cytological analysis suggested that *M. grisea* has 6 chromosomes (Leung and Williams 1987), but only one pair of related strains was examined. The relatively small size of fungal chromosomes makes cytological analyses using light microscopy difficult. In addition, such analyses require a sexual cycle in the organism under study because they rely on visualization of condensed pachytene chromosomes (Leung and Williams 1987; Raju 1980). Many *M. grisea* strains of interest are infertile, or show low fertility levels. The development of pulsed-field gel electrophoresis (PFGE) has allowed the visualization of chromosome-sized DNAs from organisms where cytological analysis is not possible, either because of the size of the chromosomes or the lack of a sexual stage. In addition PFGE

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allows many strains of an organism to be compared at the same time. PFGE has been used to develop electrophoretic karyotypes for several filamentous fungi, including *Neurospora crassa*, *Aspergillus nidulans*, *Cochliobolus heterostrophus*, and *Septoria* spp. (Orbach et al. 1988; Brody and Carbon 1989; Cooley and Caten 1991; McDonald and Martinez 1991; Tzeng et al. 1992; and see Mills and McCluskey 1990, for a review) and has been used in conjunction with parasexual analysis to examine karyotypes of fungi imperfecti (Talbot et al. 1991).

Talbot et al. (1993) have examined the electrophoretic karyotype of *M. grisea* rice pathogens from the United States representing a diverse collection of pathotypes. They found karyotype diversity, even among fungal strains that appeared by DNA fingerprint analysis to be clonally related (Levy et al. 1991). Skinner et al. (1993) report karyotype studies on two parental strains in an *M. grisea* mapping population in order to assign linkage groups to electrophoretically separated chromosomes. Our report extends the scope of these analyses to include karyotype studies of both fertile and infertile pathogens of diverse grasses, as well as laboratory strains developed for improved fertility. We demonstrate that many infertile strains, but not fertile strains, contain B-like minichromosomes in addition to the standard complement of larger chromosomes. Female fertility is correlated with the absence of these B chromosomes. Chromosome size polymorphisms and rearrangements appear common, especially among infertile field isolates of the rice pathogen. Interestingly, however, some strains from diverse geographic areas and with diverse host specificities have retained similar genome organizations. We demonstrate that these strains are

capable of intermating and producing abundant viable ascospores. Electrophoretic karyotyping has been used to support and extend genetic mapping efforts with the rice blast fungus.

RESULTS

Survey of laboratory strains and field isolates.

To determine the range of sizes of *M. grisea* chromosomes, preparations of fertile laboratory strains and field isolates were separated and compared to chromosomes of *Schizosaccharomyces pombe* and *Neurospora crassa* (Fig. 1, Table 1). As in *N. crassa*, most *M. grisea* chromosomes are larger than 3.5 megabase pairs (Mb). In Figure 1, a series of related rice pathogenic laboratory strains, chosen for high fertility, are shown between the *N. crassa* and *S. pombe* marker chromosome DNAs. These laboratory strains include 6043 and 4224-7-8, the parental strains of the genetic cross (serial number 4360) used for production of an RFLP map (Sweigard et al.

Table 1. *Magnaporthe grisea* strains

Strain	Host	Origin/Source/Comments
O-17	Rice	Ken60-19, Japan, H. Yaegashi
O-42	Rice	Japan, H. Kato
O-128	Rice	P-2, Japan, M. Henry
O-135	Rice	China, CNRRI, Y. Shen
O-184	Rice	Texas, U.S.A., M. A. Marchetti
O-219	Rice	Ivory Coast, Africa
O-245	Rice	Philippines, J. M. Bonman
O-250	Rice	India, J. M. Bonman
O-254	Rice	Korea, J. M. Bonman
O-259	Rice	Egypt, S. Kamel
O-282	Rice	Brazil, A. S. Prabhu
O-283	Rice	Brazil, A. S. Prabhu
Guy11	Rice	Fr. Guiana, J. L. Notteghem
G-1	<i>Digitaria</i> spp.	West Va, U.S.A, F. M. Latterall
K76-79	Weeping love-grass	Japan, H. Yaegashi
WGG-FA40	Finger millet	Japan, H. Yaegashi
G-68	<i>Pennisetum</i> spp.	IRRI, A. Obusan
G-71	Finger millet	ICRISAT, R. Gopinath
G-81	<i>Pennisetum</i> spp.	Georgia, U.S.A., H. Wells
T1	Wheat	Brazil, A. S. Prabhu
T2	Wheat	Brazil, A. S. Prabhu
T3	Wheat	Brazil, A. S. Prabhu
T4	Wheat	Brazil, S. Igarashi
T5	Wheat	Brazil, S. Igarashi
T6	Wheat	Brazil, S. Igarashi
T7	Wheat	Brazil, S. Igarashi
G-155	<i>Rhynchetium roseum</i>	Brazil, A. S. Prabhu
G-156	<i>Digitaria</i> spp.	Brazil, A. S. Prabhu
G-157	Triticale	Brazil, A. S. Prabhu
4091-5-8	Weeping love-grass	Lab strain, Kang et al. 1994
4136-4-3	Weeping love-grass	Lab strain, Kang et al. 1994
6043	Rice	Lab strain, Leung et al. 1988
4224-7-8	Rice	Lab strain, Valent and Chumley 1994
4360-17-1	Rice	Lab strain, Valent and Chumley 1994
4360-R-12	Rice	Lab strain, Valent and Chumley 1994
4375-R-6	Rice	Lab strain, Valent and Chumley 1994
4396-1-Tetrad		From cross, 4091-5-8 × O-135
4396-2-Tetrad		From cross, 4091-5-8 × O-135

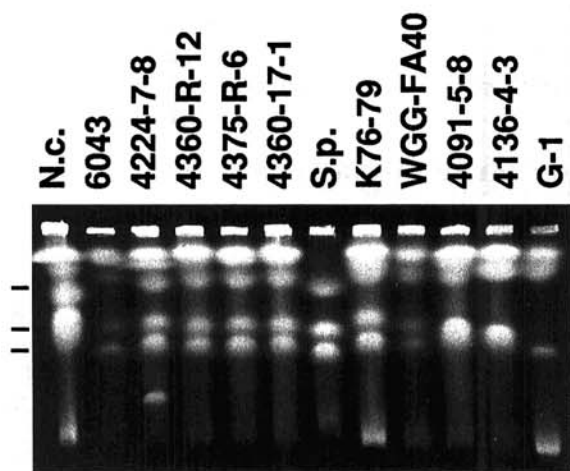


Fig. 1. Size ranges of *Magnaporthe grisea* chromosome DNAs. Chromosome DNAs from a series of rice pathogenic laboratory strains bred for high fertility were resolved between chromosome DNAs of *Neurospora crassa* (N.c.) and *Schizosaccharomyces pombe* (S.p.), which are included as size standards. They include 6043 and 4224-7-8, and three strains derived from them as described (see text). The strains to the right of *S. pombe* are pathogens of grasses other than rice. Two are fertile laboratory strains (4091-5-8 and 4136-4-3), and the other three are field isolates (K76-79, WGG-FA40, and G-1). This CHEF gel was run at 40 V (1.45 V/cm) with a switching interval of 90 min for 178 h. The sizes of the *N. crassa* chromosomes in Mb are: 4.0, 4.6, 5.1, 5.7, 9.2, and 10.3 (Orbach 1992; Orbach et al. 1988). The sizes of the *S. pombe* chromosomes, 3.5, 4.6, and 5.7 Mb from bottom to top (Fan et al. 1988), are indicated to the left of the figure by dashes.

1993). Also shown are two highly fertile progeny of this cross, 4360-17-1 and 4360-R-12, and 4375-R-6, a strain from a cross between these two 4360 progeny. To the right of *S. pombe*, the DNAs from five strains that are pathogens of grasses other than rice are shown. These include the fertile field isolates K76-79 (a weeping lovegrass pathogen) and WGG-FA40 (a finger millet pathogen), which are the parents of 4091-5-8 and the grandparents of 4136-4-3. Also shown is field isolate G-1, an infertile pathogen of crabgrass (*Digitaria* spp.).

Among the strains related through crosses, the chromosome patterns are generally quite similar. Some chromosome size polymorphisms were observed, as exemplified by the presence of a chromosome much smaller than 3 Mb in strain 4224-7-8. RFLP analysis has revealed that this particular small chromosome resulted from an apparent translocation (see below and Sweigard et al. 1993). More subtle polymorphisms are represented by the doublet at 4 to 5 Mb. On this gel the doublet in 4091-5-8 and 4136-4-3 appears as a single broad band. However, under different electrophoretic condi-

tions, this band is resolved into two chromosome bands that are closer in size than the analogous ones present in 6043, 4224-7-8, and their progeny strains (data not shown). The crabgrass pathogen G-1, which fails to mate with any other strain, appears to have differences in chromosomal DNAs in the 4 to 5 Mb size range.

Southern hybridization analysis of the gel in Figure 1 using the *M. grisea* *TUB1*, *RSY1* and *CUT1* genes as probes (data not shown) indicated that each gene hybridized to a different sized chromosome within an individual strain. Among strains, the *TUB1* and *CUT1* genes hybridized to similar sized chromosomes in most cases, with *TUB1* hybridizing to a chromosome slightly greater than 4.6 Mb, and *CUT1* hybridizing to a much larger chromosome. In contrast, the *RSY1* gene hybridized to the top bands in 6043, the two 4360 strains and 4375-R-6, but to the second, compressed band in 4224-7-8. As discussed below, the presence of *RSY1* on different sized chromosomes in 6043 and 4224-7-8 is due to their differing by a translocation involving this chromosome.

To expand our analysis, the chromosome DNAs of *M. grisea* field isolates collected from around the world were separated (Figs. 2 and 3). Examination of 10 rice pathogens

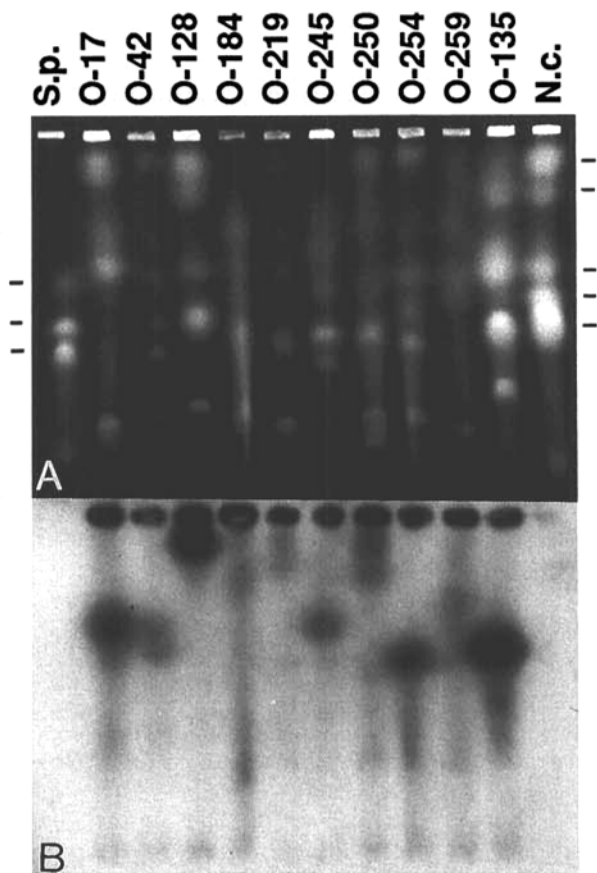


Fig. 2. Chromosome length polymorphisms (CLPs) among field isolates that infect rice. **A**, The origins of the rice pathogens are listed in Table 1. Preparations of chromosomal DNAs were electrophoresed at 40 V with a switching interval of 90 min for 94 h, followed by a regime (also at 40 V) with a 140-min switching interval for 147 h. *Neurospora crassa* (N.c.) and *Schizosaccharomyces pombe* (S.p.) are included as size standards. The sizes of the *S. pombe* chromosomes (3.5, 4.6, and 5.7 Mb from bottom to top) are indicated on the left and the sizes of the *N. crassa* chromosomes (4.0 and 4.6 Mb unresolved, 5.1, 5.7, 9.2, and 10.3 Mb) are indicated on the right. **B**, Southern blot analysis with the *ILV1* gene as a hybridization probe highlights CLPs in the chromosome carrying this gene.

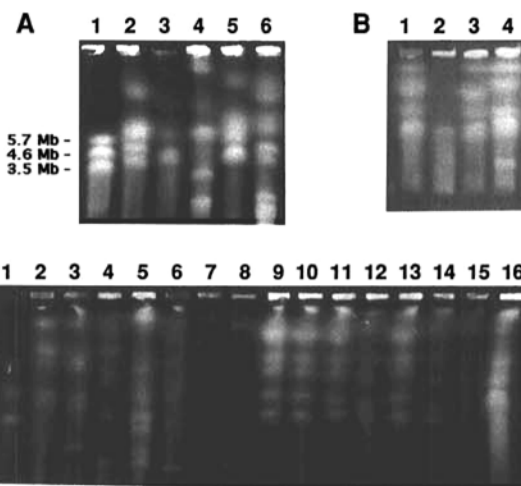


Fig. 3. Electrophoretic karyotypes of *Magnaporthe grisea* pathogens of diverse grasses. **A**, Chromosomal DNAs were electrophoresed at 35 V with a switching interval of 140 min for 193 h. *Schizosaccharomyces pombe* chromosomes (lane 1) were run as a size standard with the sizes indicated to the left of the figure. The *M. grisea* strains are: a Japanese weeping lovegrass pathogen K76-79 (lane 2), a Japanese finger millet pathogen WGG-FA40 (lane 3); a Philippine *Pennisetum* pathogen G-68 (lane 4), an Indian finger millet pathogen G-71 (lane 5) and a U.S. *Pennisetum* pathogen G-81 (lane 6). **B**, Chromosomal DNAs from the hermaphroditic rice pathogen Guy11 (lane 1), and from fertile laboratory strains 4091-5-8 (lane 2, degraded), 6043 (lane 3), and 4224-7-8 (lane 4). Samples were loaded on a 0.8% gel and electrophoresed at 36 V with a switching interval of 120 min for 168 h. Although no size standards are shown on this particular gel, sizes can be inferred from sizes of chromosomal bands in 6043 and 4224-7-8 relative to standards in Figure 1. **C**, Preparations of chromosomal DNAs from Brazilian pathogens of rice, wild grasses, and wheat. The gel was electrophoresed at 35 V, with a switching interval of 90 min for 94 h, followed by a second regime using a switching interval of 140 min for 145 h. Again, *S. pombe* chromosomes (lane 1, positions marked) and *N. crassa* chromosomes (lane 16) are included as size standards. The *M. grisea* strains are: 4091-5-8 (lane 2); O-135 (lane 3); O-282 (lane 4); O-283 (lane 5); G-155 (lane 6); G-156 (lane 7, very faint); G-157 (lane 8, faint); T-1 (lane 9); T-2 (lane 10); T-3 (lane 11); T-4 (lane 12); T-5 (lane 13); T-6 (lane 14); and T-7 (lane 15).

from eight countries (Fig. 2A) revealed the existence of numerous chromosome length polymorphisms (CLPs). These CLPs are further illustrated by hybridization of the resolved chromosome DNAs with the *ILV1* probe (Fig. 2B). All of these rice pathogens contain chromosomes in the size range less than 3 megabases, which are not present in most of the strains of Figure 1. Under the conditions used for the gel in Figure 2A, the larger chromosomes of *M. grisea* are resolved. They are about the same size as the larger *N. crassa* chromosomes of which the two largest are 9.2 and 10.3 Mb in length. The maximum number of bands resolved in this gel are the 7 bands observed for O-42. The second and third largest bands appear faint because they are in a zone of maximal resolution which expands the bands (Vollrath and Davis 1987), but are visible in an overexposure of this gel (data not shown).

Comparison of chromosome-length DNAs of some *M. grisea* pathogens of grasses other than rice (Fig. 3A) again revealed the presence of chromosome size polymorphisms which are readily apparent in the lower size range. Two *Penisetum* pathogens, G-68 and G-81, (lanes 4 and 6), had chromosomes less than 2 Mb in size while two *Eleusine* pathogens, WGG-FA40 and G-71 (lanes 3 and 5), and an *Eragrostis* pathogen, K76-79 (lane 2), did not (see also Fig. 1). Examination of the karyotype of the female-fertile rice pathogen Guy11 (Fig. 3B, lane 1) revealed that, in contrast to the result with other rice pathogens, it lacks chromosomes smaller than 2 Mb.

Electrophoretic karyotypes for strains collected from different hosts in one geographic region were analyzed and compared to those of strains 4091-5-8 (Fig. 1) and O-135 (Fig. 2). Separation of chromosome DNA preparations of *M. grisea* pathogens of rice, wild grasses, and wheat (*Triticum aestivum* L.) that were collected in Brazil revealed quite different karyotypes among the strains with different host specificities (Fig. 3C). It is notable that the wheat pathogens, T-1 to T-7 (Fig. 3C lanes 9-15), collected at several different locations over a period of 3 years had identical karyotypes at this level of resolution. The karyotypes of the wheat pathogens are quite different from the karyotypes of the two Brazilian rice pathogens examined, O-282 and O-283 (Fig. 3C lanes 4 and 5). The wheat pathogen karyotype looks more similar to that of the wild grass pathogens, G-156 and G-157 (Fig. 3C lanes 7 and 8). As with O-135 and the other rice pathogens shown in Figure 2A, the Brazilian rice pathogens and one of the wild grass pathogens, G-155 (Fig. 3C lane 6), contain mini-chromosomes. Mini-chromosomes are absent from the other two wild grass pathogens and from all the wheat pathogens, as is true with the highly fertile mating type tester strain 4091-5-8 (Fig. 3C, lane 2).

Fertile strains lack small chromosomes.

All strains examined in Figures 1, 2, and 3 have been tested for fertility. The *Eleusine* pathogens WGG-FA40 and G-71, the wild grass pathogens G-156 and G-157, the wheat pathogens, T-1 through T-7, the rice pathogen Guy11, and the fertile laboratory strains 4091-5-8, 4136-4-3, 6043, 4224-7-8, 4360-R-12, 4360-17-1, and 4375-R-6 are all fertile hermaphrodites that cross either as males or as females with strains of the opposite mating type. All other strains are female sterile. Some of the female sterile strains, such as the rice pathogen O-135, are fertile as males in crosses with highly fertile mating type testers. Other strains, such as G-1, G-68, G-81, and many rice pathogens, are totally infertile, failing to cross with any other strain. Except in the case of 4224-7-8 (discussed below) all female fertile strains lack small chromosomes. Most of the infertile or poorly fertile, female-sterile strains contain small chromosomes.

The percentage of viable ascospores produced in a cross is of interest because a high level of ascospore viability is an indicator of the similarity in genome organization between two strains. For example, when a reciprocal translocation is present between two strains, ascospore viability drops to 50% or below (Perkins and Barry 1977). Viability of ascospores for the most fertile strains among those analyzed are shown in Table 2. The excellent viability of ascospores seen for these crosses suggests that these laboratory strains and field isolates from diverse hosts and diverse geographic locations have retained very similar genome organizations.

Further characterization of small chromosomes.

Approximately half of the strains characterized in Figures 2 and 3 contain chromosomes less than 3 Mb in size. Further resolution of these small chromosomes was achieved by using electrophoretic conditions that allow maximum separation of DNAs between 200 and 1,200 kb in size (Fig. 4A). Up to 4 small chromosomes have been resolved in the strains we have surveyed. These chromosomes vary considerably in size among the different strains. Furthermore, not all of the small chromosomes are resolved in this gel. For example, a mini-chromosome in the rice pathogen O-135 (whose size is approximately 2 Mb) is not resolved under the conditions used here.

All mini-chromosomes tested in rice pathogens hybridize to the rice pathogen-specific middle repetitive DNA sequence MGR586, which is found on all larger chromosomes (data not shown; Hamer et al. 1989). We were interested in determining whether low copy number DNA sequences found on mini-chromosomes were unique to these chromosomes or might be present on larger chromosomes in some strains. Chromo-

Table 2. Viability of ascospores in crosses of highly fertile strains

Mat1-2 Parent		Mat1-1 parent		Viable ascospores (%)
Strain	Origin and host	Strain	Origin and host	
4091-5-8	Lab strain, weeping lovegrass	4136-4-3	Lab strain, weeping lovegrass	95
4091-5-8	Lab strain, weeping lovegrass	4375-R-6	Lab strain, rice	95
4091-5-8	Lab strain, weeping lovegrass	T-5	Field isolate, wheat	85
G-71	Field isolate, finger millet	4136-4-3	Lab strain, weeping lovegrass	95
G-71	Field isolate, finger millet	T-5	Field isolate, wheat	90
Guy11	Field isolate, rice	4136-4-3	Lab strain, weeping lovegrass	70
Guy11	Field isolate, rice	T-5	Field isolate, wheat	70
4091-5-8	Lab strain, weeping lovegrass	O-135	Field isolate, rice	25-50

some-specific clones of the O-135 mini-chromosome were isolated following its purification from a preparative CHEF gel. A small number of clones were obtained and all but one of these contained middle repetitive DNA sequences. The clone that lacked repetitive sequences, pCB715, hybridized to two bands in genomic Southern of strain O-135. This clone, as expected, hybridized to the mini-chromosome of strain O-135, but it failed to show homology to the larger chromosomes of strains O-135 and 4091-5-8. This clone also failed to hybridize to the larger chromosomes of other strains tested, including several rice pathogen field isolates, and the grass

pathogens K76-79, WGG-FA40, G-68, G-71, and G-81 (data not shown). Thus, the pCB715 insert appears to be mini-chromosome specific.

To determine whether small chromosomes in different strains may be related, pCB715 was used as a probe for a blot of the gel shown in Figure 4A. In Figure 4B it is seen that this probe hybridizes to many, but not all, of the small chromosomes in these strains. For example, it hybridizes to the 3 small chromosomes of G-68, but only to 2 of the 3 found in G-81. No hybridization is observed to the smallest G-81 chromosome, which is about 680 kb in size. The pCB715 probe does not hybridize to the small chromosome in strain 4224-7-8 (results not shown). It should also be noted that the probe hybridizes with different intensity to the different small chromosomes, which cannot be accounted for by the different amounts of DNA present in each lane. For example, the strongly hybridizing chromosome of G-81 (approximately 950 kb) appears to contain about the same amount of DNA as the more weakly hybridizing band of about 800 kb in G-68. This may indicate that sequences homologous to pCB715 either vary in copy number on the different small chromosomes or that there is some sequence divergence between the copies on different chromosomes. Other probes isolated from the O-135 small chromosome need to be hybridized to further determine the relatedness of these small chromosomes.

Inheritance of the O-135 mini-chromosome.

We investigated the inheritance of the small chromosome of the Chinese rice pathogen O-135 through a series of back-

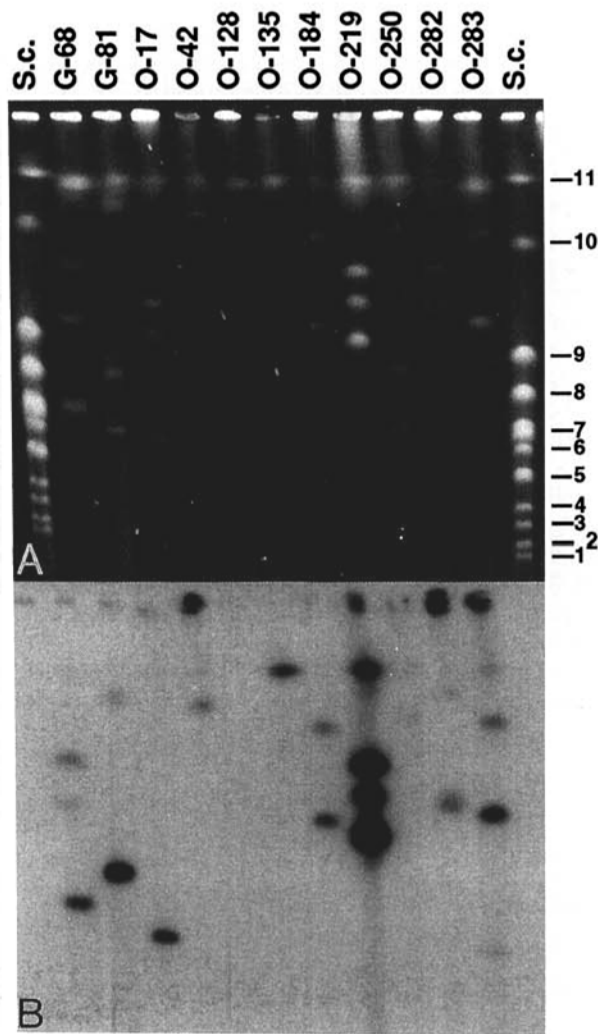


Fig. 4. Resolution of mini-chromosomes in *Magnaporthe grisea* field isolates. **A**, To separate chromosomes between 200 and 1,200 kb, this 0.8% agarose gel was electrophoresed at 150 V with a switching interval ramped linearly, from 90 to 180 s, for 33 h. *Saccharomyces cerevisiae* strain AB1380 is included as a size standard in lanes 1 and 13 (Link and Olson 1991). The *S. cerevisiae* chromosomes are separated into 11 bands as indicated by the numbered lines to the right of the gel. The sizes of the chromosomes (in kb) represented by the lines are: 240 (1); 280 (2); 350 (3); 440 (4); a 590 doublet (5); 680 (6); a triplet of 755, 810 and 840 (7); 950 (8); 980 (9); 1,120 and 1,130 (10); 1,640 and 2,500 (11). (David Burke, personal communication). The *M. grisea* strains include two *Pennisetum* pathogens (G-68, G-81) and rice pathogens from around the world (Table 1). **B**, The separated chromosomes were transferred to a nylon membrane and probed with pCB715, a mini-chromosome-specific clone obtained from the Chinese rice pathogen O-135.

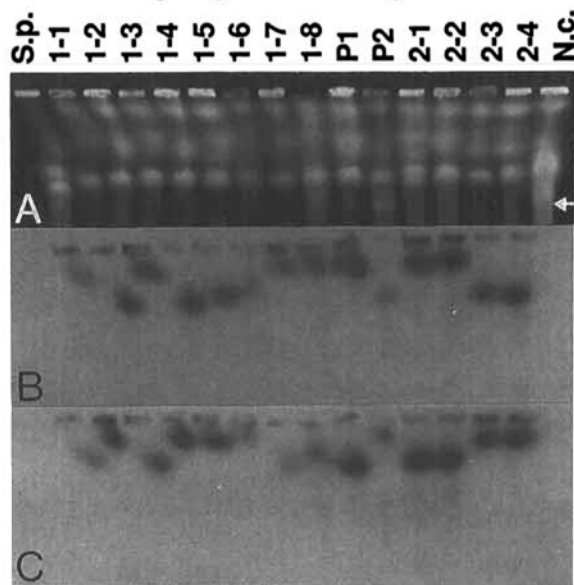


Fig. 5. CHEF analysis of two tetrads from the 4091-5-8 x O-135 cross. **A**, The chromosome DNAs of all eight ascospore progeny from one tetrad, 4396-1, and four progeny representing the meiotic products of a second tetrad, 4396-2, were resolved. *Schizosaccharomyces pombe* (S.p.) and *Neurospora crassa* (N.c.) were included as size standards. Parental strains 4091-5-8 and O-135 are identified as P1 and P2, respectively. Progenies from tetrad 4396-1 are labeled 1-1 through 1-8 and those from tetrad 4396-2 are labeled 2-1 through 2-4. The position of the O-135 mini-chromosome band is indicated with a white arrow. This gel was run at 40 V with a switching interval of 90 min for 95 h. The switching interval was then changed to 140 min and the gel run for an additional 21 h before it floated in the chamber and was stopped. The gel was transferred to a nylon membrane and the filter was probed with the *ILV1* gene (**B**) and the *RSY1* gene (**C**).

crosses undertaken to identify *M. grisea* genes for pathogenicity and virulence (Valent et al. 1991). In this backcrossing scheme, strain 4091-5-8, which lacks a small chromosome, was mated with O-135, and progeny with the highest level of fertility were backcrossed to O-135, the recurrent parent. Progeny selected for high fertility at each of four backcross generations, did not contain the mini-chromosome (data not shown). To analyze further the inheritance of this chromosome, chromosome DNAs of nine random progeny from the O-135 × 4091-5-8 cross were separated by CHEF. Only two of the nine inherited the small chromosome (data not shown). The mini-chromosome specific clone from O-135 (pCB715) was used as a hybridization probe against progeny containing and lacking the small chromosome. Strains with the small chromosome exhibited hybridization to pCB715, and hybridization was localized to the small chromosome. Strain 4091-5-8 and progeny that did not contain the small chromosome did not hybridize to pCB715. CHEF analysis was also done on two complete tetrads (serial numbers 4396-1 and 4396-2) from the O-135 × 4091-5-8 cross (Fig. 5). The small chromosome of O-135 is absent from all 16 progenies from these two tetrads (Fig. 5 and data not shown).

Another set of 18 progeny from the cross between O-135 and 4091-5-8 were tested for the presence of the mini-chromosome sequence by hybridization to pCB715 using genomic Southern analysis. Only 4 of these strains contained homology to pCB715. Thus, in both random spore and tetrad analysis, the mini-chromosome in O-135 is not inherited in a Mendelian manner.

Hybridization analysis of separated chromosomes.

Cloned genes and RFLP mapping probes were hybridized to the separated chromosomes of various strains to identify chromosome homologs. Figure 6 shows separation of chromosome-sized DNAs from the RFLP parental strains and the rice pathogen O-135. This gel and comparable gels were blotted and probed repeatedly with DNA sequences of inter-

est. Typical results are shown in Figure 6B-E, in which RFLP mapping clones were used as probes (Sweigard et al. 1993). The rDNA probe (Fig. 6B) hybridizes to the second largest chromosome bands in the RFLP parental strains, but to a larger chromosome band in the rice pathogen O-135. Cos125 (Fig. 6C), which is linked to the *RSY1* gene, appears to be involved in two translocations (see below). Cos193 (Fig. 6D) hybridizes to the largest chromosome in all strains tested. Cos243 (Fig. 6E) is located on the third largest chromosomal DNA band in several strains tested. Cos156 (Fig. 6F) is genetically linked to the mating type locus, *MAT1*, and is consistently located on the smallest of the normal complement of chromosomes. Similar analyses were done for multiple probes that identify physical markers and known genes in the RFLP map, in order to verify the linkage group assignments made in the segregation analysis (Sweigard et al. 1993).

A schematic diagram summarizing the chromosomal location of various cloned genes and sequences in strains 6043, 4224-7-8, and O-135 is shown in Figure 7. This information is compiled from multiple hybridization experiments. In several cases in Figure 7, a single symbol indicates loci for which genetic analysis has linked two markers in addition to all the relevant data from CHEF analysis. For example, we originally detected the apparent linkage of the *TUB1* and *LYS1* loci by CHEF analysis, and the linkage was confirmed genetically by a cross between a benomyl resistant strain and a *lys1-1* auxotroph (data not shown). This linkage was independently confirmed in the RFLP mapping cross using strains from a completely different genetic background (Sweigard et al. 1993).

Chromosome rearrangements identified using CHEF.

CHEF analysis indicated that there are chromosome length polymorphisms between the RFLP mapping parental strains 6043 and 4224-7-8 (Figs. 1, 3B, and 6A). Strain 4224-7-8 has two chromosomal DNAs that do not correspond to similar sized bands in 6043. In addition to a "mini-chromosome"-size

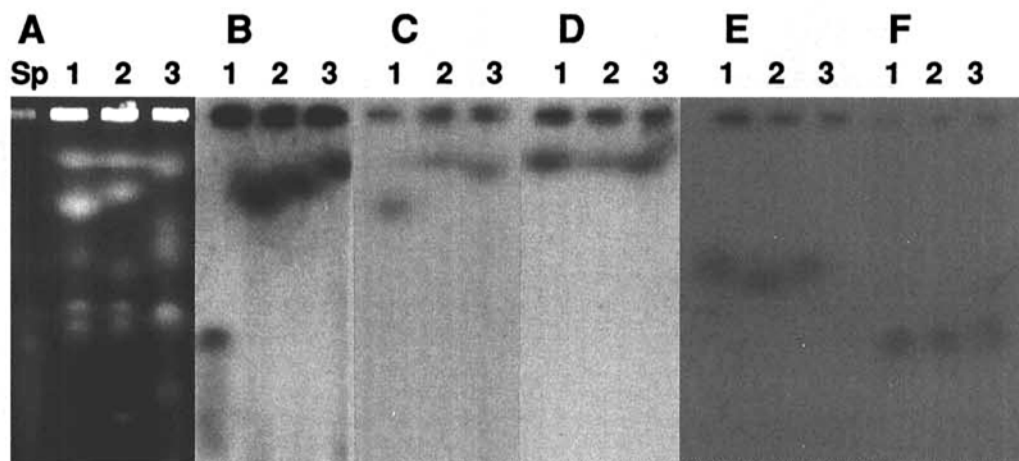


Fig. 6. Hybridization analysis of resolved *Magnaporthe grisea* chromosome DNAs. A, CHEF separation of chromosome-sized DNAs from the RFLP mapping parents 6043 (lane 1) and 4224-7-8 (lane 2) and from the rice pathogen O-135 (lane 3). *Schizosaccharomyces pombe* (S.p.) is included as a size standard. Preparations of chromosomal DNAs were loaded on a 0.8% gel and electrophoresed at 40 V with a switching interval of 90 min for 134 h, followed by a regime at 36 V with a 140-min switching interval for 149 h. B-E, Resolved chromosome DNAs in lanes 1, 2, and 3 of "A" were transferred to a membrane and hybridized with an *Neurospora crassa* rDNA cosmid and with cosmids that detect RFLP markers in the *M. grisea* map. Hybridization probes are: B, *N. crassa* rDNA clone 5:3E; C, cos125; D, cos193; E, cos243; F, cos156.

band, 4224-7-8 contains an apparently very large chromosome band identified in analyses using electrophoretic conditions that allow for the separation of DNAs around 10 Mb in size. Specifically, under the electrophoretic conditions used in Figures 3B and 6, the *RSY1* gene hybridizes to the largest chromosome band in strain 4224-7-8, which migrates as if it were significantly larger than the *CUT1* hybridizing band. However, under the electrophoretic conditions used in Figure 1, the *RSY1* gene hybridizes to a second, compressed band in 4224-7-8 that migrates faster than the *CUT1* hybridizing band. Size inversion and lateral band spreading of large DNA molecules under some conditions is well documented (Gunderson and Chu 1991). This apparent inversion of migration of the *RSY1* hybridizing chromosomal DNA relative to the *CUT1* hybridizing band in 4224-7-8 suggests that this chromosome is larger than all other chromosomes in strains 4224-7-8 and 6043. In contrast, in 6043, the *RSY1* hybridizing band migrates similarly to the *CUT1* hybridizing band under all electrophoretic conditions, indicating that these two chromosomes in 6043 are similar in size. Mapping analysis identified a relatively large linkage group with markers hybridizing to this largest chromosome of strain 4224-7-8 (Sweigard et al. 1993).

CHEF analysis supported the RFLP mapping data suggesting that the parental strains 6043 and 4224-7-8 differ by a reciprocal translocation involving the *RSY1* containing chromosome. Indications of a translocation came from the following data: (i) Genetically linked RFLP markers from one end of the largest linkage group, including cos125 (Fig. 6C) and *RSY1*, hybridize to the largest 4224-7-8 chromosome band and to the second largest chromosome band in 6043 (6043 chromosome 2c, Fig. 7). (ii) Five linked markers from the opposite end of this same linkage group, including cos193 (Fig. 6D) and cos245, contained sequences that hybridize to the largest chromosome band in 6043 (6043 chromosome 1) as well as the largest 4224-7-8 band. (iii) Finally, sequences homologous to a cosmid clone, A12G1, which segregates as a normal Mendelian marker in the RFLP mapping cross (Sweigard et al. 1993), hybridizes to the mini-chromosome-size band in 4224-7-8 and to the second largest, cos125/*RSY1* containing band in 6043 (J.A. Sweigard and B. Valent, unpublished results). Therefore, the very large chromosome in 4224-7-8 contains sequences from both chromosomes 1 and 2c in 6043. Remaining sequences from 6043 chromosome 2c hybridize to the 4224-7-8 mini-chromosome-size band (Sweigard et al. 1993).

A second example of a translocation was identified in the cross between the hermaphroditic weeping lovegrass pathogen, 4091-5-8, and the female sterile rice pathogen O-135 (Valent et al. 1991). Viability of ascospores from this cross ranged up to 50% on repeated occasions (Table 2). Strains 4091-5-8 and O-135 contain CLPs for both the *RSY1* and *ILV1* containing chromosomes (Fig. 5). The *RSY1* probe hybridized to a chromosome DNA of approximately 6 Mb in 4091-5-8 and one of about 9 Mb in O-135. The *ILV1* probe hybridized to a chromosome DNA of about 8.5 Mb in 4091-5-8 and one of about 5.7 Mb in O-135. Through both tetrad analysis and random ascospore isolation, progeny were found to contain either the *RSY1* and *ILV1* chromosomes of O-135, or the *RSY1* and *ILV1* chromosomes of 4091-5-8, suggesting that these chromosomes do not assort independently. Figure 5

illustrates the cosegregation of the two pairs of parental chromosomes in two complete tetrads. In addition, the rDNA appears to be located on the same chromosome as *ILV1* in 4091-5-8 and in many other strains (Fig. 7 and unpublished data), but it appears to be located on the same chromosome as *RSY1* in O-135. These data suggest that a reciprocal translocation exists between O-135 and 4091-5-8 involving the chromosomes that contain *ILV1* and *RSY1*.

The *Pennisetum* pathogen G-68 apparently contains a chromosomal rearrangement that distinguishes it from most other strains. G-68 appears to lack chromosome-sized DNAs in the range of 3.5 to 4.6 Mb (see Fig. 3A, lane 4). Southern hybridization analyses with several CHEF gels indicated that the linked *TUB1* and *LYS1* genes hybridize to a chromosome in this size range in all strains except G-68 (Fig. 7 and unpublished data). In G-68, both genes hybridized to the chromosome DNA band at the upper limit of resolution in the gel shown in Figure 3A. This chromosome DNA is larger than the *N. crassa* linkage group V chromosome, which is estimated to be 9.2 Mb (Orbach et al. 1988; Orbach 1992). The nature of this translocation has not been studied in detail using additional probes to determine which chromosomes are involved.

DISCUSSION

CHEF electrophoresis was used to analyze the number and size of chromosomes in field isolates of *M. grisea* that were collected from a variety of hosts and geographic locations. While some chromosome number variability seems apparent, it is not as great as previously reported (Kameswar and Crill 1981). We have resolved 5 to 6 chromosome bands greater than 3 Mb in length in most strains examined. Chromosome

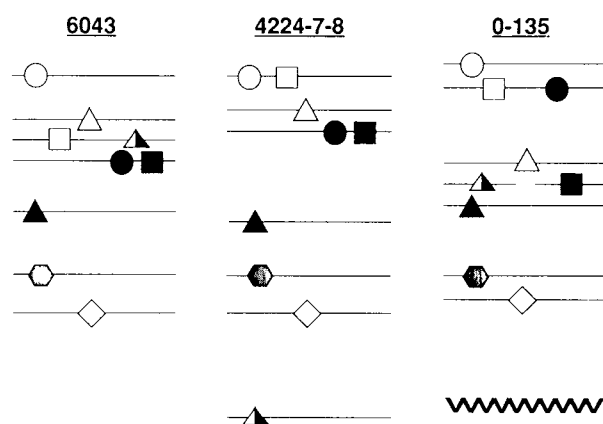


Fig. 7. Schematic drawing of gene and physical marker locations on chromosomal bands from the gel in Figure 6. Markers that show linkage through genetic analysis and that always hybridize to the same size chromosomal band are indicated by a single symbol. Symbols are: ○ = cos193 and cos245; □ = *RSY1* gene and cos125; ■ = *ILV1* gene; open triangle = *CUT1* gene; ● = rDNA; filled triangle = cos243; shaded hexagon = *TUB1* and *LYS1* genes; open diamond = cos156; black and white triangle = cosmid A12G1; wavy line = mini-chromosome-specific clone, pCB715. In the karyotype diagrammed for 6043, cos193/cos245 mark Linkage Group (LG) 1. Three comigrating chromosome bands follow: LG2c carrying *RSY1*/cos125; LG2b carrying *ILV1*; and LG2a carrying *CUT1*. RFLP marker cos243 marks LG3, *TUB1*/*LYS1* mark LG4 and cos156 marks LG5 (Sweigard et al. 1993).

bands in different size ranges were resolved by changing the conditions of electrophoresis. We now believe that the typical haploid chromosome complement of *M. grisea* strains is seven "A" chromosomes, based on the several strains that have been subjected to CHEF analysis and those that have been used to construct RFLP maps (Farman and Leong 1995; Skinner et al. 1993; Sweigard et al. 1993). The largest *M. grisea* chromosome DNAs are as large as the two largest *N. crassa* chromosomes, which are thought to be about 9.2 and 10.3 Mb in size (Orbach et al. 1988; Orbach 1992). *M. grisea* and *N. crassa* are unusual among fungi that have been electrophoretically karyotyped by having such large chromosomes (Mills and McCluskey 1990).

In our study, variation in the electrophoretic karyotypes of field isolates that infect rice appeared to be higher than the variation of karyotypes among the limited number of field isolates we tested that infect other gramineous species such as finger millet, weeping lovegrass, and wheat. Talbot et al. (1993) have conducted a detailed investigation of karyotypic variation within rice pathogen populations from the Southern United States. An extensive population study using DNA fingerprinting techniques (Levy et al. 1991) previously demonstrated that MGR586 DNA fingerprint patterns were sufficiently stable to distinguish clonal lineages among these U.S. rice pathogens. However, karyotypes varied significantly within and between lineages, obscuring the relatedness established by MGR586 fingerprinting. Talbot et al. (1993) suggested that the relatively high karyotype variability among rice pathogens reflects the lack of sexual reproduction among these populations in the field. Given the typical variability in karyotype between rice pathogens seen in this study and in the one by Talbot et al. (1993), the occurrence of a rare female fertile rice pathogen, strain Guy11 (Leung et al. 1988; Notteghem and Silué 1992), which retains a karyotype similar to that of highly fertile strains that infect other grasses is interesting.

In sharp contrast to the karyotype variation that appears typical among rice pathogens isolated from the field, wheat pathogens show remarkable karyotype conservation (Fig. 3C). Wheat blast became a serious problem for farmers in Brazil during the mid-1980's (Urashima et al. 1993). The uniformity in karyotype of wheat pathogens isolated over a 3-year period in different locations in Brazil, along with a high level of sexual fertility, suggested that strains that infect wheat have arisen from a fertile subpopulation of *M. grisea* strains and not from rice pathogens indigenous to the area. This conclusion is consistent with results of MGR586 fingerprint analyses that demonstrated that the wheat pathogens were not derived by mutation from strains that infect rice (Valent and Chumley 1994). It now appears that wheat pathogens are most closely related to *M. grisea* pathogens of *Eleusine* spp. (Urashima et al. 1993).

The excellent viability of ascospores seen in crosses between some unrelated *M. grisea* field isolates and laboratory strains (Table 2) suggests that a subpopulation of *M. grisea* strains from diverse hosts and geographic regions have similar genome organizations. These highly fertile hermaphroditic field isolates include a pathogen of *Eleusine* spp. from India (G-71), a rice pathogen from French Guiana (Guy11), and wheat pathogens from Brazil (T-1 - T-7). These field isolates are interfertile, with ascospore viability at 70% or above, and they produce abundant viable ascospores in crosses with the

unrelated laboratory strains 4091-5-8 or 4136-4-3, the two strains we typically use as mating type testers due to their high fertility (Kang et al. 1994). The genome organization of this subset of field isolates and laboratory strains resembles the pattern of strain 6043 in Figure 7. Strain 6043 is the product of a breeding program to isolate highly fertile rice pathogens, and is the progeny of a cross between Guy11 and a descendant of 4091-5-8 (Leung et al. 1988). The similarity in genome organization between Guy11 and 4091-5-8 may have contributed to the fertility of the cross that generated 6043. A more extensive analysis of other non-rice pathogens to determine how prevalent karyotype similarity is among these strains, many of which are female fertile, is warranted.

Two genetic crosses pursued due to interesting phenotypic differences between the strains showed ascospore viability that consistently failed to exceed 50%. The RFLP mapping cross parental strains, 6043 and 4224-7-8, were chosen because they differed in at least four genes controlling host specificity (Sweigard et al. 1993). All four genes were mapped and two of these, the host species specificity gene *PWL2* (Sweigard et al. 1995), and the avirulence gene *AVR2-YAMO* (M. J. Orbach, L. Farrall, F. G. Chumley and B. Valent, unpublished results), have been cloned based on their map positions. Extensive crosses involving the weeping lovegrass pathogen 4091-5-8 and the rice pathogen O-135 identified interesting genes controlling pathogenicity and host specificity (Valent et al. 1991). We have demonstrated that strains 6043 and 4224-7-8 differ by a translocation involving chromosomes 1 and 2c from 6043, and strains 4091-5-8 and O-135 differ by a translocation involving chromosomes 2b and 2c. Thus, the presence of a single translocation in each cross can account for most of the decreased viability of ascospores. Further genetic analysis will be facilitated by selection of laboratory strains with the same genome structure.

The chromosomes of *M. grisea* can be grouped into two general types: those larger than 2,000 kilobases (kb) that segregate in a Mendelian manner and carry known genes of the fungus, and smaller, apparently dispensable chromosomes ranging in size from 500 kb to 2,000 kb. Approximately half of the strains analyzed in this study contained mini-chromosomes less than 2 Mb in size, in addition to a full complement of larger chromosomes. Considerable variability was observed in the numbers and sizes of small chromosomes among the various *M. grisea* strains that contained them. Of the 14 rice pathogen field isolates examined from diverse geographic locations, all but one contained one to four of these small chromosomes. These small chromosomes may be related because most contain DNA sequences homologous to pCB715, which are found on the mini-chromosome of O-135, but not on the normal complement of chromosomes. The only rice pathogen field isolate we have analyzed that does not contain a mini-chromosome is the female fertile strain Guy11. Among 10 field isolates pathogenic on grasses other than rice, four contained small chromosomes. All four of these strains are female sterile, while five of the six strains lacking small chromosomes are female fertile.

Unlike field isolates, most strains derived through genetic crosses in the laboratory, such as 6043, 4091-5-8, 4136-4-3, and 4375-R-6, lack small chromosomes. This may be because mini-chromosomes are poorly transmitted through meiosis. For example, among progeny from a cross between O-135

and 4091-5-8, only 22% (6/27) of random progeny tested received the O-135 mini-chromosome, suggesting that it is inherited in a non-Mendelian manner. Even though O-135 was used as the recurrent parent in a backcrossing scheme (Valent et al. 1991), its mini-chromosome did not appear in many of the backcross progeny. In addition, in an analysis of two complete tetrads (Fig. 5), none of the progeny inherited the mini-chromosome. Those strains lacking the mini-chromosome are perfectly viable and most are good pathogens. We thus propose that the mini-chromosomes are dispensable, "B"-type chromosomes (Jones and Rees 1982). They fit the criteria that distinguish B chromosomes in a number of ways. They are smaller than the standard chromosomes, they are nonessential for growth, they are inherited in a non-Mendelian manner, and they contain sequences not present on the larger chromosomes.

B chromosomes are found in many plant and animal species (Jones and Rees 1982) and have recently been reported in fungi (Miao et al. 1991; Tzeng et al. 1992). Most B chromosomes contain no genes of known function. The B chromosome in *N. haematococca* that carries a gene for detoxification of the phytoalexin, pisatin, is an exception. No functional genes have been defined in the *M. grisea* B chromosomes, although we do note a correlation between the presence of B chromosomes and lack of female fertility. Jones and Rees (1982) observe that fertility problems associated with B chromosomes are common, with higher numbers of B chromosomes correlated with decreasing fertility. The lack of accumulation of the mini-chromosome in the O-135 × 4091-5-8 backcross breeding line may be due not only to its low heritability but also to the criteria used to select the parent to cross with O-135 for each generation. These strains were selected for female fertility and none of them contain the mini-chromosome. Fertility was also tested for some of the progeny that were analyzed from the cross between O-135 and 4091-5-8. All six strains that contained the mini-chromosome were female sterile while among those tested that lacked the chromosome, 11 of 15 were female fertile. Thus, although the B chromosome is correlated with female sterility, its absence does not guarantee female fertility. Female fertility is a complex trait in fungi and would be expected to be controlled by many genes. Our evidence suggests that one factor that may inhibit female fertility is the presence of the B chromosome.

The laboratory strain 4224-7-8 contains a chromosome in the size range of those suggested to be B chromosomes, and in fact, the 4224-7-8 small chromosome appears smaller in size than the one found in the rice pathogen O-135 (Fig. 6A). The strain 4224-7-8 at first appeared to be an exception to the rule that strains that contain mini-chromosomes are female sterile. However, the 4224-7-8 small chromosome has several properties that distinguish it from the other small chromosomes. The small chromosome of strain 4224-7-8 was inherited from its female parent, 4157-1-1. In the 4224-7 tetrad, this small chromosome segregates 1:1 and in coupling with the very largest chromosome. The 4224-7-8 small chromosome has no homology to the O-135 mini-chromosome specific probe pCB715. And finally, sequences homologous to the cosmid clone A12G1, which behaves as a normal Mendelian marker in the RFLP map and which hybridizes to the normal complement of chromosomes in all other strains tested, hybridize to this smallest chromosomal band in 4224-

7-8. Thus, we believe that the small chromosome in 4224-7-8 is an "essential" A-type chromosome that has resulted from a translocation, and not a dispensable B-like chromosome.

Genetic analysis of genes responsible for pathotype structure among field isolates of the rice blast pathogen is still hampered by the low level of fertility of most rice pathogens from the field. Karyotyping is a valuable tool to guide further attempts to improve genetic analysis of the rice blast fungus.

MATERIALS AND METHODS

Strains.

The strains used in this study are listed in Table 1, and are available in the laboratory of B. Valent at DuPont. Strain designations are as follows: strains named O- are field isolates that infect rice and strains named T- are field isolates that infect wheat. Field isolates that infect grasses other than rice or wheat are named G-. Strains previously published under another name are indicated by that name. The rice pathogen Guy11 was generously provided by J. L. Notteghem (CIRAD/CA, Montpellier, France) and the laboratory strain 6043 was generously provided by H. Leung (Washington State University, Pullman).

Preparation of fungal chromosome DNAs.

Mycelial cultures of *M. grisea* were grown in 100 ml of liquid medium containing 1% glucose, 0.3% yeast extract, and 0.3% enzymatic casein hydrolysate. Inoculation was achieved by placing about 25 cm² of mycelia from a petri dish culture in a Waring blender and fragmenting it with two 20-s pulses. The cultures were grown for 2 days by shaking (200 rpm) at room temperature. Mycelia were harvested by vacuum filtration, weighed, and resuspended in 10 ml of 1 M sorbitol per gram of mycelia, wet weight. To generate protoplasts, Novozym 234 was added at 2 ml of a 20 mg/ml solution per 3 g of wet weight mycelia and the cultures were shaken (100 rpm) for 90 min at room temperature. Protoplasts were purified by filtering successively through cheesecloth and Nytex nylon mesh that has a 25 micron pore size. The protoplasts were pelleted at 2,000 × g for 10 min. The protoplasts were washed by resuspension in 1 M sorbitol and then pelleted again. After two washes, the protoplasts were counted using a hemacytometer and repelleted. Protoplasts were finally resuspended in 1 M sorbitol at 3.3 × 10⁹/ml. To embed the protoplasts in agarose, they were mixed with molten 1.0% low melting-temperature agarose in 1 M sorbitol, 50 mM EDTA at a ratio of 1 volume protoplasts to 2 volumes of agarose solution, and pipetted into a Teflon mold to solidify into an agarose/protoplast plug. These plugs were treated with proteinase K in NDS buffer as described previously (Orbach et al. 1988). After lysis of the protoplasts, the chromosome DNA/agarose plugs were washed with 50 mM EDTA 3 times for 30 min at 50°C and then stored in TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) at 4°C.

Neurospora crassa, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* chromosome DNA preparations were prepared as described previously (Orbach et al. 1988).

Electrophoresis and hybridization of chromosome DNAs.

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was performed using units constructed by

DuPont shops based on plans provided by Chu et al. (1986) or using a Bio-Rad CHEF-DRII unit. All gels were 0.7% agarose gels (BRL UltraPure LE) run in 0.5× TBE buffer at 9°C as described (Orbach et al. 1988) unless otherwise specified. Electrophoretic conditions for each gel mentioned in this report are described in the figure legends.

After electrophoresis, chromosome DNAs were transferred to Hybond N (Amersham) hybridization membrane as described by Vollrath et al. (1988), except that DNA fragmentation prior to denaturation was by acid depurination (two treatments, 10 min each, in 0.25 M HCl) instead of UV irradiation. Hybridizations were performed as described (Orbach et al. 1988) using probes prepared by the random hexamer priming method of Feinberg and Vogelstein (1983). After autoradiography, probes were removed from the membranes by incubation in 0.4 M sodium hydroxide for 30 min at 45°C. The membranes were then rinsed in 0.2× SSPE, 0.1% SDS, 0.2 M Tris-Cl (pH 7.5) for 30 min at 45°C and rehybridized with different probes.

DNA probes for hybridization.

Several *M. grisea* genes of known function were used as hybridization probes. These were beta-tubulin (*TUB1* carried on plasmid pCB635), acetolactate synthase (*ILV1* on pCB573), scytalone dehydratase (*RSY1* on pCB640), cutinase (*CUT1* on pCB606) (Sweigard et al. 1992), a lysine biosynthetic gene (*LYS1* on cosmid CP866E1) and rDNA (on *N. crassa* pSV50 cosmid 5:3E). Cosmid clones cos125, cos156, cos193 (cosegregating with cos72 in the published map), cos243, cos245, and A12G1 identify RFLPs segregating in the map produced by Sweigard et al. (1993). Plasmid pCB715 contains a fragment from the O-135 mini-chromosome that was cloned following extraction of the chromosome from a preparative CHEF gel. Mini-chromosome DNA from O-135 was resolved on a 1.0% low melting point agarose gel (BRL UltraPure) and cut from the gel with a scalpel. Gel slices were washed 4 times in TE for 30 min at 4°C and then equilibrated in 3 changes of 1× BRL React #3 buffer for 30 min at 4°C. Gel slices were then melted at 65°C, cooled to 37°C and digested with *EcoRI*. Fragments were cloned into *EcoRI*-digested pUC118.

Assessing the fertility of *M. grisea* crosses.

Crosses were performed on oatmeal agar plates (Valent et al. 1991), and results were confirmed by at least one additional round of testing. Viability of ascospores was determined as follows: In a fertile *M. grisea* cross, mature asci exude directly through the perithecial neck and immediately release their ascospores. These ascospores accumulate in a viscous liquid drop on the perithecial neck and, for a short period after release, they can be collected using a small inoculating loop and 0.25% gelatin solution (Sigma, No. G-0510). Collected ascospores were rinsed into a 50- to 200-μl droplet of gelatin solution on a 2 YEG agar plate, and the drop was spread with a small glass spreader to separate individual ascospores. The percentage of viable ascospores was determined 24 h later using a stereomicroscope at 80× magnification. Ascospore germination determined in this manner was confirmed by releasing intact asci from the bulbs of maturing perithecia and spreading them onto the surface of an agar plate. These asci were examined microscopically to determine

if some asci had missing or badly deformed ascospores that would be missed in the procedure described above. Germination of the ascospores within these asci was checked 24 h after release from the perithecia. Viable ascospore counts were taken over a period between 13 and 20 days after a cross was initiated.

ACKNOWLEDGMENTS

We are grateful to all individuals listed in Table 1 for sharing fungal strains. We are also grateful to David Burke (Department of Human Genetics, University of Michigan) for sharing unpublished data on the sizes of the chromosomal DNAs in *S. cerevisiae* strain AB1380. We thank James A. Sweigard (DuPont, Wilmington, DE) and Deanna Funnell-Baerg (Department of Plant Pathology, University of Arizona) for a critical reading of the manuscript.

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