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Molecular Evidence for Chromosome Transfer Between Biotypes of *Colletotrichum gloeosporioides*

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Two genetically distinct biotypes (A and B) of *Colletotrichum gloeosporioides* that cause different anthracnose diseases on the legumes of *Stylosanthes* spp. have been identified in Australia. All virulent isolates of these biotypes are anamorphic. Laboratory pairings of nitrate reductase (*nit*) mutants representing the two biotypes indicated that biotypes A and B form separate vegetative compatibility groups (VCGs). We have investigated the mechanisms leading to a major karyotype polymorphism in this fungus. All biotype A isolates studied to date carry a 2-Mb chromosome while some biotype B isolates carry an apparently dispensable but homologous 1.2-Mb chromosome. A biotype B-like field isolate termed Bx was recently obtained from *S. guianensis* in Northern Australia. The Bx isolate was shown to carry two homologous chromosomes, 1.2 Mb and 2 Mb in size. These two chromosomes lacked two repeat sequences that were present on other chromosomes in biotype B and these repeats appeared to be monomorphic in biotype B and Bx. This suggests the 1.2-Mb and 2-Mb chromosomes may be recent additions to the Bx genome. Analysis of the DNA sequence organization of the 2-Mb and 1.2-Mb chromosomes in Bx by means of chromosome-specific DNA markers indicated that these chromosomes were each identical to the 2-Mb and 1.2-Mb sized homologous chromosomes in biotype A and some biotype B isolates, respectively. Extensive analysis of the rest of the genome of Bx by restriction fragment length polymorphisms, random amplified polymorphic DNAs, and minichromosome profiling indicated that the background genome of Bx was like that of biotype B. No other biotype A-like markers outside of the 2-Mb chromosome were identified in Bx. Pairing of *nit* mutants indicated that Bx was part of the biotype B VCG. The results therefore indicate that a dispensable 2-Mb chromosome in the biotype B isolate Bx most probably originated by a relatively recent "horizontal" transfer from biotype A. The results suggest that the occasional transfer of specific chromosomes may occur between apparently genetically isolated clonal lines of asexual pathogens.

Additional keywords: genetic variation, horizontal gene transfer, karyotype polymorphisms.

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Many important fungal phytopathogens appear to lack a sexual cycle. Intraspecific genetic recombination in these asexual fungi can potentially still occur by means of heterokaryosis and the parasexual cycle (Glass and Kuldau 1992; Leslie 1993). However, in many instances either mycelial or vegetative incompatibility has developed to restrict the extent of parasexual genetic recombination that can occur within fungal species. Because of these constraints on genetic recombination, asexual pathogen species often exist as genetically distinct clonal groups or lineages (McDonald and McDermott 1993; Shull and Hamer 1995). Where there is no apparent capability for genetic recombination by either sexual or parasexual means between clonal lineages then one would expect the pathogen to be very limited in its ability for environmental or pathogenic adaptation. However, in the field, many asexual fungal pathogens appear to be able to rapidly adapt in virulence to new, resistant, host genotypes. Obviously, the mechanisms generating genetic variation in asexual fungal pathogens are of importance to molecular plant-pathogen interactions. Several processes, in addition to those of the parasexual cycle, have been proposed as mechanisms generating genetic variation in asexual fungal pathogens and these include the activity of transposable elements, mitotic reciprocal translocations, and the exchange of cytoplasmic genetic elements (Kistler and Miao 1992). An unusual feature of many asexual fungal pathogens is that they demonstrate variable karyotypes (Kistler and Miao 1992). This variation in karyotype can occur even among closely related and supposedly clonal descendants (Masel et al. 1990; Talbot et al. 1993). Our work (Masel et al. 1993a) has involved a study of the mechanisms determining karyotype variation in the asexual fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. infecting the tropical legumes (*Stylosanthes* spp.).

In northern Australia, legumes (*Stylosanthes* spp.) are used extensively in improved pastures for cattle production. Anthracnose disease, caused by *Colletotrichum gloeosporioides*, has been an important constraint on the expansion of *Stylosanthes* in Australia. Two types (A and B) of anthracnose disease have been recognized on *Stylosanthes* in Australia (Irwin and Cameron 1978) and these appear to be caused by two fungal biotypes (Manners et al. 1992; Maclean et al. 1993). Biotype A infects most species of *Stylosanthes*, while biotype B infects *S. guianensis* (Aubl.) Sw. only. Sexuality has not been observed either within or between virulent isolates of the two biotypes (Ogle et al. 1986). The A and B biotypes appear to be genetically distinct on the basis of morphology,

symptomatology, double-stranded RNA, restriction fragment length polymorphisms (RFLP), and molecular karyotypes (reviewed in Manners et al. 1992). Although considerable genetic differences can be detected between biotypes using neutral markers such as both arbitrarily chosen RFLP probes or random amplified polymorphic DNA (RAPD) primers, there is near-monomorphy within each biotype (Braithwaite et al. 1989, 1990; Manners et al. 1993). These data have indicated that biotype A and biotype B represent genetically isolated clonal lineages of *C. gloeosporioides* (Manners et al. 1992).

Although DNA fingerprinting has indicated that isolates within each biotype are genetically very similar and probably clonal descendants, karyotype analysis has revealed considerable variation in the size and number of chromosomes of different isolates within each biotype (Masel et al. 1990). To begin to understand how this variation in karyotype may arise in a clonal organism we have investigated the origin of one polymorphic 1.2-Mb chromosome in biotype B (Masel et al. 1993a, 1993b). Ten independent DNA probes specific to this chromosome were identified in one strain but hybridization analysis by means of these probes demonstrated that all probe sequences or any homologues were completely absent from other biotype B isolates (Masel et al. 1993a). These results suggested that the variable occurrence of the chromosome was due to either deletion from progenitor biotype B strains or addition from an unknown source to only some biotype B strains (Masel et al. 1993a). The addition hypothesis was previously favored because the 1.2-Mb chromosome lacked repetitive DNA, which was present on all other resolvable chromosomes and which was monomorphic between all biotype B isolates regardless of whether they carried or lacked the 1.2-Mb chromosome. The origin of the 1.2-Mb chromosome has been further investigated (Masel et al. 1993a; He et al. 1995). A homologous 2-Mb chromosome was found in all isolates of biotype A that have been studied. However, it seemed unlikely that biotype A was a recent donor of the 1.2-Mb chromosome, as RFLP analysis indicated that the 1.2-Mb and 2-Mb chromosomes were quite dissimilar.

Recently, we collected new biotype B isolates from Northern Australia to investigate the possible correlation of the presence of the 1.2-Mb chromosome with specificity toward *S. guianensis* cv. Graham (He et al. 1995). Although this correlation with race specificity was not substantiated, we did identify two new isolates that carried two chromosomes homologous to the previously identified 1.2-Mb and 2-Mb chromosomes. In this current study we have compared the chromosomes in these new isolates to those previously characterized in biotypes A and B. The results provide compelling evidence that the 2-Mb biotype A-like chromosome is present in an essentially biotype B-like genetic background. We present evidence that biotypes A and B represent distinct vegetative compatibility groups (VCGs) and are apparently incapable of heterokaryosis in the laboratory. The results thus suggest that a form of "horizontal" transfer of specific chromosomes between biotypes occurs in nature and may play an important role in the generation of genetic variation in this asexual pathogen.

RESULTS

Bx isolate carries 2-Mb and 1. 2-Mb homologous chromosomes.

Two isolates (UQ395 and UQ396) were obtained in 1992 from different plants in an experimental plot of *S. guianensis* cv. Graham grown in North Queensland (He et al. 1995). Preliminary molecular analysis of these two isolates indicated that they were almost identical. The isolate UQ396 was studied in more detail and in this paper we will only present data for this isolate. The code Bx will be used to describe isolate UQ396. Previously well-characterized isolates were used to represent biotypes A and B (Braithwaite et al. 1990; Masel et al. 1990, 1993a, 1993b; He et al. 1995). Isolate A1 was used to represent biotype A. Isolates B1, B3, and B4 represented isolates of biotype B that either contain (B3) or lack (B1, B4) the 1.2-Mb chromosome. As reported previously (He et al. 1995), Bx had two chromosomes that hybridized to chromo-

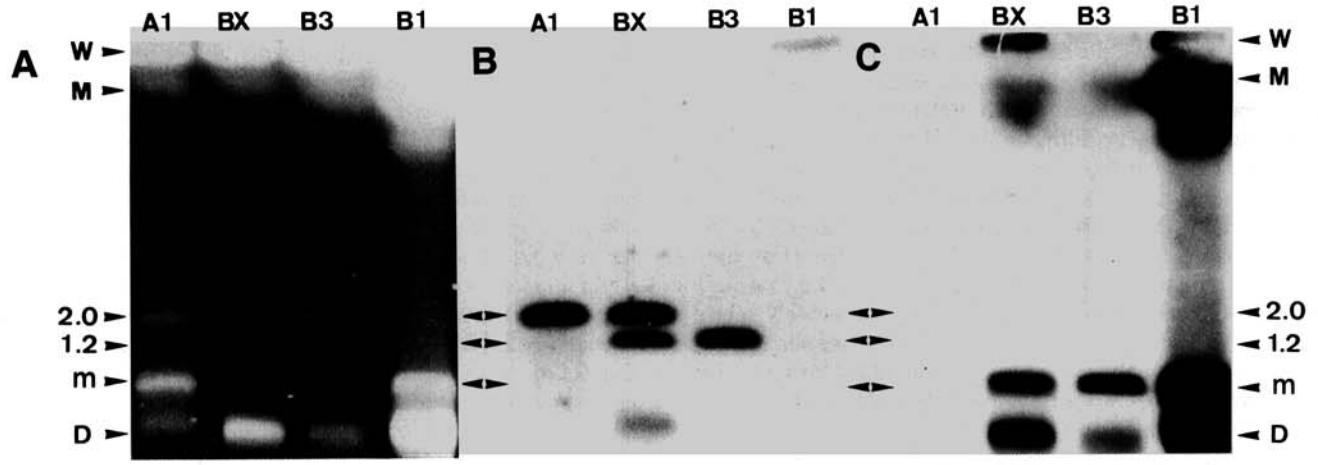

Fig. 1. A, An ethidium bromide–stained CHEF gel of chromosomes of biotype A (A1) containing the 2-Mb chromosome, biotype B isolates either containing (B3) or lacking (B1) the dispensable 1.2-Mb chromosome, and Bx carrying both 1.2-Mb and 2-Mb chromosomes. The positions of the loading wells (W), the region of large nonresolved chromosomes (M), the 2-Mb and 1.2-Mb chromosomes, the smaller nonresolved 200 to 600 kb chromosomes (m), and degraded DNA (D) are indicated. **B,** Southern blot analysis of the gel shown in **A** after hybridization to one of the clones previously isolated from the 1.2-Mb chromosome (Masel et al. 1993a). This clearly reveals the presence of the A1-like 2-Mb and B3-like 1.2-Mb homologous chromosomes in the Bx isolate. **C,** Southern blot analysis of the gel shown in **A** after hybridization to a dispersed repeat DNA probe (pCHB1) that is specific to biotype B and is present on the nonresolved large (M) and small chromosomes (m) but is absent from the 1.2-Mb and 2-Mb chromosomes.

some-specific DNA probes (Masel et al. 1993a) obtained from the 1.2-Mb dispensable chromosome of the biotype B3 isolate (Fig. 1). We compared the size of these 2 Bx chromosomes to the 1.2-Mb chromosome of B3 and the 2-Mb chromosome of A1 (Fig. 1). The chromosome-specific DNA probes hybridized to a 2-Mb and a 1.2-Mb chromosome in the Bx isolate that were identical in size to the 2-Mb A-like and 1.2-Mb B-like chromosomes observed in A1 and B3, respectively (Fig. 1). No hybridization was observed to isolates B1 and B4 (not shown) that lack these chromosomes.

2-Mb and 1.2-Mb chromosomes in Bx are similar to the corresponding chromosomes in biotypes A and B.

To investigate whether the 2-Mb and 1.2-Mb chromosomes in Bx were identical to the 2-Mb and 1.2-Mb chromosomes previously recognized in biotypes A and B, respectively, we initially used RFLP analysis with the chromosome-specific DNA probes on total DNA isolated from biotype A (isolate A1), biotype B isolates that either carried (isolate B3) or lacked the 1.2-Mb chromosome (isolates B1 and B4), and Bx (Fig. 2). The DNA was digested with four restriction enzymes and probed with 10 chromosome-specific DNA probes derived from the 1.2-Mb chromosome (Masel et al. 1993a). These experiments showed that, for each DNA probe and restriction enzyme used, the RFLP banding pattern in lanes of Bx DNA matched the sum of the bands detected in the lanes loaded with DNA from A1 and B3. The chromosome-specific DNA probes revealed no bands that were unique to Bx and no hybridization was noted to biotype B isolates B1 and B4 that lacked the 1.2-Mb chromosome. These results indicate that Bx carries the 2-Mb and 1.2-Mb chromosomal sequences of biotype A and B, respectively.

The RFLP analysis described above suggests that the 2-Mb and 1.2-Mb chromosomes observed in Bx are the same as the homologous and similarly sized counterparts in A1 and B3. To demonstrate this more directly we purified DNA of the 1.2-Mb and 2-Mb chromosomes from pulsed-field gels of Bx and B3 and A1 isolates for direct comparison. We were unable to obtain sufficient unsheared chromosomal DNA for RFLP analysis but were able to undertake a polymerase chain reaction–based analysis to compare the chromosomes. The chromosomal DNA was amplified using small arbitrary primers according to the DNA amplification fingerprinting (DAF) procedure (Caetano-Anolles et al. 1991). The amplification products were separated on agarose. To avoid bands that resulted from amplification of contaminating DNA of larger chromosomes smearing through the CHEF (contour-clamped homogenous electric field) gel, we probed Southern blots of the gels of DAF products with a cosmid clone (Cos60; He et al. 1995) that is specific to the 1.2-Mb and 2-Mb chromosomes (Fig. 3). Four experiments with distinct arbitrary primers were used for these experiments but only two primers revealed a clear polymorphism that distinguished the 1.2-Mb and 2-Mb chromosomes of the B3 and A1 strains, respectively (Fig. 3). Comparison with purified chromosomes of Bx indicated that the banding pattern of the 2-Mb chromosome of Bx was identical to that of the 2-Mb chromosome of A1 while the banding pattern of the 1.2-Mb chromosome of Bx was identical to that of the 1.2-Mb chromosome of B3. Thus, these chromosome-specific markers provide direct evidence that Bx contains the 1.2-Mb and 2-Mb chromosomes of biotypes B and A, respectively.

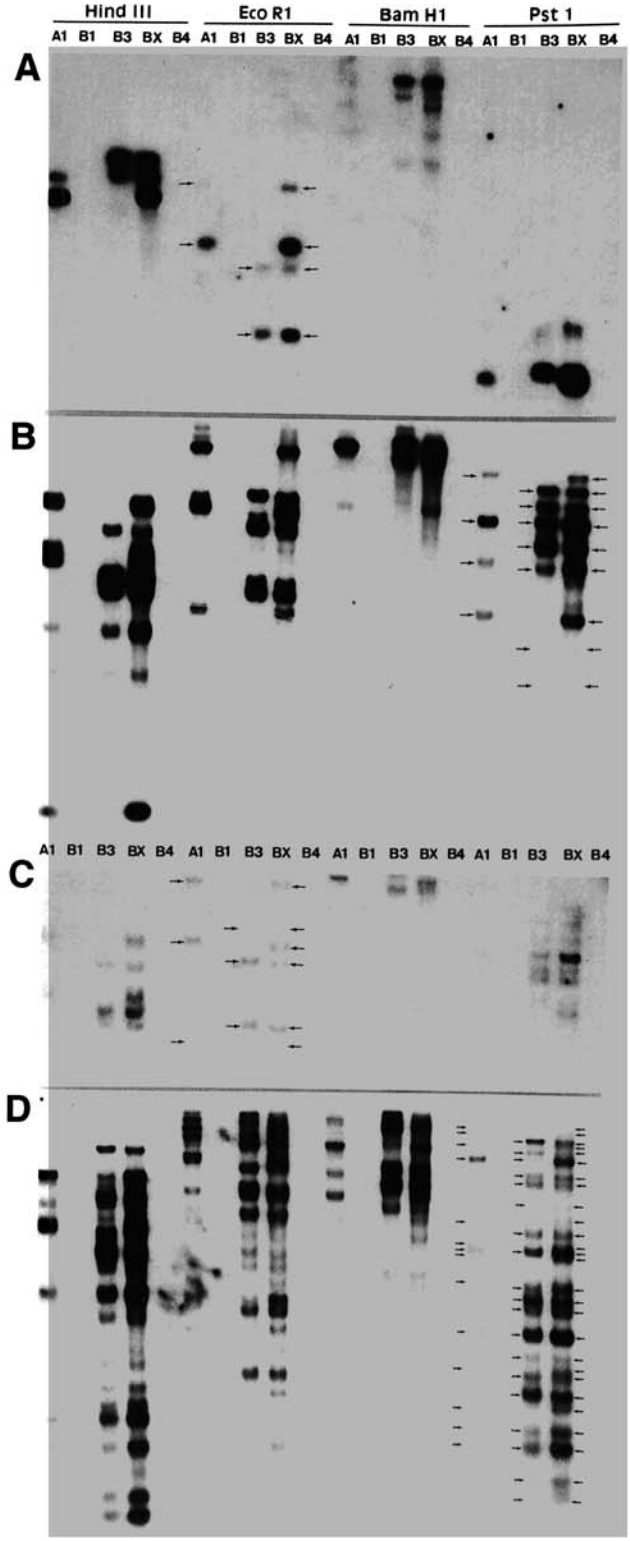

Fig. 2. Restriction fragment length polymorphism (RFLP) analysis of *Hin*dIII, *Eco*RI, *Bam*HI, and *Pst*I restriction digests of total DNA from isolate A1, which contains the 2-Mb chromosome, isolate B3, which contains the homologous 1.2-Mb chromosome, isolate Bx, which contains both of these homologous chromosomes, and isolates B1 and B4, which lack these homologous chromosomes. **A–C,** Results obtained with three distinct DNA probes isolated from the 1.2-Mb chromosome (Masel et al. 1993a). **D,** Results obtained with cosmid clone Cos60 also isolated from the 1.2-Mb chromosome (He et al. 1995). The RFLP banding patterns in the Bx lanes match the sum of those in the A1 and B3 lanes.

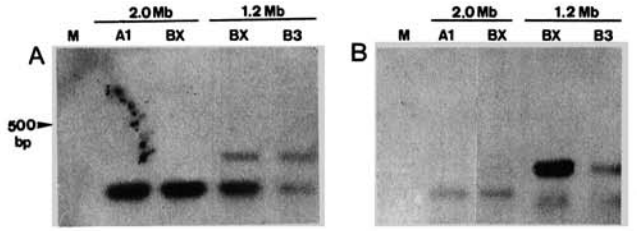

Fig. 3. A comparison of the 2-Mb chromosomes in A1 and Bx and the 1.2-Mb chromosomes in B3 and Bx by means of an adapted chromosome-specific DNA amplification fingerprinting procedure. The respective chromosomes were purified by pulsed-field electrophoresis as shown in Figure 1 and the chromosomal DNA amplified with the single arbitrary primers 5′-GGCTGCTG-3′ (**A**) and 5′-CCTGGTGG-3′ (**B**). The amplification products were separated on a 2% agarose gel, blotted onto nylon, and hybridized to Cos60, a cosmid clone isolated from the 1.2-Mb chromosome. Results with these markers indicate that the 2-Mb and 1.2-Mb chromosomes of Bx are identical to the similarly sized chromosomes in A1 and B3, respectively. M lane contains *Hin*dIII cut λ standards.

The background genome of Bx is predominantly like that of biotype B.

To assess whether the Bx strain was a dihaploid hybrid, heterokaryon, or partial recombinant between biotype A and B, a wide range of neutral markers not associated with the 1.2-Mb or 2-Mb chromosomes were used to compare the rest of the genome of Bx with the genomes of typical biotype A and biotype B isolates.

Ribosomal DNA. First, we analyzed the ribosomal rDNA genes that are carried on a large nonresolved chromosome >10 Mb in size (Masel et al. 1990). We have previously shown that the ribosomal DNA of biotypes A and B differ in a *Xba*I-generated RFLP (Braithwaite et al. 1990). When an rDNA probe from *Aspergillus nidulans* was used to detect this RFLP, the Bx strain was similar to biotype B and distinct from biotype A (Fig. 4).

Low-copy RFLPs. In the next experiment, we identified 10 distinct cDNA clones from biotype B that detected an RFLP

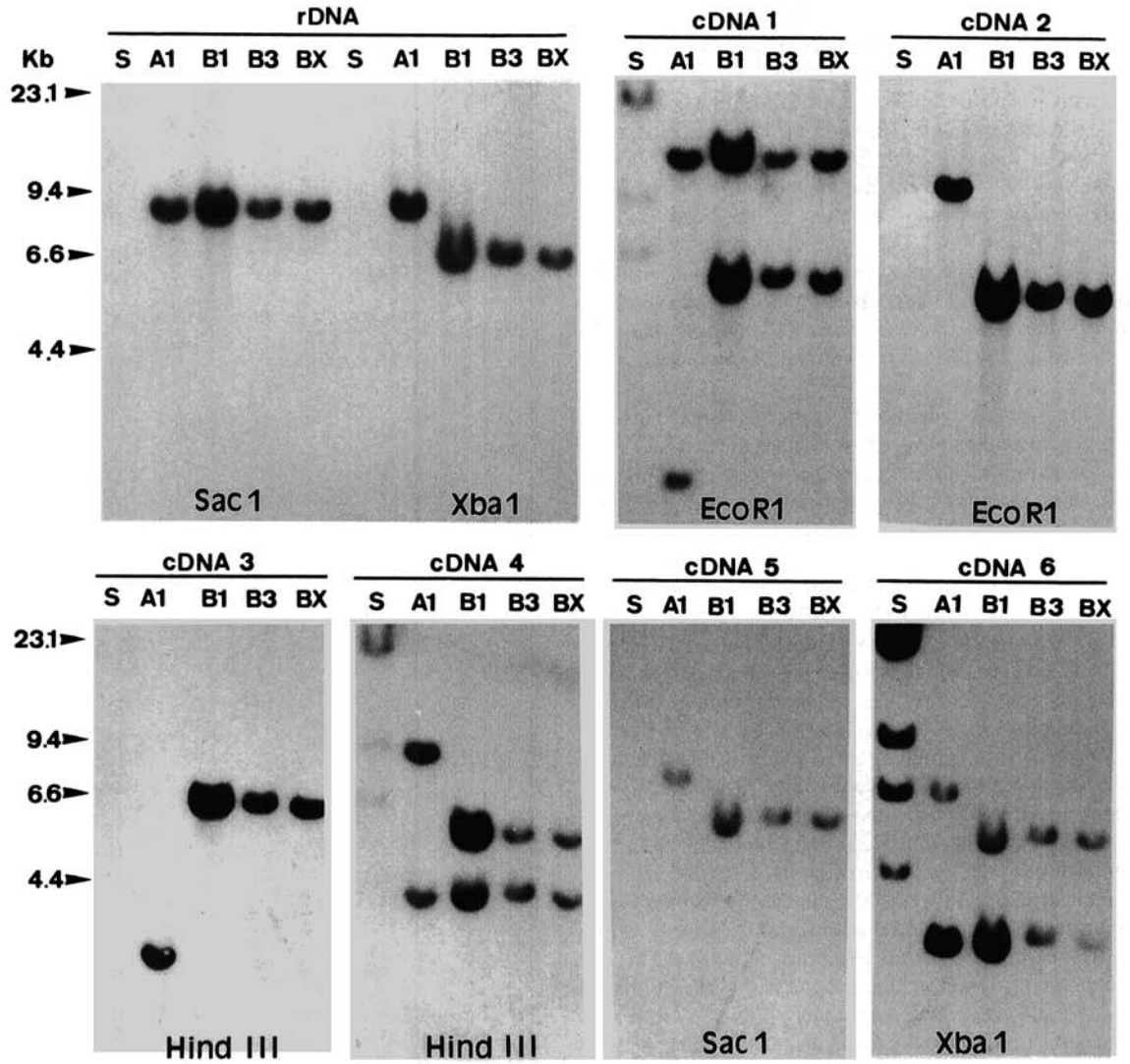

Fig. 4. Restriction fragment length polymorphism (RFLP) analysis of total DNA from isolates A1, B1, B3, and Bx with DNA probes that are not located on either the 1.2-Mb or 2-Mb chromosomes. The hybridization probes used include the clone pAR1 containing the rDNA repeat unit of *Aspergillus nidulans* (Lockington et al. 1982), which reveals an RFLP with the enzyme *Xba*I, and cDNA clones 1 to 6, which are examples of 10 distinct cDNAs that were arbitrarily selected from a cDNA library from the B3 isolate and that revealed an RFLP between biotypes A and B using one of either *Sac*I, *Eco*RI, *Hin*dIII, or *Xba*I enzymes as shown. Each of the rDNA and cDNA clones hybridized to the unresolved chromosomes at position M on the pulsed-field gel shown in Figure 1. These DNA probes show that Bx only gives a B-like RFLP pattern. A similar result was obtained with the other four cDNA clones. Lane S refers to the *Hin*dIII cut λ standards shown in kilobases.

between biotypes A and B. These cDNA clones were not located on the 2-Mb or 1.2-Mb chromosomes but were located on the larger and poorly resolved chromosomes of *C. gloeosporioides* (Masel et al. 1990). Examples of the results obtained are shown in Figure 4. In each of the 10 cases these low-copy RFLP probes indicated a biotype B genotype for Bx and no biotype A-like alleles were found in Bx by means of these DNA probes.

Repetitive DNA probes. To provide a broader comparison of the genome outside of the 1.2-Mb and 2-Mb chromosomes in biotype B isolates and Bx we also used two dispersed repeat sequence DNA probes, pKB2 (Braithwaite et al. 1990) and pCHB1 (C. He, unpublished). The pKB2 sequence is dispersely repeated on both the large chromosomes (>10 Mb) and minichromosomes (200 to 600 kb) in biotype B (Masel et al. 1990) and is not present on the 1.2-Mb chromosome (Masel et al. 1993a). In biotype A, however, pKB2 sequences are only located on the large (>10 kb) unresolved chromosomes (Masel et al. 1990). In Bx the pKB2 sequence is distributed as for biotype B and is also not detectable on the 1.2-Mb and 2-Mb chromosomes (data not shown). The clone pCHB1 carries a part of the retroelement *Cg*T1 (C. He, unpublished) that is present in biotype B and absent in biotype A. Hybridization analysis has shown that the pCHB1 repeat sequences are present on the large unresolved chromosomes of the biotype B and the Bx isolates as well as the smaller (<600 kb) minichromosomes and are absent from both the 2-Mb and the 1.2-Mb chromosomes (cf. Fig. 1C). Both of these repetitive elements have been shown to be monomorphic within biotype B (Braithwaite et al. 1990; C. He, unpublished). Hybridization of these two repeat DNA probes to Southern blots of restriction enzyme–digested DNA for biotypes A and B isolates and Bx revealed biotype B-like patterns of hybridization in the Bx strain (Fig. 5).

RAPD analysis. The genomes of Bx and the other isolates representing the two biotypes were scanned using RAPD markers. For this analysis a total of 18 arbitrary primers were used that revealed 126 bands and 83% dissimilarity between biotypes A and B. For 17 of the primers tested the Bx strain was identical in banding pattern to that of the biotype B strains (e.g., Fig. 6A). One primer amplified a 1.7-kb band from the DNA of Bx that was absent in assays of all biotype B isolates but was strongly amplified from DNA of the biotype A isolate (Fig. 6B). However, when this band was cut from the gel and hybridized to a Southern blot of a CHEF gel of biotype A and Bx it hybridized to the 2-Mb chromosome, suggesting that it did not represent a DNA sequence from another chromosome of biotype A in Bx.

Minichromosome profiles. We have shown previously that biotypes A and B can be readily distinguished by pulsed-field gel electrophoresis of minichromosomes in the size range 200 to 600 kb (Masel et al. 1990, 1993b). The Bx strain contained only 4 minichromosomes (data not shown), which is characteristic of biotype B isolates, which carry 2 to 5 minichromosomes and unlike biotype A isolates, which usually contain 8 to 10 minichromosomes (Masel et al. 1990, 1993b).

In summary, an extensive range of neutral markers that were absent from the 1.2-Mb and 2-Mb chromosomes indicated that the background genome of Bx is like that of biotype B. This indicates that Bx is not a heterokaryon or dihaploid hybrid but represents a partial recombinant carrying a biotype

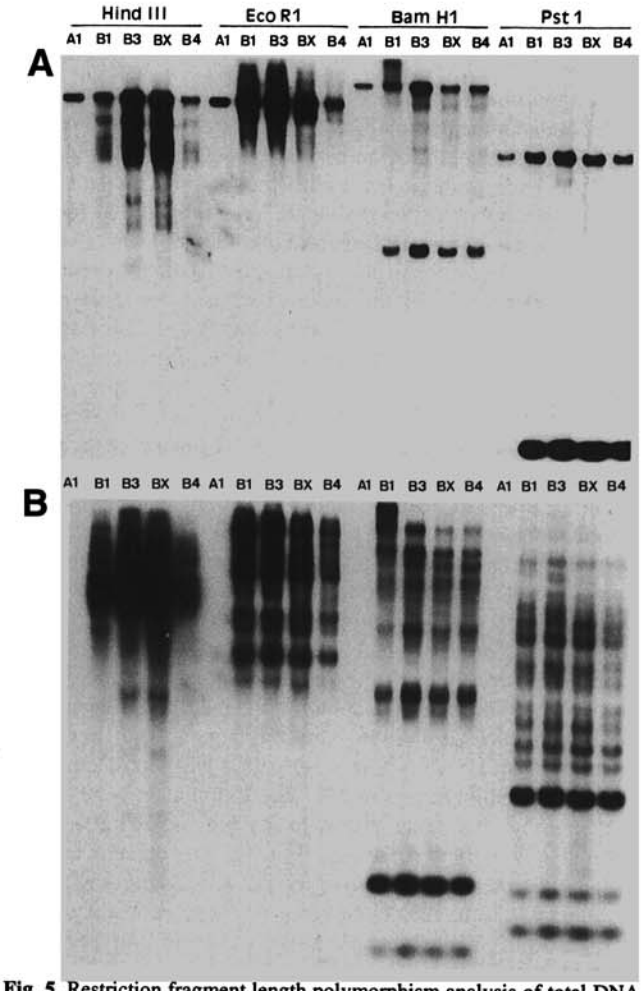

Fig. 5. Restriction fragment length polymorphism analysis of total DNA from A1, B1, B3, B4, and Bx using dispersed repeat clones pKB2 (**A,** Braithwaite et al. 1990) and pCHB1 (**B**) as hybridization probes. These DNA probes do not hybridize to either the 1.2-Mb or 2-Mb chromosomes (Masel et al. 1993a; Fig.1C). The same filters used in Figure 2 were reprobed with pKB2 and pCHB1. The Bx lane is like that of the other biotype B isolates.

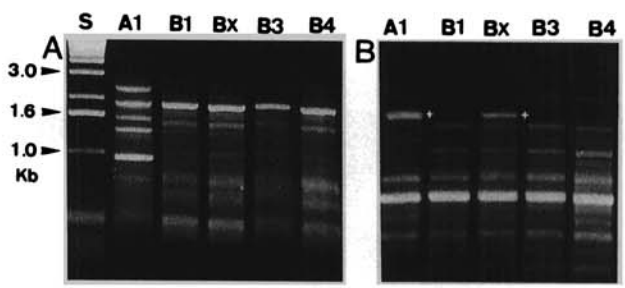

Fig. 6. Examples of random amplified polymorphic DNA (RAPD) analysis of total DNA from isolates A1, B1, B3, B4, and Bx. **A,** Result obtained with primer sequence 5′-GGAAGCCAAC-3′ where RAPD patterns of B1, B3, B4, and Bx are similar while that of A1 is highly divergent. This was typical of results obtained with 17 other arbitrarily chosen primers. **B,** Result obtained with primer sequence 5′-GTCCAT GCAG-3′. This primer amplified a band (+) present in A1 and Bx but absent in other biotype B isolates while all other bands in Bx resemble the biotype B profile. The RAPD band (+) was excised and shown to hybridize to the 2-Mb chromosome of Bx and A1 in blots of CHEF gels such as Figure 1. Lane S refers to the size markers, which are indicated in kilobases.

A-like 2-Mb chromosome in a predominantly biotype B-like genetic background.

Bx is a member of the biotype B VCG.

Previous molecular analysis has indicated that there is little or no genetic recombination between biotype A and B isolates of *C. gloeosporioides* in Australia. To directly test whether the biotypes were vegetatively compatible and capable of forming heterokaryons and possibly parasexual recombinants, we employed a method of pairing complementary auxotrophic mutants. Nitrate non-utilizing mutants of isolates representing each of 4 races of biotypes A and biotype B were generated by selection on chlorate. The mutants were grown on specific indicator media (Correll et al. 1987) and tentatively classified into mutation complementation groups representing structural nitrate reductase gene mutations (*nit1*), regulatory gene mutations (*nit3*) and molybdenum-containing cofactor mutations (*nitM*). Pairing experiments on minimal media showed that complementary mutants of either the same isolate or different isolates and races from the same biotype would produce profuse growth at the colony interface, suggesting the presence of heterokaryons and vegetative compatibility. In contrast, no effect was observed when mutants from different biotypes were paired even though they were in different complementation groups. These results demonstrate that the biotypes form two distinct VCGs. Nitrate non-utilizing mutants were also prepared from Bx and these were paired with complementary mutants from biotypes A and B. The Bx mutants were able to form putative heterokaryons with the biotype B mutants but not with any biotype A mutants (Fig. 7). These results indicate that Bx is a member of the biotype B VCG. According to current theories of the molecular basis of vegetative compatibility in other fungi this suggests that Bx and other biotype B isolates share alleles at several matching *het* loci (Glass and Kuldau 1992).

Pathogenicity of Bx.

Biotype B isolates cause disease specifically on *S. guianensis* while biotype A isolates cause limited lesions on a range of *Stylosanthes* spp. including the commercial species *S. scabra* (Irwin and Cameron 1978). Physiological races that differ on particular differential cultivars of *S. guianensis* and *S. scabra* have been defined within biotypes B and A, respectively (Irwin et al. 1986). In biotype B, all races are virulent on *S. guianensis* cv. Endeavour but races 2, 3, and 4 can be differentiated by their specific virulences on the *S. guianensis* genotypes, accession 18750, cv. Graham, and cv. Cook, respectively (Manners et al. 1993). Monosporic cultures of the Bx isolate caused type B disease on *S. guianensis* differential cvs. Endeavour, Graham (Fig. 8), and Cook, but not on *S. guianensis* accession 18750. This suggests that Bx represents a new race with broader virulence than the races described previously.

Because of the presence of the biotype A-like chromosome in Bx, pathogenicity assays were also undertaken to determine its virulence on the biotype A host *S. scabra.* Two independent inoculations of mature glasshouse-grown plants of *S. scabra* cvs. Fitzroy and Seca with the Bx isolates at conidial concentrations of 10^6 per ml, following standard inoculation procedures (Irwin et al. 1986), revealed no disease symptoms or any evidence of fungal growth and sporulation. Inoculation experiments were also undertaken using a recently described contained inoculation procedure (He et al. 1995). In this assay high humidity is maintained throughout the infection process in an enclosed container. In two out of three independent experiments, the Bx strains caused severe leaf chlorosis of the leaves of both *S. scabra* cvs. Fitzroy and Seca when inoculated at 10^7 conidia per ml (Fig. 8). Leaf chlorosis was not caused by the biotype B isolate B3 (UQ62; Fig. 8) on *S. scabra* in these experiments but biotype A isolate A3 (UQ14; Fig. 8) produced some chlorosis as well as lesions. In one of the three independent experiments, however, chlorosis was either weak or absent among replicates with Bx. When severe chlorosis was observed, microscopic analysis indicated that although appressoria had formed and some of these appeared to have penetration pegs there was no evidence of mycelial growth or sporulation either on or within the inoculated leaves. These results suggest that the Bx strains are not pathogenic or capable of completing their life cycle on *S. scabra* but may have a capacity to cause some deleterious physiological effects.

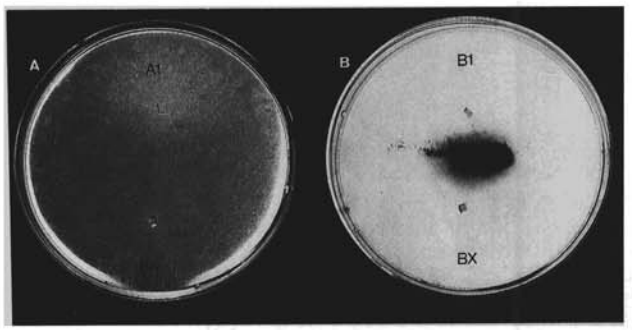

Fig. 7. Pairing of a nitrate reductase molybdenum-containing cofactor mutation (*nitM*) of the isolate Bx with nitrate reductase gene mutation (*nit1*), *nit1* mutants of isolates A1 (**A**) and B1 (**B**) on minimal media. The lush hyphal growth at the interface of B1 and Bx indicates complementation of the mutations via heterokaryosis while no indication of heterokayon formation occurs in the A1 and Bx pairing.

DISCUSSION

The laboratory analysis of vegetative compatibility of the two biotypes of *C. gloeosporioides* infecting *Stylosanthes* spp. in Australia that we have undertaken here indicated that these two pathogen groups are unable to undergo heterokaryosis. A lack of capacity to undergo either sexual or parasexual genetic recombination in these experiments is consistent with the previous molecular analysis that has shown large genetic differences between the biotypes and a high degree of monomorphy within the biotypes (Braithwaite et al. 1989, 1990; Manners et al. 1992). These results are consistent with the notion that biotypes A and B represent genetically isolated clonal lineages. However, the molecular analysis of the recently isolated Bx isolate reported herein provides compelling evidence that this isolate carries a biotype A-like 2-Mb chromosome in a predominantly biotype B-like genetic background. This is strong evidence that Bx represents a partial genetic recombinant between the biotypes and that a limited amount of genetic material and possibly only a specific chromosome of biotype A has been retained in this recombinant. These findings have important implications for the evolution of asexual pathogens and suggest that some genetic exchange may occur between

apparently genetically isolated clonal lineages and that genetic interchange between such groups may be restricted to particular chromosomes.

The results presented in this paper have important implications for the generation of karyotype variation in phytopathogenic fungi. We have previously argued (Masel et al. 1993a) that the 1.2-Mb chromosome was a recent addition to the genome of the biotype B pathogen as it was genotype specific and lacked a dispersed repeat sequence (pKB2) that is monomorphic in the biotype. Subsequent analysis has shown that another monomorphic but distinct dispersed repeat sequence (pCHB1) is also absent from the 1.2-Mb chromosome (Fig. 2). A recent search for a possible donor of the 1.2-Mb chromosome was unsuccessful and the origin of this chromosome remains unexplained (He et al. 1995). However, the 2-Mb chromosome in Bx also lacks the repeat sequences on clones pKB2 and pCHB1 and is also highly genotype specific, and therefore probably dispensable in biotype B. This suggests that the 2-Mb chromosome in Bx may also have originated by addition. The identification of a seemingly identical 2-Mb chromosome in biotype A isolates that have a distinct genetic background appears, in this instance, to identify the donor of the 2-Mb chromosome as a biotype A or a biotype A-like strain. Therefore, current evidence strongly suggests that one mechanism generating karyotype polymorphisms in *C. gloeosporioides* may be the occasional introgression of foreign chromosomes from another genetically distinct clonal lineage. It is likely that the reasons that the 1.2-Mb and 2-Mb chromosomes are retained in the biotype B genome are that (i) they are compatible and can replicate with the rest of the biotype B genome and (ii) they may encode some selective phenotypic advantage. For example, dispensable chromosomes in *Nectria haematococca* have been shown to carry genes with possible roles in pathogenicity (Miao et al. 1991; VanEtten et al. 1994). A more detailed study of the genes encoded on these chromosomes may provide some clues as to the function of these unusual dispensable chromosomes.

So far, the 1.2-Mb chromosome has only been observed in a subgroup of race 3 isolates of biotype B (Masel et al. 1993a; He et al. 1995). For the isolation of Bx, experimental plots of the cultivar *S. guianensis* cv. Graham were established in Northern Australia to specifically isolate race 3 strains of biotype B that are virulent on this cultivar (He et al. 1995). It is therefore not unexpected that the Bx isolate and its related isolate (UQ397) carry the 1.2-Mb dispensable chromosome. We are uncertain, at present, whether transfer of the 2-Mb chromosome has only occurred to the subgroup of biotype B that already contains the homologous 1.2-Mb chromosome.

Fig. 8. Pathogenicity tests with biotype A isolate A3 (UQ14, **A** and **D**), Bx (UQ396; **B** and **E**) and biotype B isolate B3 (UQ62; **C** and **F**) on *Stylosanthes scabra* cv. Fitzroy (F; **A–C**) and *S. guianensis* cv. Graham (G; **D–F**). The results illustrate the biotype B symptom of Bx on *S. guianensis* and the chlorosis and lack of sporulation or mycelial growth of Bx on *S. scabra*. Photos taken 8 days after inoculation using the contained assay procedure described by He et al. (1995).

However, the present work indicates that the presence of the 1.2-Mb chromosome does not facilitate heterokaryosis between biotypes A and B, as *nit* mutants of isolate B3, which contains the 1.2-Mb chromosome, were compatible with other biotype B isolates but incompatible with any of the biotype A isolates.

It is not possible to conclude, from the evidence that we have accumulated so far, when or where the 2-Mb chromosome was transferred between biotypes. Circumstantial evidence suggests that the transfer may have been relatively recent. All the biotype A-like alleles specific to the 2-Mb chromosome that we have detected by means of the chromosome-specific DNA probes (Figs. 2 and 3) are conserved in the Bx isolate. There appeared to be no new polymorphic bands specific to the 2-Mb chromosome that were present in Bx but absent in either the A1 isolate or B3. This suggests that there has been no time for divergence of the 2-Mb chromosome of Bx from its presumed progenitor in biotype A. Both the 2-Mb and the 1.2-Mb chromosome in Bx lack the dispersed repeat sequences on pKB2 and pCHB1 that are monomorphic within biotype B and Bx. This suggests that both the 1.2-Mb and 2-Mb chromosomes were added to the biotype B genome more recently than the proliferation of these repetitive sequences. Furthermore, two of eight new isolates collected in 1992 were like Bx while none of 17 biotype B isolates collected in the early to mid 1980s carried the 2-Mb chromosome (A. M. Masel, unpublished data). Taken together, these data suggest that chromosome transfer may be a recent event.

Stylosanthes spp. are native plants of South America and are grown extensively in other regions of the globe. Anthracnose disease is also widespread (Lenné 1994) and it is likely that the biotypes present in Australia were separately introduced from overseas (Manners et al. 1992). It is not possible to state with certainty whether the transfer of the 2-Mb chromosome occurred in Australia. However, it seems likely that a rare recombination event could have occurred in Australia, where these two biotypes are widely distributed and could coexist on a common host, *S. guianensis*. The possibility that chromosome transfer occurred outside Australia would necessitate that the Bx strain was an introduced progenitor of the biotype B lineage and that most of this population have subsequently lost the 2-Mb chromosome and others have also lost the 1.2-Mb homologous chromosome. This scenario would also imply that the biotype A line was present in the same region of origin as Bx but was also introduced separately. This would appear to be an unlikely coincidence considering that there is considerable genetic diversity in *C. gloeosporioides* infecting *Stylosanthes* spp. outside Australia (Manners et al. 1992; He et al. 1995, 1996). However, a definitive answer to this question can probably only be arrived at when the geographic origins of biotypes A and B have been identified, and molecular analysis conducted on extensive collections of *C. gloeosporioides* from their center of diversity in South America.

In the field, particular environmental conditions favoring a rare sexual or parasexual event between biotypes could have occurred. Cisar et al. (1994) have recently shown that sexual recombination can occur between some widely divergent biotypes of *C. gloeosporioides* under particular environmental conditions. However, sexual matings with Australian biotypes A and B have never been observed. The limited amount of biotype A DNA that is retained in Bx may imply that most of the biotype A DNA is unstable in the hybrid and/or the progeny of either a sexual or parasexual cross. Alternatively, if an A/B recombinant was only vegetatively compatible with biotype B, as demonstrated here for Bx, then most of the biotype A DNA may have been lost by extensive parasexual backcrossing to other B strains. It is possible that the biotype A DNA on the 2-Mb chromosome in Bx imparts some selective advantage that underlies its retention in the Bx genome. We failed to detect any reproductive advantage in pathogenicity studies on the host *S. scabra* that can be infected by biotype A but not biotype B. The Bx isolate was able to induce some chlorosis on this host in some particular environmental conditions, which suggests that it may have some capability to cause deleterious physiological effects on plants that are nonhosts of typical biotype B isolates. However, the significance of this for the survival of the Bx genotype is unclear. This question can be addressed by a wider host range study of this isolate and also by broader sampling of its frequency in the field.

Although members of different VCGs do not usually form heterokaryons, the "horizontal" transfer of genetic information between strains from different VCGs has been demonstrated in *Neurospora* spp. (Debets et al. 1994). In this organism, the transfer of mitochondrial plasmids was shown to occur during some vegetatively incompatible interactions. Taylor and Borgmann (1994) provided evidence that a nuclear repetitive element may have been transferred from a virulent biotype to the genetically distinct and apparently genetically incompatible, weakly virulent biotype of *Leptosphaeria maculans*. The transfer of a mitochondrial plasmid has also been demonstrated between genetically incompatible fungal species (Kempken 1995) and the interspecific transfer of introduced plasmids carrying selectable marker genes has been shown to occur in mycoparasitic interactions (Kellner et al. 1993). To our knowledge there is no experimental evidence demonstrating the transfer of entire nuclear chromosomes during incompatible fungal interactions. However, we have recently shown that the transfer of a linear autonomously replicating transformation vector can occur, albeit infrequently, between the two *C. gloeosporioides* biotypes in simple mycelial pairing experiments in the laboratory (A. Poplawski, unpublished). It may be possible to extend these experiments to specific chromosomes tagged with selectable marker genes in the future to test the possibility that chromosome transfer can occur in laboratory conditions.

MATERIALS AND METHODS

Fungal isolates.

All isolates used in this study have been described previously (Braithwaite et al. 1990; Masel et al. 1990, 1993a). Isolate codes A1 to A4 represented biotype A. Isolates B1 to B4 represented biotype B and correspond to isolates B1a to B4a in Braithwaite et al. (1990). These isolates are representative of each biotype in that they represent distinct physiological races and originate from distinct locations in Northern Australia. The isolates UQ396 (Bx) and UQ395 were described by He et al. (1995). All isolates were grown from monoconidial cultures. Fungi were cultured as described previously (Masel et al. 1990).

Chromosome separations.

Samples were prepared for pulsed-field gel electrophoresis from either spores or fungal protoplasts as described by Masel

et al. (1990). Separation between the 1.2-Mb, 2-Mb, smaller "minichromosomes" of 200 to 600 kb, and larger nonresolved chromosomes (Fig. 1) was achieved using a CHEF DRII apparatus in 0.7% (wt/vol) agarose gels (Bio-Rad Laboratories, Richmond, CA) and a switch time of 1,300 s ramped to 1,600 s over 60 h at 70 V. CHEF gels were stained with ethidium bromide and blotted onto nylon filters as described by Masel et al. (1990).

Chromosome-specific DNA markers.

For the purification of DNA from specific chromosomes, CHEF gels were prepared with low melting point agarose (Bio-Rad Laboratories) and were loaded with multiple spore blocks from a single isolate. After the electrophoresis, the end lanes were cut off and stained with ethidium bromide. The bands corresponding to specific chromosomes were cut from the unstained region of the gel and the DNA recovered using β-agarase (New England Bioloabs, Beverly, MA) according to the manufacturer's instructions. To obtain molecular markers specific to particular chromosomes an adaptation of the DAF (Caetano-Anolles et al. 1991) method was employed. Approximately 5 ng of chromosome-specific DNA was amplified in a 20-μl reaction volume containing 12 μM oligonucleotide primer, 5 mM $MgCl_2$, 3 units of Stoffel fragment enzyme (Perkin-Elmer Cetus, Norwalk, CT), 10 mM Tris (pH 8.3), 10 mM KCl, 200 μM of each dNTP, and overlaid with 20 μl of paraffin oil. Amplification used the following program: 94°C for 5 min, 35 cycles of a 2-phase amplification cycle consisting of a melt phase of 94°C for 30 s and an annealing phase ramp of 52 to 48°C (7 cycles per degree descent) for 1 min. A final extension phase of 5 min at 70°C was used. The reaction products were separated by electrophoresis on a 2% (wt/vol) agarose gel and transferred by capillary blotting to Hybond N+ membrane (Amersham Int., Little Chalfont, U.K.). Chromosome-specific reaction products were revealed by hybridization to cloned chromosome-specific DNA probes as described below.

Southern hybridization analyses.

Total DNA was extracted from fungal cultures, digested with restriction enzymes, electrophoresed on agarose gels, and blotted onto nylon filters following the methods of Braithwaite et al. (1990). The following hybridization probes were used; pKB2 (Braithwaite et al. 1990; Masel et al. 1993a) and pCHB1 are dispersed repeats isolated from biotype B genomic libraries prepared in pUC19 and pBluescript SK+, respectively; Cos60 (He et al. 1995) is a cosmid clone from the 1.2-Mb chromosome of isolate B3 obtained from a cosmid library prepared in the vector SuperCos (Stratagene Inc., La Jolla, CA), 10 cDNA clones were selected arbitrarily from a λgt10 cDNA library of biotype B isolate B3 grown in culture (Masel et al. 1993c). DNA probes were labeled with α-^{32}P[dCTP] using a Megaprime labeling kit (Amersham Int.) and hybridizations and filter washings were carried out at high stringency as described by Masel et al. (1993a).

RAPD analysis.

Total DNA was extracted as described above. Polymerase chain reaction assays using single arbitrary decanucleotides (Operon Technologies Inc.) and gel electrophoresis were as described by Kazan et al. (1993).

Vegetative compatibility analysis.

Nitrate non-utilizing mutants were generated by adapting the methods of Puhalla (1985) and Correll et al. (1987). Wild-type isolates from 5- to 7-day-old cultures were inoculated onto the minimal medium (MM) of Puhalla (1985) containing 5% (wt/vol) $KClO_3$. Plates were incubated at room temperature. *Nit* mutants emerged as sparse, fast-growing sectors from the wild-type colony, usually between 7 to 14 days after inoculation. These were then subcultured onto MM lacking chlorate. To determine the site of mutation, *nit*s were grown on MM amended with different nitrogen sources (Correll et al. 1987). Plates were incubated at room temperature, and reaction results were taken 5 days after inoculation. At least two mutants from each wild-type isolate were generated for each isolate, to allow pairing in all combinations. Mutants were stored in sterile water. Pairing was done on MM by excising approximately 9 mm^2 parental agar blocks of complementary *nit* mutants (e.g., *nit1* and *nitM*) and placing them 1 to 3 cm apart. These were incubated at room temperature, and inspected each week, for up to 4 weeks, for the appearance of thickly growing mycelium between the parental blocks.

Pathogenicity tests.

Mature plants were grown for about 8 weeks in the glasshouse and were inoculated with a conidial suspension at 10^6 spores per ml in water by spraying to run off. Plants were then maintained for 48 h in the dark at 100% humidity and 24°C and then transferred to the glasshouse. Plants were inspected for disease at 10 to 14 days after inoculation. At least three plants were used per treatment and the experiment was conducted twice. A separate series of experiments was undertaken using younger plants in a fully contained pathogenicity test described by He et al. (1995). Briefly, plants were grown for 4 to 5 weeks in peat cups and then transferred in the peat cup to plastic containers. The leaves were inoculated with a 10^7 spore per ml suspension with a camel-hair brush and the container was sealed. Containers were incubated at 24°C in a 14-h diurnal light cycle in a growth chamber. Plants were inspected for symptoms 7 to 8 days later. This experiment was conducted three times with at least 3 replicate plants per treatment. For microscopy, leaves were cleared and stained as described by Sharp et al. (1990).

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