Neth. J. Pl. Path. 99 (1993) Supplement 3: 119–128

Electrophoretic karyotype analysis of Botrytis cinerea

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Accepted 10 August 1993

Abstract

The karyotypes of five strains of *Botrytis cinerea* were analysed by pulsed field gel electrophoresis (PFGE). None of the five chromosome patterns were identical and all five strains contained one or several minichromosomes of variable length. Considering that some of the chromosomal bands represent unresolved doublets, the total genome size of the five strains was estimated to vary between at least 33.9 and 39.7 megabasepairs (Mbp). Hybridization of blots of pulsed field gels with rDNA and ß-tubulin probes revealed that these hybridize with the same chromosomal band. Chromosome-specific probes were isolated hybridizing to chromosomal bands 9 and 11 of strain SAS56. The latter two probes hybridized to only two of the five strains tested. The results are discussed in relation to the genome plasticity and variability of filamentous fungi.

Additional keywords: chromosome-specific probes, minichromosomes, pulsed field gel electrophoresis.

Introduction

Significant progress in the karyotype analysis of filamentous fungi has been made by the introduction of pulsed field gel electrophoresis (PFGE) techniques, which made it possible to resolve chromosomes in a size range from 0.1 to 10 Mbp, depending on electrophoretic parameters (reviewed by Mills and McCluskey, 1990; Skinner et al., 1991). The technique is based on the periodic switching of the electric field under a defined angle during electrophoresis, forcing DNA molecules to reorientate at each switch in the electric field during migration through the gel. The time needed for reorientation is inversely correlated to the size, resulting in a lower net migration of large chromosomes versus small chromosomes. Nowadays, the most commonly used PFGE technique is the contour-clamped homogeneous electric field (CHEF) system in which chromosomes migrate in zig-zag patterns, eventually resulting in separation of chromosomes in a straight lane (Chu et al., 1986).

The use of CHEF gel electrophoresis has revealed that many fungal species include isolates or strains with highly variable electrophoretic karyotypes (reviewed by Mills and McCluskey, 1990; Skinner et al., 1991). Prominent examples of this variability are *Colletotrichum gloeosporioides* (Masel et al., 1990), *Ustilago hordei* (McCluskey and Mills, 1990), *Nectria haematococca* (Miao et al., 1991a) and *Septoria tritici* (McDonald and Martinez, 1991), where numbers and sizes of chromosomes differ between almost all strains analysed. This genome plasticity indicates that fungi are apparently genetically very flexible, but it raises serious questions about their genetic stability.

The karyotype of *Botrytis cinerea* Pers.: Fr., a ubiquitous plant pathogen with a very wide host range, has not been studied extensively. Several examples have been reported of genetic variability among isolates and instability within one isolate (Hansen and Smith,

1932; Grindle, 1979; Lorenz, 1983; Van der Vlugt-Bergmans et al., 1993). Nevertheless, a light microscopic study of mitotic chromosomes of five *B. cinerea* isolates, obtained from different hosts, indicated that these all have the same number of chromosomes, i.e. 16 (Shirane et al., 1988, 1989). In order to obtain tools for a molecular genetic characterization of this fungus, we have initiated the electrophoretic separation of chromosomes of five *B. cinerea* strains. By hybridization analysis four DNA probes have been assigned to specific chromosomal bands. Two of the probes hybridize to all strains, whereas two others differentiate between strains.

Materials and methods

Growth of B. cinerea strains and isolation of protoplasts. Table 1 presents the origin and the year of isolation of the strains of *B. cinerea* used in this study. Strains were grown for 2 weeks on PDA supplemented with 300 g/l of homogenized tomato leaves. Conidia were harvested by suspending in B5 medium (Duchefa, Haarlem, the Netherlands) with a droplet of 5% Triton X100, transferred to a flask containing 100 ml of B5 medium and cultures were grown overnight at 20 °C under continuous shaking. Mycelium was collected on Miracloth filter, washed on the filter with 0.8 M $MgSO_4$/ 20 mM MES/ 1 mM EDTA, pH 5.8 (MME buffer) and transferred into 20 ml of MME buffer containing 5 mg/ml Novozym 234 (Novo). The sample was incubated at 28 °C under very gentle shaking until complete disintegration of the mycelium (usually 30–90 min). The resulting protoplast suspension was adjusted to 1 M $MgSO_4$, filtered over Miracloth and centrifuged for 10 min at 2000 rpm to remove large cell debris. The supernatant, containing the protoplasts, was collected and 2 volumes of 1 M NaCl/ 20 mM MES/ 1 mM EDTA, pH 5.8 (NME buffer) were added. The protoplasts were pelleted by centrifugation for 15 min at 3500 rpm. The protoplasts were washed twice by resuspending them in NME buffer, followed by centrifugation. Finally the pellet was resuspended in STE buffer (0.8 M sucrose/ 25 mM Tris-HCl/ 50 mM EDTA, pH 7.5) at a concentration of 5×10^7 protoplasts per ml.

Table 1. Strains of *Botrytis cinerea*, their origin and the year of isolation.

Strain	Origin	Year of isolation
SAS56	monoascospore strain[1]	
Bc7	tomato	1972
Bc21	rose	1990
Bc25	rose	1990
Bc26	rose	1990

[1] Described in Faretra et al. (1988b).

Preparation of plugs for CHEF gel electrophoresis. To a protoplast suspension (5×10^7/ ml), one volume of a warm (50 °C) 1.2% low-melting-point (LMP) agarose solution in STE buffer was added. The sample was mixed and aliquots of 300 µl were poured in a CHEF casting mould (BioRad) placed in ice. Solidified plugs were incubated in 0.5 M EDTA/ 0.1 M Tris-HCl/ 1% lauroylsarcosine, pH 9.5 with 2 mg/ml Proteinase K or Pronase E at 50 °C under gentle shaking for 48 h. The plugs were subsequently washed five times with 0.05 M EDTA, pH 8.0 and stored at 4 °C in 0.05 M EDTA, pH 8.0. To check the quality of the DNA samples, small slices of a plug were electrophoresed for 2 h

on a standard 0.8% agarose gel in TAE buffer (Sambrook et al., 1989). Migration of a substantial part of the DNA from the slots into the gel indicated that the quality was sufficient to be used for CHEF gel electrophoresis.

CHEF gel electrophoresis. Plugs containing chromosomal DNA were inserted into the slots of a 1% agarose gel (Chromosomal Grade, BioRad) in 0.5 × TAE buffer (Sambrook et al., 1989). The slots were filled with 1.2% LMP agarose in STE buffer. The gel was placed in the CHEF electrophoresis tank (CHEF-DRII system, BioRad) in a cold room (6 °C) and electrophoresis was performed for 3 days at 100 V. To resolve large chromosomes of *B. cinerea*, the electric field was switched every 3000 sec for 8 h, followed by switching every 500 sec for 64 h. To resolve *B. cinerea* minichromosomes, the electric field was switched every 900 sec for 24 h, followed by switching every 240 sec for 48 h. The temperature of the electrophoresis buffer during the run was 8–9 °C. After electrophoresis the gel was stained for 1 h in 0.5 × TAE containing 1 µg/ml ethidium bromide and destained for 1 h in 0.5 × TAE.

To determine the size of the *B. cinerea* chromosomes, well defined size standards from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (BioRad) were co-electrophoresed. The sizes of *B. cinerea* chromosomes were estimated in relation to the size standards by assuming a linear inverse correlation between migration (in cm) and the chromosome size (in Mbp) (Vollrath and Davis, 1987).

Blotting, hybridization and cloning procedures. Blots of CHEF gels were made by standard Southern blot procedures (Sambrook et al., 1989) and were hybridized with radioactively labeled probes as described by Church and Gilbert (1984). Blots were washed in 0.2 × SSC/ 0.2% SDS at 65 °C. Radioactive probes were obtained by the oligolabeling method described by Hodgson and Fisk (1987). Standard DNA cloning and isolation techniques were performed as described in Sambrook et al. (1989). The genomic library of *B. cinerea* strain SAS56 was kindly provided by Dr M. Kusters-van Someren and Dr J. Visser, Dept. of Genetics, Wageningen Agricultural University, the Netherlands.

Results

Electrophoretic karyotypes. The chromosomes of five strains of *B. cinerea* (Table 1) were analysed by CHEF gel electrophoresis. Fig. 1 shows a stained gel run under conditions for resolution of chromosomes varying in size between 4 and 2 Mbp. The vast majority of chromosomal bands migrated in between the smallest *S. pombe* marker of 3.5 Mbp and the largest *S. cerevisiae* marker of 2.2 Mbp, indicating that the sizes of the *B. cinerea* chromosomes fall within a rather narrow range, when compared to chromosomes of many other fungi (Skinner et al., 1991). In SAS56 9 chromosomal bands (indicated by arrows) could be distinguished in this size range. The intensity of some bands was higher, suggesting that they contain multiple unresolved chromosomes. The electrophoretic karyotypes of the other four strains differed significantly from the pattern of SAS56 and from each other. The karyotypes of strains Bc21, Bc25 and Bc26, which were all isolated from the same host species in the same year (Table 1), did not show more similarities to each other than to the other two strains, SAS56 and Bc7 (Fig. 1, Table 2).

Fig. 2 shows a stained gel run under conditions to resolve small chromosomes (<1 Mbp). In this run, all the chromosomes resolved in Fig. 1 comigrate to one position, yielding a broad band of extremely high intensity. Moreover, at the bottom of the gel, at least one minichromosome of variable size was visible in each lane. SAS56 contains two minichromosomes of 270 and 220 kbp (indicated as CHR10 and CHR11, respectively),

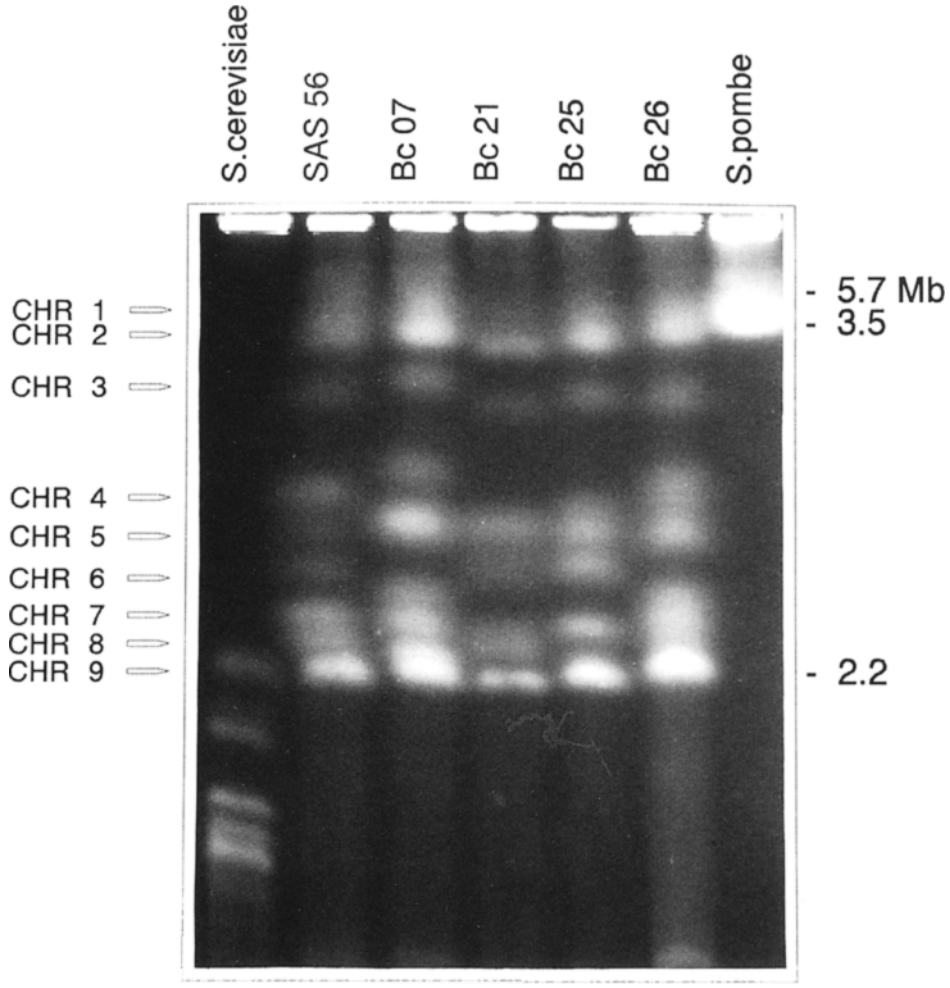

Fig. 1. CHEF gel electrophoresis resolving *Botrytis cinerea* chromosomes varying in size between 4 and 2 Mbp. Strain numbers are indicated at the top. In the left margin, chromosomal bands of SAS56 are indicated by numbered arrows. In the right margin, sizes of relevant marker chromosomes of *Schizosaccharomyces cerevisiae* and *Schizosaccharomyces pombe* are indicated (in Mbp).

whereas strains Bc7, Bc21, Bc25 and Bc26 each contain a single minichromosome of 280, 250, 260 and 290 kbp, respectively. In the Bc7 lane also two fainter bands of approximately 580 and 450 kbp are visible. Table 2 presents the calculated sizes of all the chromosomal bands of the *B. cinerea* strains, as deduced by comparison with size standards. Since the intensity of some bands is higher, it is assumed that these represent at least doublets (indicated in Table 2 by asterisks). If these bands are indeed doublets (and not triplets), the total genome sizes of the five strains are estimated to range from at least 33.9 Mbp (strain Bc21) to 39.7 Mbp (strain SAS56).

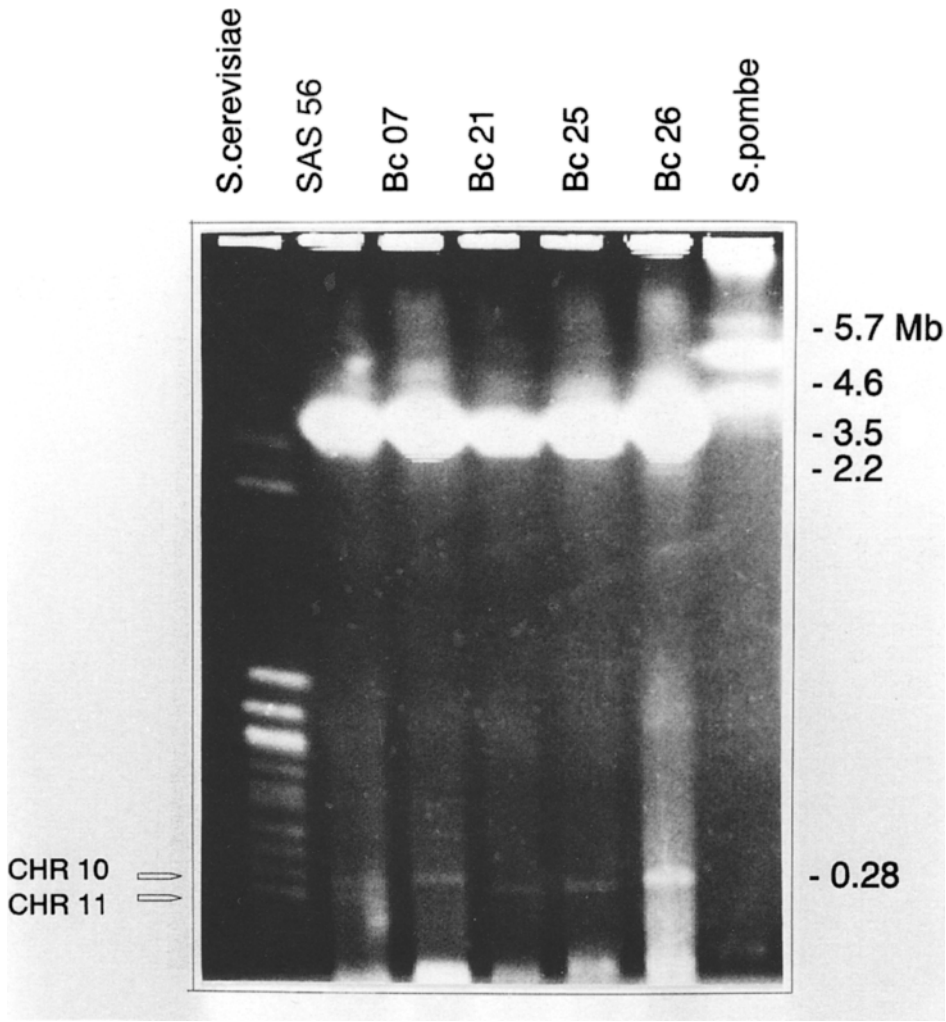

Fig. 2. CHEF gel electrophoresis resolving *Botrytis cinerea* chromosomes varying in size between 1 and 0.2 Mbp. Strain numbers are indicated at the top. In the left margin, chromosomal bands of SAS56 are indicated by numbered arrows. In the right margin, sizes of relevant marker chromosomes of *Schizosaccharomyces cerevisiae* and *Schizosaccharomyces pombe* are indicated (in Mbp).

Isolation of chromosome specific probes. In order to obtain DNA probes specific for *B. cinerea* SAS56 chromosomes, we resolved the chromosomes partially on a CHEF gel and cut out six gel slices each containing multiple chromosomal bands (data not shown). The DNA from these slices was extracted and used for two purposes. Firstly, DNAs from each gel slice were labeled and used separately as a probe on replicate filters containing 100 random lambda phages from a genomic library of SAS56. Approximately 25 plaques hybridized specifically with labeled DNA from one of the gel slices but not with labeled DNA from the other slices (data not shown). DNA from the strongest hybridizing lambda

Table 2. Estimated sizes (in Mbp) of chromosomes of five *Botrytis cinerea* strains.

	Botrytis cinera strain				
	SAS56	Bc7	Bc21	Bc25	Bc26
1	4.0	4.0	4.0	4.0	4.0
2	3.5*	3.5*	3.4*	3.5*	3.5*
3	3.3	3.3	3.2	3.3	3.3
4	2.9*	3.0	2.8*	2.8	2.9
5	2.7	2.8*	2.6	2.7*	2.8
6	2.6	2.5	2.4	2.6*	2.7*
7	2.4*	2.4*	2.3*	2.4*	2.5
8	2.3*	2.3	2.2*	2.2*	2.4*
9	2.2*	2.2*	0.25	0.26	2.2*
10	0.27	0.58			0.29
11	0.22	0.45			
12		0.28			
Total	39.7	38.3	33.9	37.4	37.6

* Asterisk indicates multiple unresolved chromosomes.

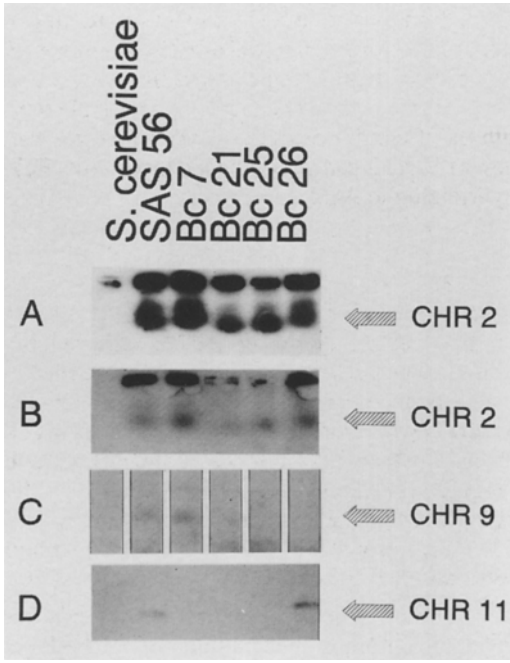

Fig. 3. Autoradiographs of Southern blots of CHEF gels, hybridized to four different DNA probes. Panels A, B and C represent relevant parts of blots from gels resolving 2–4 Mbp chromosomes (see Fig. 1), while panel D represents a part of a blot from a gel resolving 0.2–1 Mbp chromosomes (see Fig. 2). The probes were derived from (A) the *Phytophthora infestans* rDNA probe, (B) the *Botrytis cinerea* ß-tubulin gene, (C) the chromosome-specific clone AG1, and (D) the chromosome-specific clone AG2.

clone, AG1, was isolated and used as a probe on blots of CHEF gels.

Secondly, DNA from a gel slice containing both unresolved minichromosomes of SAS56 was digested with *Eco*RI and ligated into the cloning vector pGEM3 Zf(-). Transformation of the ligation mixture yielded five colonies, of which one (AG2) contained an insert of 1.2 kbp. This insert was used as a probe on blots of CHEF gels.

Allocation of DNA probes to chromosomal bands. Blots were made of the gels presented in Figs 1 and 2 and of gels electrophoresed under the same conditions. The blots were hybridized with four DNA probes: a *Phytophthora infestans* rDNA gene (Pieterse, unpublished), the *B. cinerea* β-tubulin gene (Van der Vlugt-Bergmans et al., unpublished) and the two chromosome-specific clones AG1 and AG2 described above.

The rDNA probe (Fig. 3A) appeared to hybridize with high intensity to chromosomal band 2 and with much lower intensity to chromosomal band 1. The ß-tubulin gene, which is a single copy gene in *B. cinerea* (Van der Vlugt-Bergmans et al., unpublished), also hybridized to band 2 and weakly to band 1 (Fig. 3B). The overall hybridization signal was much weaker with the ß-tubulin probe than with the rDNA probe due to the high copy number of rDNA genes.

Probe AG1 appeared to hybridize with chromosomal band 9 (2.2 Mbp) in SAS56 and with a band of the same size in strain Bc7 (Fig. 3C). It did not hybridize at all with chromosomes of strains Bc21, Bc25 and Bc26. Hybridization of probe AG1 to a Southern blot containing *Eco*RI-digested total DNA of the five strains was unsuccessful.

Probe AG2 hybridized specifically to the 220 kbp minichromosome of SAS56 and the 290 kbp minichromosome of Bc26 (Fig. 3D), but failed to hybridize to chromosomes of Bc7, Bc21 and Bc25. On a Southern blot containing *Eco*RI-digested and *Hin*dIII-digested total DNA of the five strains, probe AG2 hybridized with a single copy fragment present only in SAS56 and Bc26, and not detectable in Bc7, Bc21 and Bc25 (data not shown). When the same blot was rehybridized with the ß-tubulin probe, all five strains gave an identical hybridization pattern (data not shown). Thus it can be concluded that strains Bc7, Bc21 and Bc25 are devoid of sequences hybridizing to AG2.

Discussion

With the use of CHEF gel electrophoresis, we have shown polymorphisms in karyotypes among five *B. cinerea* strains. All the strains have distinct chromosomal banding patterns. Even strains Bc21, Bc25 and Bc26, all isolated from the same host species (rose) in 1990 show significant variations in karyotypes. This is in agreement with results of a previous study on the genetic variation among these strains by RAPD analysis, from which it was concluded that the three strains Bc21, Bc25 and Bc26 are genetically as distinct from each other as from SAS56 and Bc7 (Van der Vlugt-Bergmans et al., 1993).

Chromosome length polymorphisms appear to be a general phenomenon in many filamentous fungi (Mills and McCluskey, 1990; Skinner et al., 1991). Kistler and Miao (1992) have proposed that there is an inverse correlation between the extent of chromosomal polymorphism and the frequency with which fungi undergo meiosis, since meiosis poses a strong selection against chromosomal aberrations between the two mating partners. So far, reports on the observation of the teleomorph, *Botryotinia fuckeliana*, in the field have been scarce (de Bary, 1866; Polach and Abawi, 1975), although it can reproducibly be obtained under defined laboratory conditions (Faretra et al., 1987, 1988a). The scarcity of observations of the teleomorph in the field and the extent of chromosomal polymorphism among the five *B. cinerea* isolates studied are in support of the hypothesis of Kistler and Miao (1992).

As a first step towards the allocation of DNA markers to specific chromosomes, we hybridized four DNA probes to blots of CHEF gels. The rDNA probe and the β-tubulin probe appeared to hybridize to the same chromosomal bands. The fact that the β-tubulin gene, a single copy gene, hybridized predominantly to band 2, but also slightly to band 1, indicates that separation in this area of this particular gel has been incomplete. In view of the poor separation in this size range and the likeliness that chromosomal band 2 represents multiple chromosomes, it remains to be established whether the rDNA genes and the β-tubulin gene actually reside on the same chromosome, or on two co-migrating chromosomes.

DNA probe AG1 was specific for chromosomal band 9 of SAS56. Surprisingly, this probe hybridized to a chromosome of similar size in only one other strain (Bc7), but did not hybridize to chromosomes of the other strains. Similar observation were made in other fungi. In *Septoria tritici*, a substantial proportion of randomly selected probes, to be used for RFLP analysis, failed to hybridize completely to DNA of at least one of the strains analysed (McDonald and Martinez, 1990). Masel et al. (1993) showed that in *Colletotrichum gloeosporioides* race 3 isolates of Type B, ten DNA probes randomly distributed over a 1.2 Mbp chromosome failed to hybridize to races 1, 2 and 4 of Type B, suggesting the absence of this complete chromosome in the latter races. Thus the probes isolated were chromosome and strain-specific. A horizontal transfer event from an unidentified source was proposed as a possible origin of the 1.2 Mbp chromosome (Masel et al., 1993). Regarding the *B. cinerea* AG1 probe, it discriminates between strains as well. However, to determine whether SAS56 chromosome 9, detected by probe AG1, has a homologue in the three non-hybridizing strains (Bc21, Bc25 and Bc26), it will be necessary to isolate DNA markers linked to AG1 and test such markers for hybridization to chromosomal blots of these strains.

The minichromosomes observed in all five *B. cinerea* strains analysed may be supernumerary, or "B" chromosomes. Properties of such chromosomes are their size variability, their absence in some strains and their abnormal segregation in sexual crosses (Skinner et al., 1991). Based on electrophoretic mobility it can be concluded that most minichromosomes observed in the five *B. cinerea* strains differ in size. Furthermore, with the use of probe AG2, we were able to show that sequences homologous to this probe are only present in SAS56 and Bc26, on chromosomes of different sizes, and not in the other three strains. Since AG2 is the only probe available for this chromosome, it cannot be established yet whether the whole chromosome is missing from Bc7, Bc21 and Bc25, or whether these strains have homologous chromosomes that only lack the sequence hybridizing to AG2. More markers linked to AG2 are required to answer this issue. Furthermore, the progeny from a cross between SAS56 (containing the 220 kbp minichromosome) and SAS405 (not containing this chromosome and not hybridizing to AG2, data not shown) will be analysed for the inheritance of the sequences hybridizing to AG2, in order to investigate (possibly unusual) segregation.

The function of 'B' chromosomes in filamentous fungi remains to be determined, but the designation as 'supernumerary' might underestimate their importance. In *Nectria haematococca*, one dispensable chromosome contains a pisatin demethylase gene (*Pda6*) and a gene for maackain degradation (*Mak1*), which are proposed to be involved in pathogenicity of this fungus (Miao et al. 1991a,b; Miao and VanEtten, 1992). Besides the *Pda6* and *Mak1* genes, other pathogenicity determinants might be clustered on the same chromosome (VanEtten, personal communication). The clustering of several pathogenicity genes on one dispensable chromosome bears analogy to the *Agrobacterium tumefaciens* Ti plasmid, encoding all functions involved in crown gall formation. *Agrobacterium radiobacter* is a closely related (if not identical) bacterial species lacking this Ti plasmid

(Otten et al., 1992). Thus it is possible that fungal minichromosomes contain clusters of genes which are in some way beneficial to pathogenic competitiveness, but are dispensable under certain (saprophytic?) conditions. Such a hypothesis makes it interesting to investigate the origin, stability and inheritance of *B. cinerea* minichromosomes and to get indications about functional genes residing on these chromosomes.

Acknowledgements

The authors gratefully acknowledge Mr J. van 't Klooster for initial experiments on protoplasting and CHEF gel electrophoresis, Dr G. van den Ackerveken for advice on CHEF gel electrophoresis conditions, and Dr C. Pieterse for providing the *P. infestans* rDNA probe. Furthermore we are grateful to Dr M.A. Kusters-van Someren and Dr J. Visser for providing the genomic library of SAS56.

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