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Inheritance of chromosome-length polymorphisms in the phytopathogenic ascomycete *Botryotinia fuckeliana* (anam. *Botrytis cinerea*)

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The electrophoretic karyotype (EK) of 24 strains of *Botryotinia fuckeliana* was resolved to determine the extent of chromosomal polymorphism among them. Based on EK variation, nine different profiles with a number of bands ranging from 5 to 8 were found. Those profiles were not related to the origins of the strains, hosts, or year of isolation. Inheritance of chromosomal bands was studied by analyzing the EK of the progeny from crosses between sexually compatible *B. fuckeliana* strains that had different karyotype. The EKs of 44 monoascospore strains showed 12 new EK profiles, suggesting that chromosomal rearrangements generated after meiotic recombination provides EK variability in this fungus. Also, we report here that the degree of EK variability indicates that sexual reproduction under natural conditions might be quite important in the life cycle of *B. fuckeliana*. Hybridization analysis showed that, in most strains, the rDNA is located in both a chromosomal band of high molecular weight and different size depending on the strain and a band of low molecular weight, revealing evidence for chromosome rearrangements in this plant pathogen.

INTRODUCTION

Cytological karyotyping of many filamentous fungi has been precluded by both the very small size of their chromosomes and the dispersed, rather than condensed, nature of the chromatin during meiosis (Kohn 1992). The development of pulsed-field gel electrophoresis (PFGE) has provided a very significant and powerful new tool for determining many fungal karyotypes (Brody & Carbon 1989, Orbach 1989, Osiewacz, Clairmont & Huth 1990, Nadal, Colomer & Piña 1996). This technique has led to the discovery that most species exhibit chromosome-length polymorphisms (CLPs), revealing a high level of intraspecific, and even population-level, variability (Chu, Magee & Magee 1993). Polymorphism has been observed in both asexual and sexual fungi and most likely results from both mitotic and meiotic processes. Available data suggest that within-species CLPs are a mixture of changes involving rearrangements of linkage groups and gains or losses of noncoding sequences (Kohn 1992). This genome plasticity indicates, apparently, that fungi are genetically very flexible.

Botryotinia fuckeliana is a filamentous, heterothallic, apothecial ascomycete that causes grey mould on many economically important crops worldwide, without any apparent host specificity (Coley-Smith, Verhoeff & Jarvis 1980). The fungus exhibits a high genetic variability (Büttner *et al*. 1994), expressed in the many phenotypical differences among isolates (Grindle 1979, Di Lenna, Marciano & Magro 1981, Movahedi & Heale 1990, Chardonet *et al*. 2000, Vallejo *et al*. 2001). Variations in ploidy levels, aneuploidy and heterokaryosis are regarded as the main cause of the genetic variability in *B. fuckeliana* (van der vlugt-Bergmans *et al*. 1993, Büttner *et al*. 1994). Meiotic recombination is not thought to be greatly involved: although many isolates are able to reproduce sexually under laboratory conditions, apothecia are rarely observed in the field. Because meiotic events have been suggested as being inversely correlated to the chromosomal polymorphisms in fungi (Kistler & Miao 1992), *B. fuckeliana* would be expected to have a high level of CLPs among isolates, making it difficult for a zygote to undergo meiosis in a normal fashion. Strains with different karyotypes should be, to some extent, reproductively isolated depending on which segments of the chromosomes are variable (Kohn 1992).

We had previously resolved the electrophoretic karyotype (EK) of five *B. fuckeliana* strains (Vallejo *et al*. 1996) and, as far as we know, the karyotypes of only five other strains have been compared (van Kan,

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Goverse & van der vlugt-Bergmans 1993). Furthermore, the segregation of CLPs in *B. fuckeliana* has not yet been investigated. Our goals for this work were to determine the extent of chromosomal polymorphisms in a greater number of strains of *B. fuckeliana* and to analyze the inheritance of chromosomal bands in an effort to explain the apparently low frequency of meiosis in this organism under natural conditions, and to find possible meiotic or mitotic mechanisms for the generation of fungal CLPs.

MATERIALS AND METHODS

Fungal strains

Twenty isolates of *Botryotinia fuckeliana* were recovered from infected plant material in different regions throughout Spain: 14 from strawberries cultivated in Huelva (southwest), two from grapevine cultures located in Cádiz (south), three each from grapes cultivated in León (northwest), Málaga (south) and Alicante (east), and one from cucumber cultivated in Almería (south-east). All of them were purified by monosporic isolation and deposited in the Mycological Herbarium Collection of the University of Cádiz. Strains 2100 and 2850 were provided by the Spanish Collection of Type Cultures. Strains SAS56 and SAS405 carrying mating-type alleles *MAT1-1* and *MAT1-2*, respectively, were kindly donated by Franco Faretra (University of Bari); both were obtained from crosses of Italian field isolates (Faretra, Antonacci & Pollastro 1988a). The remaining strains used in this study were obtained by crossing either SAS56 × UCA992 (strains named UCA992-) or SAS405 × UCA9930 (strains named UCA9930-) and isolating random sets of monoascospores. All strains and their origins are listed in Table 1.

Media and culture conditions

Cultures were grown in Petri dishes containing malt agar (MA) (2% malt extract, 2% agar, [%, wt/vol]) and maintained in incubators under alternating (12 h) light/dark cycles at 21(±1) °C. Fungal plugs (surface = 0.2 × 0.5 cm), harvested from the edge of the growing colony, were transferred weekly to plates of fresh medium and maintained under the same conditions. For storage, conidia were harvested in 0.01% (vol/vol) Tween 20 solution, filtered through a 30-μm nylon filter (Sefar Nytal, Mays, Barcelona) and centrifuged for 5 min at 120 ***g***. The resulting conidial pellet was suspended in a 10% (vol/vol) glycerol solution to a concentration of 10^7 conidia ml^{-1} and stored at −80 °C. For liquid cultures, 1 ml of this suspension was used as inoculum in a modified Czapek–Dox medium (2.5% $NaNO_3$, 0.5% KCl, 0.5% $MgSO_4 \cdot 7H_2O$, 0.01% $FeSO_4$, 1% K_2HPO_4, [%, wt/vol]) supplemented with 0.1% (wt/vol) yeast extract and 5% (wt/vol) glucose. Cultures were incubated on a rotary shaker at 200 rpm at 21(±1) ° for 24 h. Individual ascospores were collected on water agar WA (20 g technical agar, Cultimed, Panreac, Barcelona per litre distilled water).

Preparation of intact chromosomal DNA

Protoplasts of *Botryotinia fuckeliana* were formed as previously described (Vallejo *et al.* 1996). The protoplast

Table 1. Strains of *Botryotinia fuckeliana*, their origin and electrophoretic profile obtained under the PFGE conditions used in this study.

Strain	Electrophoretic profile	Host	Origin (year of isolation)/parents
UCA991	V	*Vitis vinifera*	Málaga, Spain (1991)
UCA992	III	*V. vinifera*	Jerez de la Frontera (Cádiz), Spain (1992)
UCA9930	III	*V. vinifera*	Puerto de Sta. María (Cádiz), Spain (1993)
UCA994	VII	*V. vinifera*	Alicante, Spain (1994)
UCA995	VIII	*V. vinifera*	León, Spain (1993)
UCA996	III	*Cucumis sativus*	Almería, Spain (1996)
UCA101	VIII	*Fragaria vesca*	Huelva, Spain (1997)
UCA102	III	*F. vesca*	Huelva, Spain (1998)
UCA103	III	*F. vesca*	Huelva, Spain (1997)
UCA104	III	*F. vesca*	Huelva, Spain (1998)
UCA105	III	*F. vesca*	Huelva, Spain (1997)
UCA106	II	*F. vesca*	Huelva, Spain (1998)
UCA107	III	*F. vesca*	Huelva, Spain (1997)
UCA108	III	*F. vesca*	Huelva, Spain (1997)
UCA109	III	*F. vesca*	Huelva, Spain (1997)
UCA110	I	*F. vesca*	Huelva, Spain (1997)
UCA111	VIII	*F. vesca*	Huelva, Spain (1998)
UCA112	III	*F. vesca*	Huelva, Spain (1998)
UCA113	VI	*F. vesca*	Huelva, Spain (1997)
UCA114	III	*F. vesca*	Huelva, Spain (1997)
2100	VII	*Vicia faba*	Spanish Collection of Type Cultures (1979)
2850	III	*Crosus sativa*	Spanish Collection of Type Cultures (1987)
SAS56	IV	Ascospore progeny	Italy. Faretra *et al.* (1988a)
SAS405	IX	Ascospore progeny	Italy. Faretra *et al.* (1988a)

suspension was filtered through a 30 μm nylon filter (Sefar Nytal, Mays, Barcelona) to remove the undigested mycelium. Protoplasts were pelleted by centrifugation at 1500 ***g*** for 15 min, washed three times with 0.7 M KCl, 50 mM EDTA (pH 8) (KE), and suspended in the same solution. The protoplast suspension was then mixed with 1 vol of 1% (wt/vol) low-melting-point agarose (Bio-Rad Laboratories, Richmond, CA) in KE for a final concentration of 10^8 protoplasts ml^{-1}. The mixture was allowed to solidify in Bio-Rad (Hercules, CA) plug moulds. Solidified agarose plugs were incubated in ESP buffer (0.5 M EDTA [pH 9], 1% [wt/vol] lauroylsarcosina, 10 mg ml^{-1} proteinase K) at 50 ° for 24–48 h. Afterwards, plugs were washed in 50 mM EDTA (pH 8) and stored at 4 ° in the same buffer.

Pulse-field gel electrophoresis

Karyotypes were determined by contour-clamped homogeneous electric field electrophoresis (CHEF) with a CHEF-DRII apparatus (Bio-Rad). Plugs containing chromosomal DNA were loaded onto 0.8% (wt/vol) PFGE agarose (Bio-Rad) gels in 1 × TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8]). Electrophoresis was carried out using a pulse-time gradient of 250–900 s at 3 V cm^{-1} for a total time of 65 h at 14 ° with continuous circulation of buffer. Gels were stained with 0.5 μg ml^{-1} ethidium bromide for 30–45 min, destained in 1 × TAE buffer for 1 h, and photographed under UV illumination.

Southern blot analyses

The chromosomal DNA separated by pulse-field gel electrophoresis was transferred to nylon filters (Hybon-NX; Amersham International, Bucks) as described by Sambrook, Fritsch & Maniatis (1989) for 24 h. The membrane was allowed to hybridize with digoxigenin-11-dUTP probe. The probe used for chromosome hybridization (pDm238) was a 12-kb *Eco*RI-restriction fragment, which contained the coding region of the 18S, 5.8S and 28S rDNA genes and the ITS (internal transcribed spacers) from *Drosophila melanogaster*. The fragment had been previously cloned in the plasmid pBR322 and maintained in *Escherichia coli* JM109 (Roiha *et al.* 1981). Plasmid DNA mini-prep was performed by alkaline lysis (Birnboim & Doly 1979). Inserts were released by digestion with *Eco*RI and purified from low-melting-point agarose (Bio-Rad) gels. DNA probe was labeled with the random priming method using the DIG High Prime DNA Labeling and Detecting Starter Kit II (Roche Diagnostics, Mannheim). Prehybridization and hybridization were performed at 40 ° overnight according to the manufacturer's recommendations. The membrane was washed twice in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature for 5 min and then twice with 0.5 × SSC, 0.1% (wt/vol) SDS at 65 ° for 15 min. Immunological detection was performed as recommended by the manufacturer, and membranes were exposed to X-ray film for 30 min at room temperature.

Mating type assay

Eight strains were crossed with reference strains of known mating type (SAS56 and SAS405) as described by Faretra, Antonacci & Pollastro (1988b). Strains were grown on MA in Petri dishes at 15 ° in the dark for 30 d and then at 0 ° for a further 30 d in the dark. Crosses were set up by transferring 15 sclerotia to narrow-necked glass McCartney bottles containing 9 ml of sterile distilled water and adding 3 ml of a dense suspension of microconidia for fertilization. Microconidial suspensions were prepared in sterile distilled water from the dishes used to produce sclerotia. All strains were mated in reciprocal crosses and controls included unspermatized and self-fertilized sclerotia. Bottles were incubated under alternating (12 h) light at 11 °. Apothecia were collected within 3–6 months after spermatization. Single apothecia were squashed in 250 μl sterile distilled water and mixed thoroughly to liberate ascospores. Samples (15 μl) of ascospore suspensions were spread on WA Petri dishes. Ascospores were, then, allowed to germinate, transferred individually to MA Petri dishes, and cultured until conidiation. Conidia of each monoascospore strain (MS) were collected and stored in 10% (vol/vol) glycerol solution at −80 °C. Apothecia that were not used at that time were stored in the same conditions.

RESULTS

Electrophoretic karyotypes

We reported earlier on the electrophoretic karyotyping (EK) of five *Botryotinia fuckeliana* strains (Vallejo *et al.* 1996). To broaden the comparison, additional *B. fuckeliana* strains were tested for their EKs. To this end, the intact chromosomal DNA of 24 strains of *B. fuckeliana* was released from mycelial protoplasts in agarose plugs and subjected to CHEF electrophoresis. Chromosome-sized DNAs were resolved under a single set of empirically derived running conditions (see Materials and methods), which have been improved with respect to earlier reports in order to achieve a more accurate EK profiles of the strains. Highly reproducible EKs were generated for each of the 24 *B. fuckeliana* strains, and the number of DNA bands did not change for any isolate, despite repeated subculturing and sample preparation. Strains were compared at the karyotype level, and several differences in both DNA bands number and size were observed. However, some strains had the same electrophoretic profile (Table 1).

The number of resolvable bands for 24 strains ranged from 5–8. The median band number was 6, with a modal value of 6. Care has to be taken when comparing

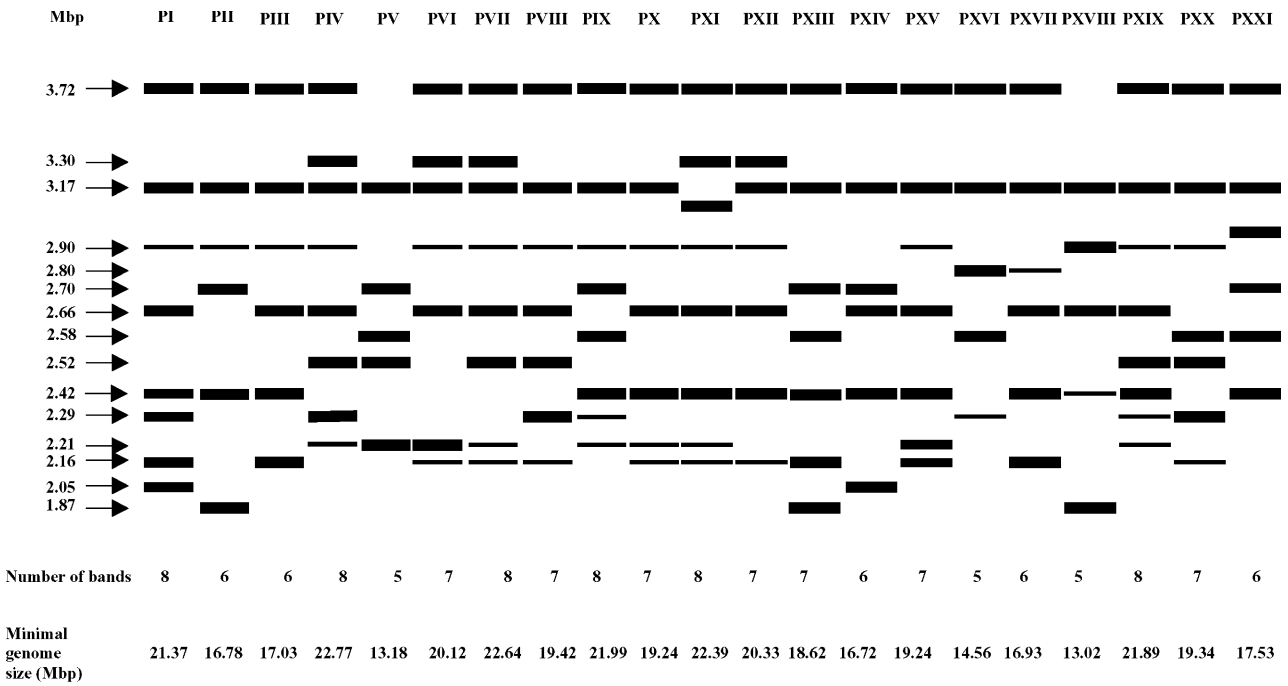

Fig. 1. Schematic representation of different electrophoretic karyotype profiles (in roman numerals) obtained by PFGE from 68 *Botryotinia fuckeliana* strains.

karyotypes of different strains on PFGE gels because: (1) bands of the same size from different strains may not represent the same chromosome; and (2) chromosomes of different sizes from different strains may be homologous. Therefore, when comparing EKs from different strains, we did not consider it appropriate to identify bands of the same size as the same chromosomal bands. Among 24 strains tested, nine different PFGE profiles were obtained under the electrophoretic conditions used (Fig. 1, Table 1). Profile III was most prevalent and was characterized by six bands. The size of each band, estimated using *Hansenula wingei* and *Schyzosaccharomyces pombe* chromosomes as standards, ranged from 1.87 to 3.72 Mbp. Band of size 3.17 Mbp was present in all strains tested; bands of 3.72 and 2.90 Mbp were also present in all of them, except for UCA991 (profile V). The relative intensity of ultraviolet fluorescence from some bands after ethidium bromide staining suggested that they might contain two or more chromosomes. Efforts to resolve those bands into more than one chromosome by varying the voltage, pulse interval, and time of run were unsuccessful.

Based on the previous descriptions, the minimal size of the genome (MSG) of *B. fuckeliana* ranged from 13.18 (profile V) to 22.77 Mbp (profile IV), with a mean MSG of 18.38 ± 0.48 Mbp (mean $\pm$ SE), although because of difficulty in resolving some chromosomal bands, the estimated genome size could possess an error factor of several Mbp.

EKs of strains isolated from strawberries cultivated in Huelva (south-east Spain) displayed 1–2 additional bands lighting up with very low intensity. The size of these bands ranged from 57 to 200 kb, being much smaller than those described above. They may represent supernumerary chromosomes, which have been previously described in many filamentous fungi (van Kan *et al*. 1993, Leclair *et al*. 1996, Covert 1998), although it is also possible they correspond to fragments of initially larger chromosomes.

The band having the highest mobility was earlier identified as mitochondrial DNA (mtDNA) in 5 *B. cinerea* strains (Vallejo *et al*. 1996). Variation in the intensity of this band indicated that several chromosomes or chromosomal fragments could be co-migrating with the mtDNA. For analytical and descriptive purposes, we termed this the 'mtDNA-band'.

Segregation of DNA polymorphisms

Random sets of ascospores were selected from two crosses between strains with different EKs (SAS56 $\times$ UCA992 and SAS405 $\times$ UCA9930, respectively), in order to analyze segregation of electrophoretic DNA bands and assess whether meiosis provides the EK variation observed in *Botryotinia fuckeliana* strains. To this end, 30 monoascospore strains (MSs) from each cross were grown to conidiation and used to prepare protoplasts. Some MSs from the crosses did not produce protoplasts and changes in the concentration of the enzyme (Lysing enzymes, Sigma-Aldrich Chemic, Steinheim, Germany) were unsuccessful in removing cell walls of the mycelium. Thus, we analyzed EKs of 16 MSs from SAS56 $\times$ UCA992 and 28 MSs from SAS405 $\times$ UCA9930. Like the EKs of the 24 strains described previously, EKs of 44 MSs were highly reproducible.

PFGE profiles displayed by the progeny from SAS56 (profile IV) $\times$ UCA992 (profile III) are shown in Table

Table 2. PFGE profiles displayed by the ascospore progeny from the crosses between *Botryotinia fuckeliana* strains showing different electrophoretic karyotypes.

	Progeny	
Profile	SAS56 (Profile IV) × UCA992 (Profile III)	SAS405 (Profile IX) × UCA9930 (Profile III)
II		UCA9930-15; UCA9930-23
III	UCA992-7; UCA992-10; UCA992-12 UCA992-17; UCA992-19	UCA9930-2; UCA9930-27; UCA9930-31
IV	UCA992-2; UCA992-5; UCA992-6 UCA992-15	
VII		UCA9930-11; UCA9930-18; UCA9930-19
X	UCA992-1; UCA992-13; UCA992-16	UCA9930-8; UCA9930-9; UCA9930-24; UCA9930-26
XI	UCA992-3; UCA992-14	
XII	UCA992-18; UCA992-20	UCA9930-25
XIII		UCA9930-21
XIV		UCA9930-3; UCA9930-10; UCA9930-13
XV		UCA9930-12; UCA9930-16; UCA9930-22
XVI		UCA9930-20
XVII		UCA9930-29
XVIII		UCA9930-7
XIX		UCA9930-5
XX		UCA9930-14
XXI		UCA9930-1; UCA9930-17; UCA9930-28

2. Nine of them were identified as profile III and IV. Seven MSs from this cross had electrophoretic profiles that had not been observed in the 24 strains previously analyzed. These profiles were named profile X, XI and XII (Fig. 1 and Table 2). Data obtained from the EKs of the progeny were statistically analyzed for the segregation of bands that were present in the EK of one of their parents and absent in the other one by Chi-square test (χ^2) (Table 3). A 3.30-Mbp band and a 2.21-Mbp band segregated in a 1:1 ratio showing a Mendelian segregation, indicating that those bands were composed of only one chromosome. However, segregation of 2.52-Mbp, 2.42-Mbp, 2.29-Mbp, and 2.16-Mbp bands was 3:1, indicating that two or more chromosomes might be present in those bands. A 3.17-Mbp band that was present in both parental strains was not observed in 2 out of 16 MSs belonging to the progeny of this cross. PFGE of these 2 descendants corresponded to profile XI, on which two new bands, that were not displayed by either of the two parental strains, were observed (Fig. 1). The molecular weight of such bands (3.30 Mbp and 3.02 Mbp) suggested that they contain chromosomes that initially co-migrated in a 3.17-Mbp band and that were separable after meiosis.

PFGE profiles displayed by the progeny from SAS405 (profile IX) × UCA9930 (profile III) are shown in Table 2 and were identified as: profile II, III, VII, X and XII. Fifteen MSs from this cross had electrophoretic profiles that had not been previously observed in either their parents or the remaining strains analyzed in this study. These profiles were named XIII, XIV, XV, XVI, XVII, XVIII, XIX, XX and XXI (Fig. 1, Table 2). The electrophoretic profile of strain SAS405 differed from that of strain UCA9930 in: (1) four bands (2.70, 2.58, 2.29 and 2.21 Mbp) being present in the former and absent in the latter; and (2) two bands (2.66 and 2.16 Mbp) being absent in the former and present in the latter. Among these, only the 2.16-Mbp band showed a Mendelian segregation (Table 3), so that the five other bands probably contained more than one chromosome. Three bands that were observed in the EKs of both parental strains, were absent in the EKs of several descendants (Fig. 1): (1) a band of 3.72 Mbp was absent in profile XVIII; (2) a band of 2.90 Mbp was absent in profiles XIII, XIV, XVI, XVII and XXI; and (3) a band of 2.42 Mbp which disappeared in profiles VII, XVI and XX. Some new bands that were not observed in the parental strains, showed up on the EKs of some progeny (Fig. 1). Specifically, (1) a 3.30-Mbp band appeared in profiles VII and XII; (2) a 2.94-Mbp band in profile XXI; (3) a 2.80-Mbp band in profiles XVI and XVII; (a) a 2.52-Mbp band in profiles VII, XIX and XX; and (5) a 1.87-Mbp band in profiles II, XII and XVIII.

When the 12 new profiles found in the progeny of the crosses are included, 21 profiles were found for 68 strains, with the number of bands ranging from 5–8, a median of 6.5, and a modal value of 6. Bands of size 3.72 and 3.17 Mbp were observed in 66 out of 68 strains tested. The 2.90-Mbp band were observed in most EKs, except in the ten strains displaying profiles V, XIII, XIV, XVI, XVII and XXI.

The most frequently observed profile was III, with six chromosomal bands, and most of the variability was found for the smallest chromosomes.

Southern blot analyses

Southern blots were hybridized with a restriction fragment containing the rDNA genes from *Drosophila melanogaster*. Hybridization was to a 3.17-Mbp band and to the mtDNA-band for: (1) two strains used in

Table 3. Segregation of chromosomal bands in the progeny from crosses between *Botryotinia fuckeliana* strains with different karyotypes obtained by PFGE.

Cross 'female × male'	Bands (Mbp)	Parental strains phenotype 'female × male'	Ascospore progeny + : −	Values for a 1:1 segregation[b]
SAS56 × UCA992	3.30	+ × −[a]	6:10	0
SAS56 × UCA992	2.52	+ × −	4:12	4*
SAS56 × UCA992	2.42	− × +	12:4	4*
SAS56 × UCA992	2.29	+ × −	4:12	4*
SAS56 × UCA992	2.21	+ × −	5:11	0.25
SAS56 × UCA992	2.16	− × +	12:4	4*
SAS405 × UCA9930	2.70	+ × −	8:20	5.1*
SAS405 × UCA9930	2.66	− × +	20:8	5.1*
SAS405 × UCA9930	2.58	+ × −	6:22	9.1**
SAS405 × UCA9930	2.29	+ × −	5:23	11.5***
SAS405 × UCA9930	2.21	+ × −	8:20	5.1*
SAS405 × UCA9930	2.16	− × +	17:11	1.2

[a] Bands present (+) or absent (−) in the PFGE karyotype of the strains.
[b] Segregation: * $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$.

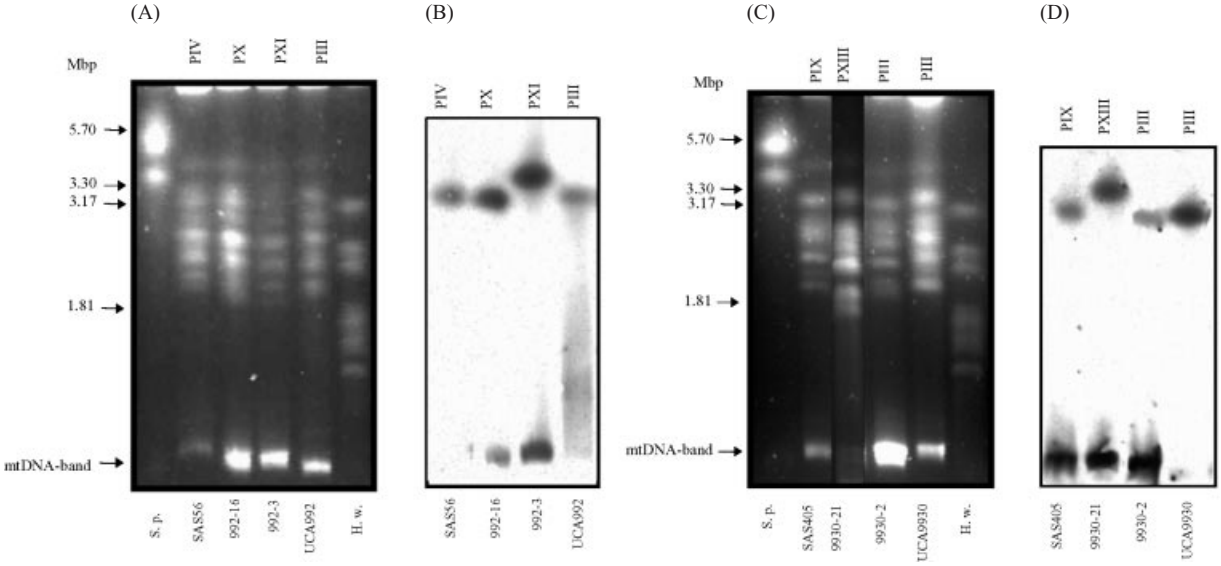

Fig. 2. Chromosomal polymorphisms in *Botryotinia fuckeliana* strains revealed by both PFGE after ethidium bromide staining (A and C) and by hybridization to a rDNA probe (B and D). Names in each lane correspond to strains listed in Table 1. Run conditions were as described in Materials and methods and sizes of the bands were estimated using *Hansenula wingei* (H. w.) and *Schyzosaccharomyces pombe* (S. p.) chromosomes as standards. (A) Electrophoretic karyotype of two parental strains (SAS56 and UCA992) and two monosascospore strains obtained from that cross, showing EK variability among them and with respect to their parents. (B) Autoradiogram of panel A, showing variability in the location of the rDNA genes. (C) Electrophoretic karyotype of two parental strains (SAS405 and UCA9930) and two monosascospore strains obtained from that cross. (D) Autoradiogram of panel C.

crosses (SAS56, in which a very weak signal was detected, and SAS405, in which a strong signal was detected) (Fig. 2); and (2) most of the strains not involved in the crosses.

In UCA992 (used as parent strain), hybridization was to a 3.17-Mbp band but not to the mtDNA-band (Fig. 2B). In most of the strains belonging to the progeny from the cross SAS56 × UCA992, hybridization was to a 3.17-Mbp band and to the mtDNA-band, but exceptions were also found, indicating chromosomal rearrangements after meiosis: (1) in strain UCA992-3 hybridization was to a 3.30-Mbp band (Fig. 2B); and (2) in strains UCA992-13 and UCA992-18 no hybridization to the mtDNA-band was observed.

In UCA9930 (used as parent strain), the rDNA probe strongly hybridized to a 3.17-Mbp band, whereas a very weak hybridization signal was observed on the mtDNA-band, which quite probably contained co-migrating chromosomes (Fig. 2D). In the progeny from SAS405 × UCA9930, hybridization with the rDNA fragment occurred to both the mtDNA-band (all analyzed MSs), and either a 3.17-Mbp band (89.3% of the MSs analyzed) or a 3.30-Mbp band (10.7% of the

Table 4. Sexual crosses between *Botryotinia fuckeliana* strains.

♀/♂	SAS		UCA								Unspermatized control
	56	405	991	992	9930	994	995	996	2100	2850	
SAS											
56	−	+	+	+	−	−	−	+	+	+	−
405	+	−	−	−	+	+	+	+	+	−	−
UCA											
991	+	−	−								−
992	+	−		−							−
9930	−	+			−						−
994	−	+				−					−
995	−	+					−				−
996	−	−						−			−
2100	+	+							−		+
2850	+	−								−	−

♀, female parent; ♂, male parent.
−, no production of apothecia.
+, production of apothecia.

MSs tested), confirming the occurrence of chromosomal-length polymorphisms (Fig. 2D). Interestingly, the latter case was observed in strain UCA9930-21, in which a 3.30-Mbp band was very difficult to be visualized under UV light on PFGE gels stained with ethidium bromide as a band different from that of 3.17 Mbp (Fig. 2C). Therefore, this 3.17 Mbp band contained, at least, two co-migrating chromosomes that could not be distinguished as different bands before hybridization.

Sexual crosses

Reciprocal crosses, unspermatized, and self-fertilized controls between eight strains carrying unknown mating type alleles and two MSs of known mating type (SAS56 and SAS405) resulted in 54 different crosses as shown in Table 4. Mating types were determined by the successful production of apothecia. The number of apothecia ranged from 1–12 per sclerotium and these emerged 1–6 months after spermatization. All strains were sexually compatible with one of the references strains. As a result, UCA9930, UCA994 and UCA995 belong to mating type *MAT1-1*, and UCA991, UCA992 and 2850 contain the idiomorph *MAT1-2*. UCA996 and 2100 produced apothecia when crossed with both SAS56 and SAS405 and were classified as mating type *MAT1-1/2*. Curiously, UCA996 and 2100 only produced normal apothecia when used as spermatizing partners (i.e. apothecia were arised from sclerotia of either reference strain), but their own sclerotia were aberrant regarding apothecia production when spermatized by either reference strains or by themselves: (1) sclerotia of the former did not produce apothecia; and (2) only 20% of the sclerotia of the latter germinated carpogenically, unlike the remaining strains in which 90% of sclerotia involved in successful crosses produced mature apothecia. Also, malformations were detected in most apothecia produced by sclerotia of strain 2100.

DISCUSSION

Karyotypic analysis has been widely used to characterize fungal strains. CLPs have been implicated as a source of variability in most asexually reproducing organisms. Despite the absence of sexual events, genetic heterogeneity in these organisms is more the rule than the exception (Sebens & Thorne 1985), with a great deal of evidence in some fungi (Kohn 1992).

In this study we analyzed the EKs of 24 strains of *Botryotinia fuckeliana*. These strains were isolated from different origins to determine the karyotypic diversity among populations of this species. For the 24 strains tested, we found nine different EK profiles which did not follow any correlation with the host, year of isolation, or phenotypical characteristics. The number of different bands observed on the PFGE gels ranged from 5–8, and most of the variability was restricted to the smallest chromosomes, as has also been reported for *Sclerotinia sclerotiorum* (Fraissenet-Tachet, Reymond-Cotton & Fèvre 1996) and *Fusarium oxysporum* (Alves-Santos *et al*. 1999). CLPs were observed in several strains, but many of them displayed the same EK profile, unlike other fungal species which show a higher EK variability (Kinscherf & Leong 1988, Skinner, Budde & Leong 1991, Zolan 1995). However, care has to be taken when considering the extent to which the EK of this fungus is variable: A higher karyotypic resolution could result in a higher degree of EK variation. Efforts to obtain more bands on the PFGE gels of our strains have been unsuccessful. Two observations suggest, however, that variability in the EK of the isolates may not be much higher than that found under the PFGE conditions used in this work: (1) the relative intensity of some bands is similar in most strains that display the same profile; and (2) the nine profiles obtained for the 24 strains do not differ too much from one another.

The apparent low degree of CLP observed in *B. fuckeliana*, and the generally acknowledged view that

meiosis is inversely correlated to CLPs (Kistler & Miao 1992), suggest that sexual events could take place in *B. fuckeliana* under natural conditions. This is in agreement with Giraud *et al*. (1997), who demonstrated genetic recombination in this fungus and pointed out that only sexual reproduction could exert the selection pressure required to keep the equal frequencies of the mating types found worldwide for *B. fuckeliana*. Controversy has remained for years regarding this issue: (1) *B. fuckeliana* apothecia have only rarely been observed in the field (Gregory 1949), however, the lack of observation of a sexual stage in nature does not prove its absence (Burt *et al*. 1996); and (2) on the other hand, although production of sexual forms in the laboratory for a given organism is not proof of its occurrence in the field (Baker 1989), most of the *B. fuckeliana* strains that have been tested for mating type have been fertile (Groves & Drayton 1939, Groves & Loveland 1953, Faretra & Pollastro 1996, Pollastro *et al*. 1996, Weeds, Beever & Long 1998), which proves that the species has kept its capacity to reproduce sexually. EK uniformity has been found in isolates of *Fusarium moniliforme* (Yan & Dickman 1992), all of which have the ability to undergo meiosis in nature, whereas high EK variability was found among infertile strains of *F. oxysporum* (Boehm, Ploetz & Kistler 1994) and most fungi that reproduce asexually (Zolan 1995). Likewise, a low degree of EK variability supports the idea that *B. fuckeliana* probably reproduces sexually in the field.

Strains with different karyotypes should be, to some extent, reproducibly isolated (Kohn 1992). However, several exceptions have also been reported, and it is unclear to what extent CLPs can inhibit meiosis (Zeigler 1998). Thus, we made crosses between strains with different EK profiles, in the belief that these crosses could help us resolve this question. The crosses showed that the strains we used are fertile, and that chromosomal rearrangements did not affect the capacity to reproduce sexually. Strains with different karyotypes are not genetically isolated from one another. Furthermore, we cultured 100 ascospores; all of them were viable. Similar results had been previously found for *Ustilago hordei* (McCluskey & Mills 1990) and *Saccharomyces cerevisiae* (Ono & Ishino-Arao 1988), in which strains with different EKs were demonstrated to undergo meiosis and produce viable spores. We resolved the EKs of 44 MSs obtained from random sets of ascospores in order to analyze the contribution of meiotic recombination to the EK variability in this species. Only 17 EK profiles out of 44 MSs were coincident with any of the nine profiles described earlier. Among these, five had changed with respect to those of their parents. The recovery of the parental EKs in 12 MSs is much higher than expected if bands assorted independently at meiosis. Therefore, the chromosomes do not appear to assort independently for reasons that are not known, and further investigations are needed before reaching any final conclusion about this point. The remaining MSs were grouped in 12 new profiles, some of which showed new chromosomal bands. New chromosome sizes have been previously reported in EK analysis of the progeny of several fungi (Plumer & Howlet 1993, Martin 1995, Hüsgen *et al*. 1999), and have been attributed to crossing-over between homologous chromosomes having two or more heterologous regions (Ono & Ishino-Arao 1988). Also, some bands (a 3.17-Mbp band for cross SAS56 × UCA992 and bands of 2.16, 2.21, 2.29, 2.58 and 2.70 Mbp for cross SAS405 × UCA9930) that were observed in the EKs of both parental strains were lost in the EKs of some of the descendants. However, the chromosome may only appear to be lost: the appearance of a chromosome with a new size may (1) result in a new karyotypic band, or (2) co-migrate with other/s chromosome/s in a band different from the previous size. In these cases, the chromosome with the new size would apparently disappear in the EKs of some descendants. Co-migration of chromosomes took place in the EKs of the parental strains used in this work, as it has been shown by segregation analysis of the bands that were present in only one of the parents for each cross. Some of those bands did not show a 1:1 segregation, indicating that they were composed of two or more chromosomes (Table 3) and supporting the aneuploidy hypothesis proposed for *B. fuckeliana*. Büttner *et al*. (1994) reported variation in ploidy amongst field isolates but on the other hand, cytological data reported by Shirane *et al*. (1989) indicated that *B. fuckeliana* strains were haploid. All together this represents strong evidence that some strains might not be haploid and that aneuploidy and differences in ploidy levels are present in this species. Our results indicate that, although variations in the DNA content are present, they do not seem to be highly extended in *B. fuckeliana* populations. Since variations in ploidy could limit the possibility of crossing-over at meiosis, the maintenance of those events in a low proportion in field populations could be the result of the balance between EK variation which increases the variability between *B. fuckeliana* isolates during clonal propagation, and the genetic advantages derived from sexual reproduction. The observation of 12 new EKs in the tested progeny suggests that chromosomal rearrangements generated after meiotic recombination provides EK variability in *B. fuckeliana*. Other mechanisms such as translocations or interchanges between non-homologous chromosomes could also provide new chromosomal bands, but since all ascospores analyzed in this work were viable, conventional crossing-over at meiosis seems to be a more plausible mechanism for generating EKs. Some MSs, however, shared the same EK profile as strains isolated from nature. Taking all the evidence together, it can be concluded that: (1) the fungus probably reproduces sexually in the field; and (2) these sexual events are responsible for the degree of EK variation. Similar formation of CLPs have been reported in other species,

for example, in meiotic progeny of field populations of *Leptosphaeria maculans* (Plummer & Howlett 1993, 1995). On the other hand, we detected no changes in the EK of a given strain during two years of repeated subculturing and sample preparation. This suggests that mitotic growth does not provide EK variability in *B. fuckeliana*, unlike other fungal strains as FOM124 of *Fusarium oxysporum* in which karyotypic variability during clonal reproduction has been recently reported (Davière, Langin & Daboussi 2001).

Under the PFGE conditions used in this work, EK variability is not as high as that found for phenotypic characteristics among strains. Strains with the same EK profile analyzed in this work were highly variable for several other phenotypic features (Vallejo *et al*. 1996, 2001; unpublished observations). Morphological, physiological, and pathogenic variability has been widely reported for *B. fuckeliana* (Grindle 1979, Di Lenna *et al*. 1981, Chardonet *et al*. 2000) and has been considered to be due to heterokaryosis, aneuploidy and a variable level of ploidy among strains (van der Vlugt-Bergmans *et al*. 1993, Büttner *et al*. 1994). It is possible that within a single EK profile a higher degree of variability might be present, and that, very probably, the reservoir of genetic variability within the heterokaryotic mycelium of *B. fuckeliana* has been generated by sexual reproduction. Asexual reproduction would contribute to spread that variation among clonal descendants, thus providing the phenotypical variability found in this species.

We tested for the presence of hidden variability in *B. fuckeliana* strains by analysing the presence, location, and variability of the rDNA gene cluster. Hybridization with the rDNA probe detected: (1) a single band of high molecular weight but different in size depending on the strain; and (2) a band of low molecular weight (mtDNA-band), revealing evidence for chromosome rearrangements. A variable location of the rDNA loci in bands of high molecular weight was also found in *Sclerotinia sclerotiorum*, and was considered to be due to translocation and crossing-over during homologous recombination (Fraissenet-Tachet *et al*. 1996). Ono & Ishino-Arao (1988) identified other causes for chromosome rearrangement, such as deletions, duplication, and insertion. Among the latter, transposons, plasmids, viral DNA, mitochondrial DNA, and DNA of a completely heterologous origin are the most plausible sources. In *Fusarium oxysporum*, EK differences result from chromosomal translocations, large deletions, and even more complex rearrangements (Davière *et al*. 2001). In the genome of *B. cinerea*, a retroelement related to the gypsy class of LTR-containing transposons (Diolez *et al*. 1995), as well as double-stranded mycoviruses (Vilches & Castillo 1997, Castro *et al*. 1999) have been identified. These could be involved in chromosomal rearrangements in the fungus. In our laboratory we have recently cloned the *gdh*A gene of *B. cinerea* and found it hybridizes to both a chromosomal band and the mtDNA band (Santos *et al*. 2001), which could also be considered as evidence of chromosome rearrangements in the genome of this pathogen.

Chromosome breakage has been reported to be an explanation for variability in the location of rDNA sequences in *Mucor circinelloides* (Díaz-Mínguez, López-Matas & Eslava 1999), and *Neurospora crassa* (Butler 1992). In the latter, rDNA was found to be a site for chromosome breakage and mapped to the terminal region of the chromosome. It has been suggested that the rDNA gene cluster in *B. cinerea* is close to the end of a specific chromosome (Taga & Murata 1994). Thus, chromosomal breakage can also be a source for differences in hybridization of the rDNA probe on EKs of *B. fuckeliana*. Random breakage of the original rDNA-containing chromosome would lead to a shortening of the chromosome and the translocation of different rDNA segments to other chromosomes. Such an event could result in the hybridization of the rDNA probe to the mtDNA-band in several strains. The high intensity fluorescence of the mtDNA-band stained with ethidium bromide suggested that the band might be composed not only of mtDNA, but also of some other DNA, perhaps from fragments of the initially larger chromosomes. Strains not hybridizing to the mtDNA-band would not have undergone rDNA breakage. Either breakage or transposition events would result in the location of the rRNA genes on a single chromosome, as reported for most fungi analyzed (Debets *et al*. 1990, Masel *et al*. 1990, Kayser & Wöstemeyer 1991, Osiewacz & Ridder 1991).

We conclude that chromosomal rearrangements generated after meiotic recombination do provide variability in EKs of *B. fuckeliana* and that sexual reproduction under natural conditions might be quite important in the life cycle of the fungus. In *B. fuckeliana*, total variability is very likely due to the relative contributions of sexual and asexual reproduction, which would affect the genetic structure of populations, whereas EK variation seems to be mostly due to meiosis.

It has been suggested that the mode of reproduction of microorganisms in natural conditions can only be determined by population genetics (Tibayrenc *et al*. 1991). We show here that the EK can be used to make inferences about the role of meiotic recombination in *B. fuckeliana*.

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