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Analysis of the electrophoretic karyotype of *Flammulina velutipes*

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Abstract The karyotype of *Flammulina velutipes* (Curt.: Fr.) Sing. was analyzed electrophoretically using contour-clamped homogeneous electric field gel electrophoresis and hybridization with DNA probes. The chromosomal DNA from the monokaryon (Fv-4K) and the dikaryon (Fv-4) were resolved into six and eight bands, respectively. The sizes of the chromosomes ranged from 1.9 to 6.0 megabase (Mb) pairs. Each of the separated bands of chromosomal DNA was identified by use of five cloned probes. The number of these chromosomes was estimated to be 6 and 12, respectively; and the size of the entire genome was estimated to be about 20.1 and 38.6 Mb, respectively. From a comparison of the hybridization patterns, the existence of allelic chromosomes of different sizes was deduced in the Fv-4 strain.

Key words *Flammulina velutipes* · Electrophoretic karyotype · Chromosome · CHEF

Introduction

Flammulina velutipes (Curt.: Fr.) Sing. is a well-known edible mushroom that is cultivated extensively all over the world. Nevertheless, relatively little is known about its genetics, and not much breeding information is available.

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In a previous study we isolated genes that were expressed at high levels during the formation of fruiting bodies by differential screening.[1] A novel gene, the *FDS* (*Flammulina velutipes* differentiation-specific) gene, was studied in terms of its structural features and its flanking sequences.[2] Now we are interested in identifying the localization to the genome of genes isolated by differential screening of *F. velutipes*.

In the basidiomycetes the determination of chromosome numbers by cytological methods is difficult because of the small size of the nuclei and the chromosomes. With the development of contour-clamped homogeneous electric field (CHEF) electrophoresis, small fungal chromosomes can be separated electrophoretically. This technique has also provided an alternative method for studies of the structural organization of chromosomal DNA from the basidiomycetes such as *Lentinus edodes*,[3] *Pleurotus cornucopiae*,[4] *P. ostreatus*, *P. pulmonarius*, and *P. salmoneostramineus*.[5]

The electrophoretic karyotypes of *Saccharomyces cerevisiae*,[6] *Schizosaccharomyces pombe*,[7] *Candida albicans*,[8,9] and *Trichoderma reesei*[10,11] have been studied extensively, but gene mapping information is still rather limited.

In this study we determined the electrophoretic karyotype of a monokaryon (Fv-4K) and a dikaryon (Fv-4) of *F. velutipes*. All of the separated bands of chromosomal DNA were identified by Southern hybridization with five previously characterized and cloned probes.[1,2] Using a combination of the CHEF system (Bio-Rad) and Southern blot hybridization, we identified homologous chromosomes by comparing the chromosomes of Fv-4K and Fv-4. Additionally, we recognized polymorphism in electrophoretic karyotypes from variations in the size of allelic chromosomes.

Materials and methods

Strains

The dikaryotic strain (Fv-4) of *Flammulina velutipes* used in this study was a stock culture of the Hokkaido Forest

Products Research Institute. The monokaryotic strain (Fv-4k) was regenerated from a protoplast of the dikaryotic strain (Fv-4) and maintained on a potato-dextrose-agar (PDA) medium.

Preparation of chromosomal DNA

Mycelia that had been grown in a MYG liquid medium (0.5% malt extract, 0.5% yeast extract, 2% glucose) for 3–4 weeks at 25°C in darkness were collected on gauze. The mycelia were quickly frozen in liquid nitrogen and ground to powder. A chilled extraction buffer [1 mM PMSF, 0.5% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol] was mixed with the powder. The mycelial debris was removed by filtration through four layers of gauze and one layer of Miracloth. The filtrate was centrifuged at 2000*g* at 4°C for 20 min. The pellet was resuspend in a fresh solution of 4 mM spermidine, 1 mM spermin, 10 mM EDTA-Na_2, 10 mM Tris-HCl, 80 mM KCl, 1 mM PMSF, 0.5% (v/v) Triton X-100, and 0.1% (v/v) 2-mercaptoethanol and was centrifuged again. The washing step was repeated four times under the same conditions.

The final pellet was washed three times with 0.7 M mannitol solution and resuspended in this solution. The suspension was mixed with an equal volume of 0.8% low-melting-point agarose solution, and the mixture was poured into a plug mold (Bio-Rad) and allowed to solidify on ice for 30 min. Each plug was washed three times with buffer A [0.5 M EDTA-Na_2 (pH 9.5), 0.05 M Tris-HCl, 1% *N*-lauroylsarcosinate] and incubated in buffer A, which contained proteinase K (2 mg/ml) for 24 h at 50°C. The plugs were then stored at 4°C until use.

Electrophoresis

Separation of the chromosomes was performed by CHEF electrophoresis with a CHEF DrII system (Bio-Rad). The running gel was made from 0.8% agarose (chromosomal-grade agarose; Bio-Rad) and 0.5× TBE buffer (10 × TBE: 0.9 M Tris-borate, 20 mM EDTA-Na_2 pH 8.0). Gels were electrophoresed at 14°C in circulating 0.5× TBE buffer with a running voltage (v)/switch interval (s)/running time (h) of 100v/360s/12h, 75v/660s/48h, 75v/1800s/32h and 50v/3600s/28h.[4]

The gels were stained in ethidium bromide 0.1 mg/ml for 40 min and destained in 0.5 × TBE for 90 min. Chromosomal DNA of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used as size markers (Bio-Rad).

Southern hybridization

DNA probes were obtained from the cDNA library that had been used for differential screening described previously.[1,2] The following fragments of cDNA were used as probes for Southern hybridization: B1-4 (700 bp), *FDS* (743 bp), A2-7 (713 bp), A1-14 (680 bp), and B1-2 (710 bp). To minimize fragmentation of chromosomal DNA, gels were irradiated for 5 min with ultraviolet (UV) light (302 nm).

Alkalized DNA was transferred to a Hybond-N^+ membrane (Amersham) as described by the manufacturer. Prehybridization and hybridization were performed as described by Azuma et al.[1] Hybridization was allowed to proceed, being carried out overnight at 42°C. The membranes were subsequently washed twice in 2× SSC (3 M NaCl 0.03 M Na-citrate, pH 7.0) that contained 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min and at 68°C for 30 min followed by 1× SSC that contained 0.1% SDS at 68°C for 30 min.

Radioactivity was recorded on the imaging plate of a Bio-imaging Analyzer (Fujix BAS 2000) with an exposure time of 4 or 12 h. Membranes were used for two or three successive hybridizations after removal of the probe.

Results

Resolution of chromosomal DNA by CHEF

To separate the chromosomes of the Fv-4K and Fv-4 strains, various combinations of switching intervals, voltages, durations, and concentrations of agarose in the gel were examined using the CHEF gel electrophoresis system (data not shown). The DNA of the Fv-4K and Fv-4 strains were separated into six and eight bands, respectively. For the Fv-4K strain, bands of ethidium bromide-stained DNA were estimated to be chromosomes of 6.0, 3.7, 3.4, 2.9, 2.2, and 1.9 Mb. For the Fv-4 strain, they were estimated to be 6.0, 3.7, 3.4 (doublet), 2.9, 2.6, 2.2, 2.0, and 1.9 Mb (Fig. 1, Table 1). For the Fv-4 strain, the relative intensities of fluorescence in UV light of the bands after staining with ethidium bromide suggested that some of them were doublets. Chromosomes of 3.4 Mb seemed not to be separated completely because a pair of similarly sized DNAs was observed. Chromosomes of 3.8 and 3.2 Mb were not clearly seen because of the low intensity of the fluorescence. These assumptions were supported by Southern hybridization.

Table 1. Chromosome size of the monokaryon (Fv-4K) and dikaryon (Fv-4) of *Flammulina velutipes*

Chromosome no.	Size (Mb)	
	Fv-4K	Fv-4
I	6.0	6.0
II		3.8
III	3.7	3.7
IV		3.5
V	3.4	3.4
VI		3.4
VII		3.2
VIII	2.9	2.9
IX		2.6
X	2.2	2.2
XI		2.0
XII	1.9	1.9

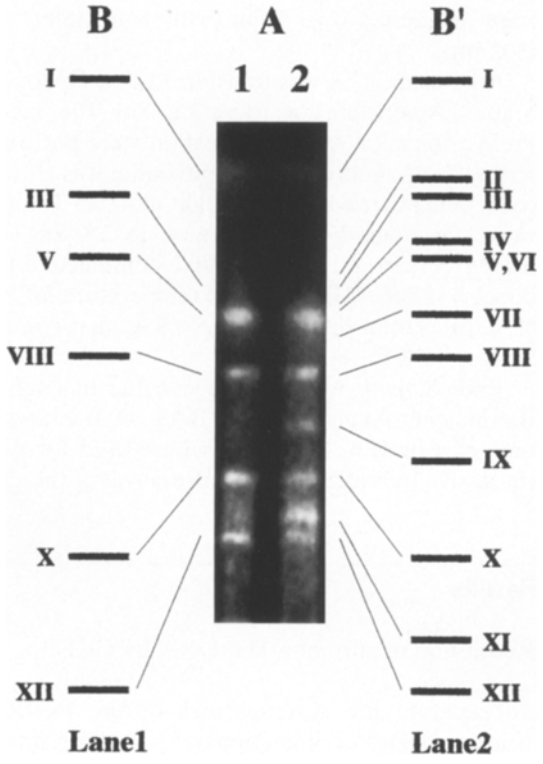

Fig. 1. Contour-clamped homogeneous electric field (CHEF) electrophoresis (*A*) and the profiles of bands of chromosomal DNA obtained by the combination of CHEF electrophoresis and hybridization (*B*, *B'*) of DNA from *Flammulina velutipes*. The number of chromosomes of the monokaryon (Fv-4K, *lane 1*) and dikaryon (Fv-4, *lane 2*) are indicated

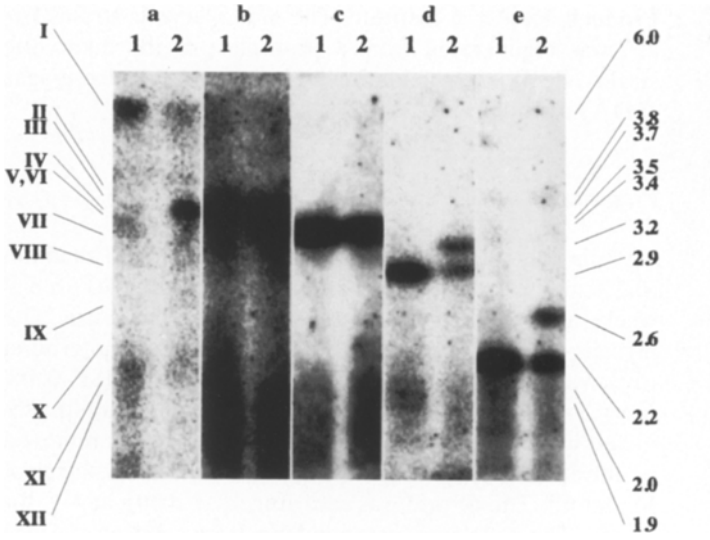

Fig. 2. Southern hybridization analysis of CHEF-resolved chromosomal DNA from *Flammulina velutipes* with the following probes: *a*, B1-4; *b*, FDS; *c*, A2-7; *d*, A1-14; *e*, B1-2. The number of chromosomes are indicated on the *left* of the figure, and the sizes of he chromosomes are indicated on the *right*. *Lane 1*, monokaryon (Fv-4K); *lane 2*, dikaryon (Fv-4)

Table 2. Mapping of five cloned genes that hybridized to the chromosomes of the monokaryon (Fv-4K) and dikaryon (Fv-4)

Chromosome no.	Hybridized probe	
	Fv-4K	Fv-4
I	B1-4	B1-4
II		B1-4
III	*FDS*	*FDS*
IV		*FDS*
V	A2-7	A2-7
VI		A2-7
VII		A1-14
VIII	A1-14	A1-14
IX		B1-2
X	B1-2	B1-2
XI		*FDS*
XII	*FDS*	*FDS*

Chromosomal localization of cloned genes

To obtain additional information about the chromosomes we performed a Southern hybridization using five cloned probes. The probes were obtained from differential screening for genes that are specifically expressed during the differentiation of fruiting bodies.[1] Some of the results of Southern hybridization are shown in Fig. 2. Each probe hybridized with at least one band of chromosomal DNA from the Fv-4K and Fv-4 strains (Fig. 2, Table 2).

The probe B1-4 hybridized with a weak signal to a band of 6.0 Mb (I) from the Fv-4K strain. For the Fv-4 strain it hybridized to the bands of 6.0 Mb (I) and 3.8 Mb (II). Additionally, for the Fv-4 strain, chromosome II was observed as a faint band after staining ethidium bromide, but the hybridization with the B1-4 probe yielded a clear signal. The *FDS* probe hybridized to a chromosome of 3.7 Mb (III) in the Fv-4K strain. Furthermore, the *FDS* probe hybridized to a DNA of 1.9 Mb (XII). For the Fv-4 strain, the *FDS* probe hybridized to 3.7 Mb (III) and 3.5 Mb (IV) DNAs of homologous chromosomes and to 2.0 Mb (XI) and 1.9 Mb (XII) DNAs of homologous chromosomes. The A2-7 probe hybridized to DNA of 3.4 Mb (V) in the Fv-4K strain and to the 3.4 Mb (doublet, V and VI) DNA in the Fv-4 strain.

The A1-14 probe hybridized to DNA of 2.9 Mb (VIII) in the Fv-4K strain and to DNA of 3.2 Mb and 2.9 Mb DNA of homologous chromosomes VII and VIII in the Fv-4 strain. Chromosome VII gave an unclear band with ethidium bromide staining, but hybridization with the A1-14 probe gave a clear signal that corresponded to chromosome VII. The B1-2 probe hybridized to DNA of 2.2 Mb (X) in the Fv-4K strain. For the Fv-4 strain, the B1-2 probe hybridized to homologous chromosomes of 2.6 Mb (IX) and 2.2 Mb (X).

Hence, bands of chromosomal DNA from the Fv-4K strain were estimated to represent 6.0 Mb (chromosome I), 3.7 Mb (III), 3.4 Mb (V), 2.9 Mb (VIII), 2.2 Mb (X), and 1.9 Mb (XII). The total genome size of Fv-4K was estimated to be approximately 20.1 Mb in length (Table 1). The chromosomes in the Fv-4 strain were estimated to be 6.0 Mb (I), 3.8 Mb (II), 3.7 Mb (III), 3.5 Mb (IV), 3.4 Mb (doublet, V

and VI), 3.2 Mb (VII), 2.9 Mb (VIII), 2.6 Mb (IX), 2.2 Mb (X), 2.0 Mb (XI), and 1.9 Mb (XII) in length. The total genome size of the Fv-4 strain was estimated to be approximately 38.6 Mb in length (Table 1).

The combination of the electrophoretic separation of chromosomes and Southern blotting indicated that the Fv-4K and Fv-4 strains have 6 and 12 chromosomes, respectively.

Discussion

Pulsed-field gel electrophoresis is applicable to DNA molecules of much higher molecular weight and separates them with greater resolution than does conventional electrophoresis. It has already proved valuable for analyses of the genomes of *Aspergillus nidulans*,[12] *Candida albicans*,[13] *Neurospora crassa*,[14] and *Trichoderma reesei*.[15] However, it is difficult to characterize weakly stained chromosomal bands.

Our results of Southern hybridization allowed more exact determination of chromosome numbers after pulsed-field gel electrophoresis in both a monokaryon and a dikaryon. In the dikaryon (Fv-4), the chromosome of 3.4Mb appeared to be a doublet after ethidium bromide staining. However, the *FDS* probe hybridized to homologous chromosomes III and IV of 3.7 and 3.5Mb DNA, respectively; and the A2-7 probe strongly hybridized to the 3.4Mb DNA, the assumed homologous chromosomes V and VI. These three overlapping bands could be distinguished based on the results of Southern hybridization. The weakly stained chromosomes of 3.8Mb (II) and 3.2Mb (VII) in the dikaryon nevertheless gave strong and specific hybridization signals with the B1-4 and A1-14 probes.

In the dikaryon, the two bands recognized by each of the five probes represented homologues of the same gene. Differences in banding patterns between the monokaryon and dikaryon can be assumed to have been derived from the considerable size heterogeneity of allelic-chromosome pairs. Each band recognized by the five cloned probes in the monokaryon (Fv-4K) is represented by homologues of the same gene in the dikaryon (Fv-4). The combination of electrophoretic separation of chromosomes and Southern hybridization revealed the numbers and sizes of chromosomes, as well as an indication of the location of the genes used as probes in this study.

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