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SHORT COMMUNICATION

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Electrophoretic karyotype of *Flammulina velutipes* and its variation among monokaryotic progenies

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Abstract The karyotype of *Flammulina velutipes* (Curt. : Fr.) Sing. was investigated using contour-clamped homogeneous electric fields (CHEF) gel electrophoresis. A parental dikaryotic stock, JA, was resolved into at least eight chromosomal DNA bands ranging from 1.4- to 4.9-megabase (Mb) pairs. Overall, little size variation was found among monokaryotic strains with a few major exceptions. Among 13 monokaryotic progenies examined, 11 strains were resolved into at least eight chromosomal DNA bands in a manner similar to the parent dikaryon, whereas the other 2 were resolved into at least seven chromosomes lacking the 2.1-Mb chromosome possessed in the former. A slightly larger size variation was found in a chromosome carrying ribosomal DNA. An estimated haploid genome size of this stock was 24.0 Mb or more.

Key words CHEF · Electrophoretic karyotype · *Flammulina velutipes* · Monokaryotic progenies

Flammulina velutipes (Curt. : Fr.) Sing., "Enokitake," is economically one of the most important edible mushrooms in Japan. Many reports have described the genetics and breeding of this fungus (Brodie 1936; Takemaru 1961; Tonomura 1978; Kitamoto et al. 1993; Nakai et al. 2000). However, the karyotype including the haploid chromosome numbers of this species is unclear (Takemaru et al. 1995; Nogami et al. 1997). Contour-clamped homogeneous electric fields (CHEF) gel electrophoresis enables the separation of chromosome-sized DNA and has been used for karyotype analyses of many fungi (Chu et al. 1986; Mills and McCluskey 1990), including cultivated edible mushrooms such as *Agaricus bisporus* (Lange) Sing. (Royer et al. 1992; Kerrigan et al. 1993), *Lentinula edodes* (Berk.) Pegler (Arima and Morinaga 1993), *Pleurotus* species (Sagawa and Nagata 1992; Tamai et al. 1995), and *F. velutipes* (Kim et al. 2000).

In the present report, we examined the electrophoretic karyotype of *F. velutipes* and its variation among monokaryotic progenies by using CHEF gel electrophoresis. Additionally, the size variation of a chromosome carrying ribosomal DNA was evaluated using Southern blot hybridization.

A commercial variety, "Nakano JA" (JA, hereafter), of *F. velutipes* was used as the parental dikaryotic stock. Individual monokaryotic strains (mJAs), which had been prepared by a dilution culture of basidiospores derived from JA, were also used. The stock and these strains were maintained on MYP agar slants (Bandoni and Johori 1972) that contained 7 g malt extract (Difco, Detroit, MI, USA), 1 g soytone (Difco), 0.5 g yeast extract (Difco), and 15 g agar powder (Wako, Osaka, Japan) in 1000 ml distilled water.

To prepare the intact chromosomal DNA, protoplasts were prepared from mycelia according to the method described by Kinugawa et al. (1989) with slight modifications as follows. Vegetative mycelia were cultured in 30 ml MYPG (MYP liquid media supplemented with 0.5% glucose) for 5 days at 25°C. The cultured mycelia were homogenized in a blender (10000 rpm, 5 min) and a 10-ml aliquot was inoculated into a new 20 ml 2× MYPG. Following 2 days culture with shaking at 96 rpm, the mycelia were collected by centrifugation (12000 rpm, 10 min), and incubated in 0.01% 2-mercaptoethanol at room temperature for 30 min according to the method described by Harris (1982), but without ethylenediaminetetraacetic acid (EDTA). The mycelia were washed with distilled water and incubated with an lytic enzyme solution containing 1% Cellulase "Onozuka" RS (Yakult, Tokyo, Japan) and 1% Driselase N-10-10 (Kyowa Fermentation, Tokyo, Japan) in 0.5 M $MgSO_4$ (pH 5.8) to liberate protoplasts for 2 h at 30°C. The protoplast suspension was mixed with equal volume of

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0.5 M KCl (pH 5.8) to facilitate protoplast precipitation, followed by centrifugation (2000 rpm, 10 min).

An agar plug containing 1.0–1.4 $\times$ 10^9 protoplasts per milliliter was used as a sample plug of intact chromosomal DNA for CHEF gel electrophoresis according to the method of Arima and Morinaga (1993) as follows. CHEF gel electrophoresis was carried out using the CHEF apparatus (CHEF DR II; Bio-Rad, Richmond, CA, USA). The sample plugs were inserted into wells of a gel consisting of 0.8% agarose (chromosomal grade, Bio-Rad) in 0.5$\times$ Tris-borate + EDTA (TBE) solution. Two intact chromosomal DNA from *Saccharomyces cerevisiae* Meyen *ex* Hansen and *Schizosaccharomyces pombe* Lindner were used as size markers (Bio-Rad). The electrophoresis was performed at a constant 45 V with four pulse time intervals of 50, 45, 37, and 25 min for durations of 72, 48, 48, and 24 h, respectively. These conditions were a slightly modified version of those used for *Aspergillus nidulans* (Eidam) Winter (Brody and Carbon 1989) and of *Lentinula edodes* (Berk.) Pegler (Arima and Morinaga 1993). The chromosomal DNA bands separated on the gel were detected under a UV illuminator after staining with 2 μg/ml ethidium bromide.

The internal transcribed spacer region of ribosomal RNA genes (ITS-rDNA) of the stock JA was amplified by polymerase chain reaction (PCR) using internal transcribed spacer-1 (ITS1) (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as a set of primers according to the method of White et al. (1990). The PCR product was recovered from a 1.4% agarose gel and labeled with digoxygenin-dUTP (DIG Easy Hyb; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and then was used as a probe of ITS-rDNA. The probe DNA was hybridized with CHEF-separated chromosomal DNA that had been transferred onto a nylon membrane, Hybond N (Amersham Biosciences, Piscataway, NJ, USA), as followed by Sambrook et al. (1989).

Figure 1 shows the electrophoretic karyotypes of the dikaryotic stock JA and its monokaryotic progenies mJAs resolved on a CHEF gel. The dikaryotic stock JA showed at least eight chromosomal DNA bands, each ranging from 1.4- to 4.9-megabase (Mb) pairs in size (Table 1). The chromosomal DNA bands V and VI were more densely stained on a gel than the other chromosomal DNA bands. Although neither of these two bands were resolved into any more bands under other electrophoretic conditions, it seems that each band would contain two or more chromosomes that comigrated due to similar size.

Overall, little size variation was found among monokaryotic strains with a major exception as follows. A conspicuous karyotype variation among monokaryotic strains was found in numbers of chromosomal DNA bands. The strains were divided into two karyotypes in respect to the presence or absence of a chromosome sized 2.1 Mb (Fig. 1, Table 1). Among 13 strains examined, 11 strains were resolved into at least eight chromosomal DNA bands in a manner similar to the parent dikaryon, whereas the other 2 resolved into at least 7 chromosomes lacking the 2.1-Mb chromosome that was possessed in the former. It is not clear if this difference in chromosome number detected among monokaryons was a result of meiotic recombination and binding with other chromosomes of similar size or if the 2.1-Mb chromosome was a supernumerary chromosome (Jones and Rees 1982; Covert 1998).

Small variations among monokaryotic progenies were found in the sizes of chromosomes I and V. The manner of segregation between two different sizes of the chromosome I was quite simple: four strains had a chromosome sized 4.6 Mb and nine strains had one of 4.9 Mb. This segregation ratio was consistent with an expected ratio of 1:1 ($\chi^2 = 1.92$, $0.1 < P < 0.2$) assuming that these two were a pair of homologous chromosomes. However, each pair of homolo-

Table 1. Estimated size (Mb) of chromosomal DNAs of *Flammulina velutipes* and its variation among 13 monokaryotic progenies

Chromosome	Size		No. monokaryons segregated
	JA[a]	mJAs[b]	
I	4.7	4.6, 4.9	4 and 9, respectively
II	4.4	4.4	No variation
III	4.1	4.1	No variation
IV	3.8	3.8	No variation
V	3.1	3.0–3.3	Varied continuously
VI	2.7	2.7	No variation
2.1-Mb[c]	2.1	2.1 or entirely missing	11 and 2, respectively
VII	1.4	1.4	No variation
Total size[d]	24.2	24.0–24.6	

JA, Nakano JA, the commercial variety of *F. velutipes* used in experiments
[a] Parent dikaryotic stock
[b] Monokaryotic progenies derived from JA
[c] See text
[d] A sum of each chromosome size, except the 2.1-Mb chromosome

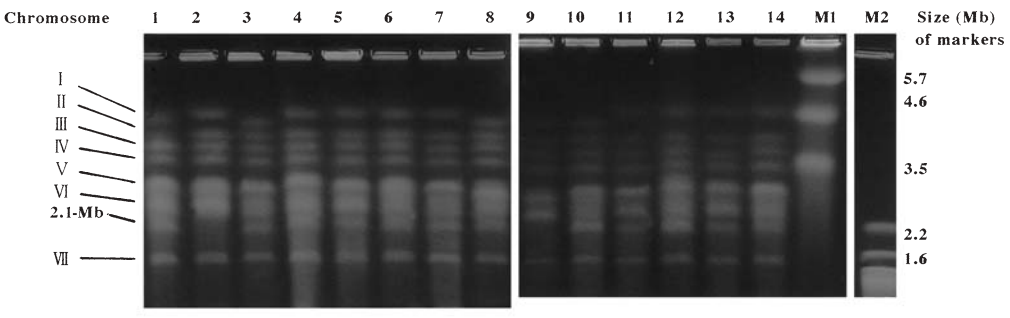

Fig. 1. Contour-clamped homogeneous electrical fields (CHEF) gel electrophoretic karyotypes of *Flammulina velutipes*. Lanes: *1*, JA; *2*, mJA1; *3*, mJA5; *4*, mJA6; *5*, mJA8; *6*, mJA10; *7*, mJA2; *8*, mJA11; *9*, mJA4; *10*, mJA7; *11*, mJA9; *12*, mJA12; *13*, mJA13; *14*, mJA14; *M1*, *Schizosaccharomyces pombe* and; *M2*, *Saccharomyces cerevisiae*

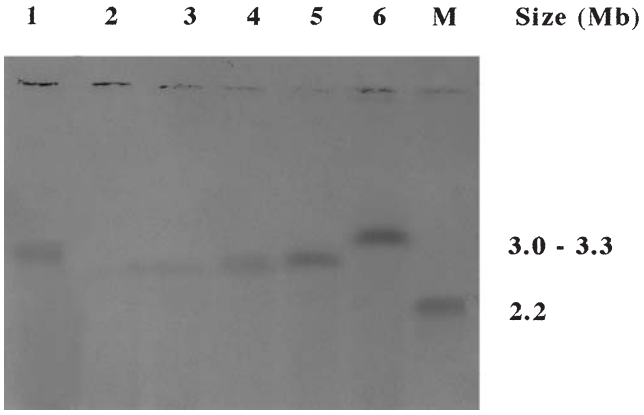

Fig. 2. Southern hybridization of CHEF-separated *F. velutipes* chromosomal DNAs probed with ITS-rDNA. Lanes: *1*, JA; *2*, mJA1; *3*, mJA4; *4*, mJA7; *5*, mJA9; *6*, mJA12; M, *Sa. cerevisiae*

gous chromosome I of the stock JA was not clearly separate on a CHEF gel (Fig. 1). On chromosome V, different sizes including those slightly larger or smaller than that of JA appeared in the monokaryotic progenies, as shown in Fig. 1 and Table 1. The probe of ITS-rDNA hybridized with chromosome V of JA and on chromosomes ranging from approximately 3.0 to 3.3 Mb of the monokaryotic progenies (Fig. 2). This result suggested that chromosome V, which carries ITS-rDNA, is a heteromorphic chromosome. The size variation of a chromosome carrying rDNA has been frequently found in other basidiomycetous species also (McCluskey and Mills 1990; Pukkila and Skrznia 1993; Rustchenko et al. 1993; Zolan 1995).

Based on these results, it is considered that the haploid chromosome number of the stock JA would be seven or more. This count is somewhat larger than those of previous reports of this fungus. Takemaru et al. (1995) and Nogami et al. (1997) reported three and six linkage groups, respectively, by using auxotrophic markers, and Kim et al. (2000) reported six chromosomal DNA bands that were resolved on a CHEF gel using Southern hybridization analysis with five DNA probes. An estimated haploid genome size, which was the sum of each chromosomal DNA band except a band of 2.1 Mb, was 24.0 Mb or more (Table 1). This estimation is larger than that of the previous report of 19.3 or 20.1 Mb by Kim et al. (2000), although somewhat smaller when compared with that of the other Eubasidiomycetes as previously reviewed (Waltz 1995; Zolan 1995).

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