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SHORT COMMUNICATION

Assignment of RAPD marker probes designed from 12 linkage groups of *Flammulina velutipes* to CHEF-separated chromosomal DNAs

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Abstract Electrophoretic karyotype analyses of *Flammulina velutipes* FSB and its monokaryotic progeny, omFSB1 and omFSB2, obtained from oidia were performed by contour-clamped homogeneous electric field (CHEF) gel electrophoresis. At least 11 chromosome-sized DNA bands (CB 1 through CB 11) for FSB, 6 bands for omFSB1, and 7 bands for omFSB2, respectively, were resolved on a CHEF gel. Southern hybridization analysis on CHEF-separated chromosomal DNA of FSB was carried out using RAPD marker probes prepared from each of the 12 linkage groups. The bands CB 1, 2, and 4 each hybridized to two or three probes for different linkage groups. The bands CB 5 and 6 both hybridized to a common probe. The bands CB 3, 7, 8, and 9 each hybridized to a single specific probe for different linkage groups. The two smallest bands (CB 10 and 11) did not hybridize with any probes.

Keywords Electrophoretic karyotype · Enokitake · Haploid genome · Linkage map · Mushroom breeding

Flammulina velutipes (Curt.: Fr.) Sing., the edible enokitake mushroom, was first cultivated using bottled sawdust media in Japan in 1928 and has become one of the economically important edible mushrooms in Japan alongside *Lentinula edodes* (Berk.) Pegler, the shiitake mushroom (Tonomura 1978). Intensive breeding that has been carried out for yield and quality has led to the establishment of several varieties including pure-white fruit-body varieties.

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Recent molecular techniques have accelerated the understanding of the genetics of this fungus (Ando et al. 2001; Sakamoto et al. 2001, 2007; Yamada et al. 2008). However, the genetic linkage map and karyotype, including haploid chromosome number and genome size of this fungus, remain unclear. Takemaru et al. (1995) and Shiomi et al. (2007) reported partial genetic linkage maps based on auxotrophic markers and two mating type factors, although traditional genetic markers such as auxotrophy or isozymes are not sufficient to provide an adequate genetic linkage map for further breeding. Recently, more effective methods to generate DNA markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), or simple sequence repeats (SSR) have been applied to construct a genetic linkage map for several crops, including cultivated edible fungi such as *Agaricus bisporus* (J.E. Lange) Pilát (Kerrigan et al. 1993; Moquet et al. 1999), *L. edodes* (Kwan and Xu 2002; Terashima et al. 2002, 2006; Miyazaki et al. 2008), *Pleurotus ostreatus* (Jacq.) P. Kumm. (Larraya et al. 2000), and *Pleurotus pulmonarius* (Jacq.) P. Kumm. (Okuda et al. 2009). In *F. velutipes*, a rough genetic linkage map consisting of 12 linkage groups was constructed based on RAPD markers, and this allowed assignment of a colony pigmentation factor (Tanesaka et al. 2007).

On the other hand, contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Chu et al. 1986) enables the separation of chromosome-sized DNA and has been used for karyotyping several fungi, as reviewed by Mills and McCluskey (1990), Waltz (1995), and Zolan (1995). By this method, electrophoretic karyotypes have been revealed for many fungi including cultivated edible mushrooms such as *A. bisporus* (Royer et al. 1991, 1992; Sonnenberg et al. 1991; Lodder et al. 1993), *L. edodes* (Arima and Morinaga 1993), *Pleurotus* species (Sagawa

and Nagata 1992; Tamai et al. 1995), and *F. velutipes* (Kim et al. 2000; Tanesaka et al. 2003; Park et al. 2010). Various reports have suggested that *F. velutipes* has at least six (Kim et al. 2000), or six or seven (Park et al. 2010), or seven or eight (Tanesaka et al. 2003) haploid chromosomes. Chromosome-specific probes have also been constructed by assignment of DNA probes prepared from a cDNA library to CHEF-separated chromosomes (Kim et al. 2000; Park et al. 2010). On the other hand, assignment using DNA probes prepared from a genetic linkage map is more effective to elucidate the karyotype involved in haploid chromosome number and genome size and for analysis of meiotic behavior, as demonstrated in *A. bisporus* (Royer et al. 1991, 1992; Kerrigan et al. 1993; Sonnenberg et al. 1996). The present study aimed to assign DNA probes designed from a genetic linkage map based on RAPD markers to CHEF-separated chromosomal DNAs of *F. velutipes*.

A brown mutant stock of *F. velutipes*, FSB, was isolated from a brown fruit body spontaneously derived from the Japanese pure-white commercial variety "ChouKin 6." Two monokaryotic strains, omFSB1 and omFSB2, generated from a population of oidia (asexual spores produced by dedikaryotization) from FSB were also used. Each of these two strains carried one of a pair of conjugate nuclei of the parent FSB (Tatsumi et al. 2004). These stocks and strains were maintained at 10°C on MYP agar slants (Bandoni and Johori 1972) consisting of 7 g malt extract, 1 g soytone, 0.5 g yeast extract, and 15 g agar powder in 1,000 ml distilled water.

To prepare intact chromosomal DNA, protoplasts were liberated from vegetative mycelia according to a method described previously (Tanesaka et al. 2003). An agar plug embedded with 5–15 × 10^8 protoplasts per milliliter was prepared as a sample plug of intact chromosomal DNA for CHEF gel electrophoresis using the CHEF DR II apparatus (Bio-Rad, USA). The sample plugs were inserted into wells of a CHEF gel consisting 0.8% chromosomal grade agarose in 0.5× TBE solution. Intact chromosomal DNA from *Saccharomyces cerevisiae* Meyen & Hansen and *Schizosaccharomyces pombe* Lindner was used as size markers (Bio-Rad). Electrophoresis was performed at a constant 45 V with four pulse time intervals of 3,000, 2,700, 2,200, and 1,800 s for durations of 108, 72, 72, and 36 h, respectively. The chromosome-sized DNAs separated on the gel were detected under a UV illuminator after staining with 2 μg/ml ethidium bromide.

Figure 1 and Table 1 show the electrophoretic karyotypes of dikaryotic stock FSB and two monokaryotic strains, omFSB1 and omFSB2, resolved on a CHEF gel. At least 11 chromosome-sized DNA bands (CB 1 through CB 11) ranging from 4.5 to 1.4 Mb were resolved in dikaryon FSB. Neighboring bands with similar sizes (CB 2 and 3, CB 5 and 6) were not well separated. Of these bands, CB 1, 2, 4, and 5 were more densely stained than the other bands. The smeared tail above 5.7 Mb detected for both monokaryons appeared to be an artifact, because such smears were not detected on other gels of FSB and monokaryon samples separated under different electrophoretic conditions. Such smears might be caused by an interaction between protoplasts at different concentrations embedded in agarose plugs and the electrophoretic conditions, e.g., switch time. In omFSB1, six bands, i.e., CB 1, 2, 4, 6, 8, and 10, and in omFSB2, seven bands, i.e., CB 1, 2, 3, 5, 7, 9, and 11, were detected. Of these bands, CB 1, 2, and 4 in omFSB1 and CB 1, 2, and 5 in omFSB2 were more densely stained than the other bands. The total genome size, obtained by simply summing sizes of individual chromosomes, was 34.8 Mb in FSB, 20.5 Mb in omFSB1, and 23.0 Mb in omFSB2 (Table 1).

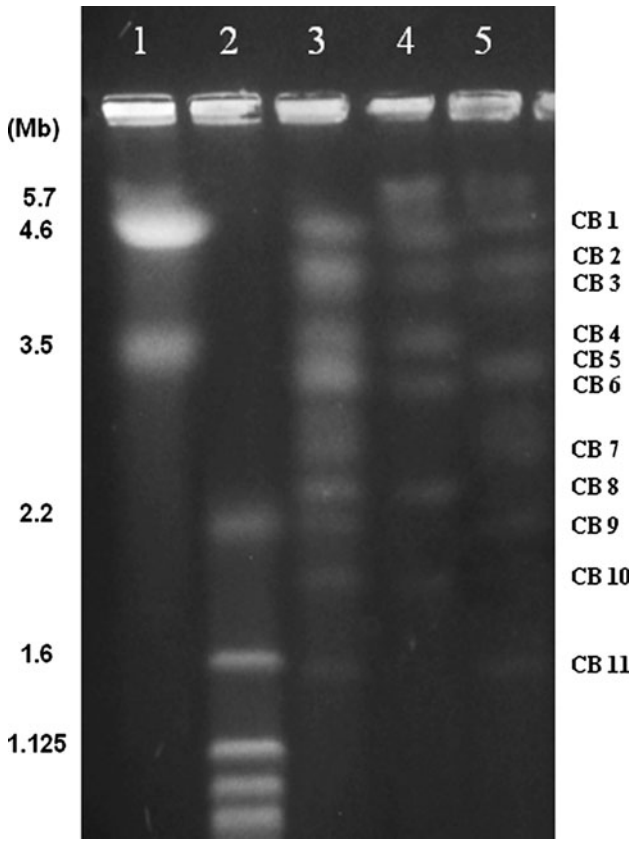

Fig. 1 Contour-clamped homogeneous electric field (CHEF) gel electrophoretic karyotypes of *Flammulina velutipes*. Lanes: *1*, *Schizosaccharomyces pombe*; *2*, *Saccharomyces cerevisiae*; *3*, FSB; *4*, omFSB1; *5*, omFSB2

Karyotype variation among stocks and strains is observed as chromosome length polymorphism (CLP) in several fungi, as reviewed by Zolan (1995). The electrophoretic karyotype of FSB presented here is slightly different from those previously reported for other cultivated

Table 1 Estimated size (Mb) of chromosomal DNA bands of *Flammulina velutipes*

Chromosome	Size			Linkage group (LG) assigned to FSB
	FSB	omFSB1	omFSB2	
CB 1	4.5	4.5	4.5	III, XI
CB 2	4.2	4.2	4.2	VIII, X
CB 3	4.0	n[a]	4.0	VI
CB 4	3.6	3.6	n	I, IV, XII
CB 5	3.5	n	3.5	V
CB 6	3.4	3.4	n	V
CB 7	2.9	n	2.9	II
CB 8	2.7	2.7	n	IX
CB 9	2.5	n	2.5	VII
CB 10	2.1	2.1	n	nh[b]
CB 11	1.4	n	1.4	nh
Total genome size-1[c]	34.8	20.5	23.0	
Total genome size-2[d]		36.4	31.7	

[a] Band not detected

[b] Not hybridized with any probe

[c] Sum of individual chromosome sizes

[d] Sum of individual chromosome sizes by weighting number of assigned linkage groups

varieties of *F. velutipes*; for example, Japanese variety "Nakano-JA" has seven or eight haploid chromosomes ranging from 1.4 to 4.9 Mb, with total sizes of 24.0–24.6 Mb (Tanesaka et al. 2003), and the karyotype from a Korean commercial variety has six or seven haploid chromosomes ranging from 1.60 to 5.84 Mb, with total sizes of 23.98–26.98 Mb (Park et al. 2010). The CLP between parental monokaryons, omFSB1 and omFSB2, presented here and also that observed in a Korean variety (Park et al. 2010) is relatively large in comparison with the CLP among monokaryotic progeny generated from basidiospores (Tanesaka et al. 2003). It is unclear whether the size differences among monokaryons are attributable to a property of individual dikaryons or to other effects, i.e., selection during the meiotic process or spore germination, or afterward to eliminate karyotype aberration among progeny.

A rough genetic linkage map consisting of 12 linkage groups (LG I–XII) of the stock FSB (Fig. 2) was previously constructed using 54 RAPD markers for 80 monokaryotic progeny of FSB (Tanesaka et al. 2007). Each linkage group consists of 2–10 markers with an average of 4.6 markers per linkage group and an interval distance ranging from 3.3 to 37.3 centimorgans (cM) with an average of 22.1 cM. The length of each linkage group ranged from 25.3 to 234.3 cM with an average of 74.7 cM per group, and the total length coverage was 896.8 cM. Figure 2 also shows the 12 RAPD marker probes, symbolized with a solid diamond on the right side of the marker name, designed from each of the 12 linkage groups and prepared as follows. Genomic DNA was extracted from mycelia using cetyltrimethylammonium bromide (CTAB) isolation buffer (Doyle and Doyle 1987) and used as a template for polymerase chain reaction (PCR) with random primers as

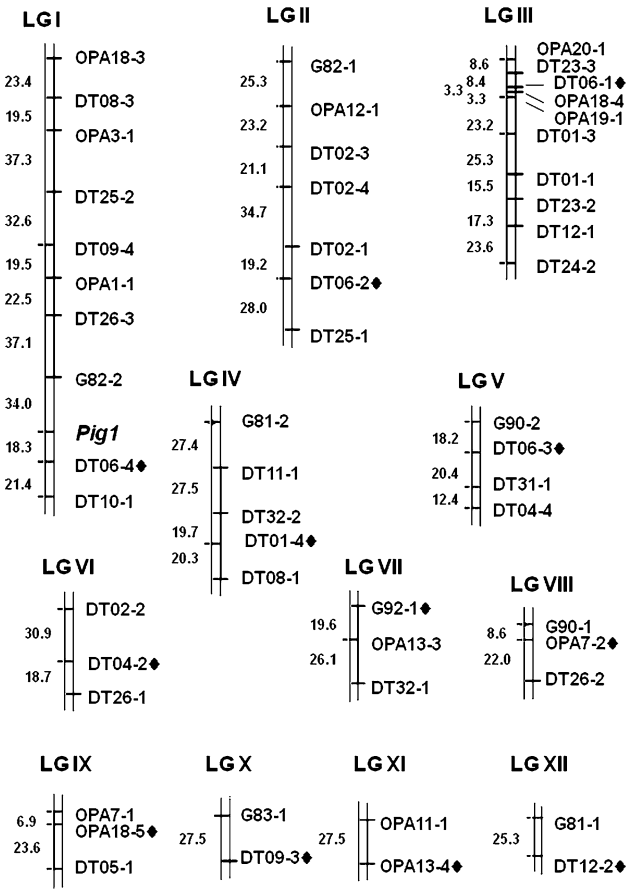

Fig. 2 Genetic linkage map of *Flammulina velutipes* FSB based on 54 random amplified polymorphic DNA (RAPD) markers and a pigmentation factor, *Pig1* (Tanesaka et al. 2007). Each linkage group (*LG*) is indicated by a *vertical line*, and RAPD markers derived from omFSB1 or omFSB2 are indicated by *horizontal bars* projecting to the left or right from a linkage group. Distances between markers are given in Kosambi centimorgans. DNA markers prepared as probes in this study are represented with a *solid diamond*

described previously (Tanesaka et al. 2007). The PCR products of interest were excised and purified from the gel, then labeled with digoxigenin (DIG)-dUTP using a PCR-based labeling kit, the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Germany), according to the manufacturer's instruction manual. The sizes of the obtained probes DT1-4 (LG IV), OPA7-2 (LG VIII), and OPA18-5 (LG IX) were 200–300 bp, and the others ranged from 500 to 1,000 bp. The probe DT06-4 (LG I) was located 18.3 cM away from the colony pigmentation factor *Pig1*. The resulting DIG-labeled probes were directly used for Southern hybridization following blotting of the CHEF-separated chromosomal DNAs onto a Hybond-N^+ membrane (GE Healthcare, Switzerland) according to an established method (Sambrook et al. 1989).

Figure 3 shows the results of Southern hybridization of the 12 probes to CHEF-separated FSB chromosomes. Two probes designed from LG III and XI both hybridized to CB 1, two probes from LG VIII and X hybridized to CB 2, and three probes from LG I, IV, and XII hybridized to CB 4. One probe from LG V hybridized to CB 5 and 6, suggesting these two bands were homologous chromosomes. The other four probes each hybridized to a single chromosomal DNA: probes from LG VI, II, IX, and VII hybridized with CB 3, 7, 8, and 9, respectively. No probes hybridized to the smallest two chromosomes, CB 10 and 11 (Table 1).

Kim et al. (2000) and Park et al. (2010) assigned several DNA probes prepared from a cDNA library to CHEF-separated *F. velutipes* chromosomes using Southern hybridization analysis. This analysis was powerful enough to construct chromosome-specific probes and also enabled detection of weak bands on gels. These approaches, however, are limited to elucidating karyotype involved in haploid chromosome number and genome size because of a limitation on size-dependent chromosome separation on CHEF gels. On the other hand, to estimate haploid chromosome number and quantitative trait loci for breeding purposes, a genetic linkage map using DNA markers is more effective, as already explained. In addition, the assignment of DNA probes designed from a genetic linkage map to CHEF-separated chromosomes is more convincing and reliable to elucidate karyotypes, as demonstrated in the common mushroom *A. bisporus* (Royer et al. 1991, 1992; Kerrigan et al. 1993; Sonnenberg et al. 1996); these workers also observed that chromosomes consisting of different linkage groups that were detected as a single band (but were actually doublets or triplets) were usually more densely stained in gels. In our study on *F. velutipes*, we also observed that three large chromosomal DNAs (CB 1, 2, and 4) that were more densely stained by ethidium bromide in both FSB and monokaryons were each assigned to two or three different linkage groups, indicating that these chromosomal DNA bands were doublets or triplets. No probes hybridized to the smallest two chromosomes, CB 10 and 11, each of which was detected in both omFSB1 and omFSB2, suggesting that they may be a pair of homologous chromosomes.

We examined the haploid chromosome number of this fungus because no hybridization data are available for monokaryons. Nevertheless, the results of the hybridization data on chromosomes of monokaryons separated by FSB and CHEF suggested that CB 4 and 8 detected in omFSB1 and CB 3, 7, and 9 detected in omFSB2 have in common no pair of homologous chromosomes or homologous regions. Although 12 linkage groups were previously calculated for FSB, our results suggest that the haploid omFSB1 has at most 10 chromosomes and omFSB2 at most 9

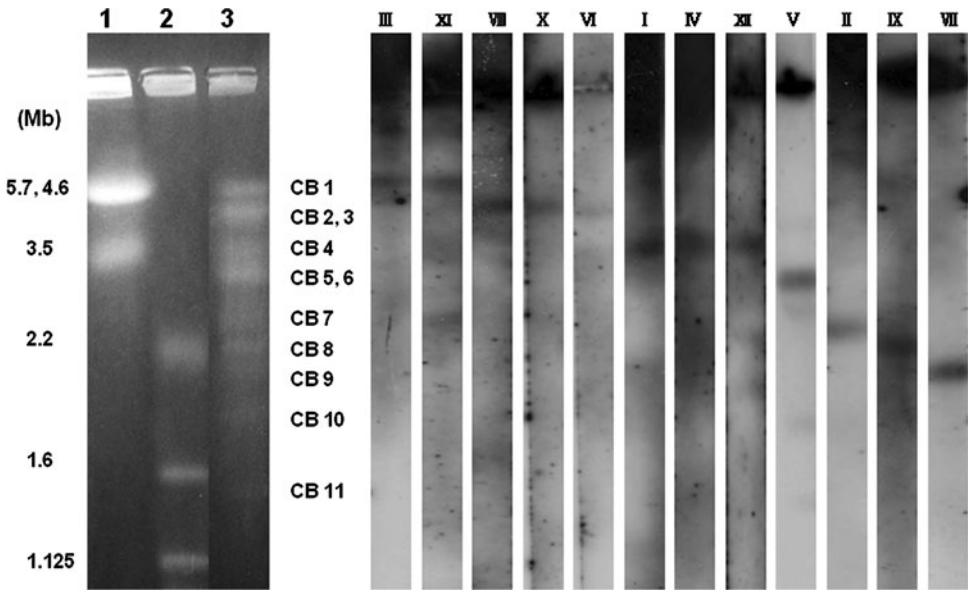

Fig. 3 Southern hybridization of CHEF-separated *Flammulina velutipes* chromosomal DNAs (*left panel*) probed with RAPD markers prepared from individual linkage groups (*right panel*). Lanes *1*, *2* and *3* in the *left panel* are the same as in Fig. 1

chromosomes. On the other hand, the large chromosomal DNAs included small linkage groups consisting of only two markers (LG X, XI, and XII). Based on these results, we assumed that each of these small linkage pairs could be linked associated with larger linkage groups assigned to the same chromosome DNA bands. We also deduced that the haploid fungus has a minimum of 7 chromosomes. Although Southern hybridization analysis was not performed on monokaryons, the haploid genome sizes could be calculated by weighting doublet or triplet chromosomal DNA bands ranging from 7 to 10 (9) chromosomes as 24.1–36.4 Mb in omFSB1 and 23.0–31.7 Mb in omFSB2 (Table 1). This size was 1.0–1.8 times greater than both the result obtained by simple summing of the sizes of separated DNAs here and that previously reported for this fungus (Kim et al. 2000; Tanesaka et al. 2003; Park et al. 2010).

Our study was also able to assign the linkage group (LG I) carrying the colony pigmentation factor *Pig1* to the 3.6-Mb chromosome band CB 4; this band is considered to be a doublet or triplet (LG I and IV, and/or XII). Such an approach of karyotyping coupled with a genetic linkage map enables molecular tagging for gene isolation.

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