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DNA Polymorphisms in *Lentinula edodes*, the Shiitake Mushroom

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DNA restriction fragment length polymorphisms (RFLPs) were examined in *Lentinula edodes* strains. Genomic DNA from strain 70 was cloned in plasmid vector pUC19, and 18 random clones containing low-copy DNA sequences were used to probe seven strains in Southern DNA-DNA hybridizations. Each cloned fragment revealed DNA polymorphism. An RFLP genotype was determined for each strain and the genetic relatedness was assessed. The coefficients of genetic similarity among the seven strains ranged from 0.43 to 0.90. The inheritance of RFLP markers was examined in single spore isolates. Homokaryons displayed a loss of polymorphic bands compared with the parent dikaryon. Hybrids constructed by crossing compatible homokaryons displayed the inheritance of RFLP markers from each parent homokaryon.

Lentinula edodes (Berk.) Pegler, the edible shiitake mushroom, is the second most important mushroom in the global mushroom market (15). World production of shiitake in 1986 was estimated at 314,000 metric tons, approximately 14.4% of total mushroom production (4). Commercial cultivation occurs either on hardwood tree logs (9, 18) or in sawdust media (14). Although this mushroom variety is of significant commercial importance, there are very few reports in the literature with regard to its genetics and breeding. Mating between homokaryons is controlled by two unlinked, heteroallelic incompatibility factors (19). Isozyme analysis revealed genetic variation among isolates of *L. edodes* (16, 17).

To expand on the genetic characterization of this fungus, it is desirable to examine genetic markers at the DNA level. Molecular markers can expedite the organization of shiitake germ plasm for future breeding efforts. In addition, molecular markers may be used to "fingerprint" commercially important strains (10). DNA restriction fragment length polymorphisms (RFLPs) are heritable differences in the lengths of DNA fragments which are generated when DNA is digested by a restriction endonuclease. Molecular markers (RFLPs) have been used extensively in genetic studies of eukaryotes, including humans (2), plants (1, 5), and fungi (3, 7, 12). The present study was undertaken to develop RFLPs as genetic markers for *L. edodes*. In addition, it was of interest to examine the utility of RFLPs in determining genetic relatedness among shiitake strains and their potential application in mushroom breeding and genetics.

MATERIALS AND METHODS

Fungus strains, hybrids, and culture. Dikaryotic mycelial cultures were obtained from various sources, listed in Table 1. Homokaryons were obtained by inoculating a dilute aqueous suspension of basidiospores on malt extract agar (MEA) and incubating it at 23 ± 2°C. Basidiospores were obtained from mushroom spore prints of the respective dikaryotic parents. Colonies resulting from the germination of single spores were transferred to MEA, and a putative homokaryotic mycelium was confirmed by the lack of clamp connections.

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Interstrain hybrids were produced by crossing compatible homokaryons. Crosses were performed by placing portions of two homokaryotic mycelial cultures approximately 1 cm apart on MEA plates. When compatible homokaryons were crossed, a vigorously growing hybrid resulted. Hybrid dikaryons were confirmed by both the presence of clamp connections on the hyphae and fruiting on sawdust media (8).

Mycelial cultures of *L. edodes* (homokaryotic and dikaryotic) were grown in liquid shaker cultures (8) at 23 ± 2°C in a sterile complex medium (the following per liter: 20 g of sucrose, 5 g of yeast extract, 5 g of malt extract, 5 g of peptone, 2 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$, pH 6). Cultures were harvested by filtration, rinsed with deionized water, frozen at −70°C, and lyophilized. The mycelium was ground to a fine powder with a mortar and pestle and used to extract total DNA.

Isolation of genomic DNA and preparation of genomic DNA library. Shiitake DNA was extracted according to the method of Zolan and Pukkila (21). Strain 70 was used to prepare a genomic library. Genomic DNA was digested with *Eco*RI (5 to 10 U/μg of DNA) and subjected to agarose gel electrophoresis on 0.7% agarose at 1.5 V/cm for 16 to 18 h. The gel was stained with ethidium bromide, and DNA fragments 500 to 2,000 bp in length were eluted from the gel and subcloned into pUC19 plasmid vector. The vector was previously digested with *Eco*RI and treated with calf intestinal alkaline phosphatase. The resulting recombinants were used to transform *Escherichia coli* JM83. Transformed white colonies, selected on the basis of antibiotic resistance, were isolated, cultured, and stored in frozen medium at −70°C.

Restriction digestion and Southern blotting. Endonuclease restriction digestion of genomic DNA was performed according to manufacturer's directions, and Southern blotting was conducted as described by Helentjaris et al. (5, 6). DNA fragments were resolved by gel electrophoresis in 0.8% (wt/vol) agarose gels. DNA was blotted on nylon membrane (MSI).

Preparation of radioactive probes and hybridization. DNA probes were labeled by nick translation with a Bethesda Research Laboratories nick translation kit and by using [^{32}P]dCTP according to the manufacturer's instructions. Labeled probes were separated from the unincorporated label with Sephadex G-50 spin columns. The specific activity of labeled probes was 1×10^7 to 5×10^7 cpm/μg of DNA. Procedures for prehybridization, hybridization, washing,

TABLE 1. Strains of *L. edodes* used in RFLP analysis

Strain no.	Source or derivation	Source no.
Dikaryons		
8	American Type Culture Collection	ATCC 48857
31	Japan	J 8b
40	Singapore	S 8
68	Pennsylvania State University	WC 305
69	Pennsylvania State University	WC 325
70	Pennsylvania State University	WC 380
77	California	V3B
Homokaryons		
8A	Dikaryon 8	
8E	Dikaryon 8	
8G	Dikaryon 8	
8H	Dikaryon 8	
8Y	Dikaryon 8	
68F1	Dikaryon 68	
68F2	Dikaryon 68	
68F4	Dikaryon 68	
68F8	Dikaryon 68	
68S4	Dikaryon 68	
68S10	Dikaryon 68	
69A	Dikaryon 69	
69B	Dikaryon 69	
69E	Dikaryon 69	
69F	Dikaryon 69	
69J	Dikaryon 69	
Hybrids		
6	68F1 × 69A	
7	68F4 × 69F	
10	68S10 × 69A	
11	68F4 × 69E	
12	8A × 69B	
14	68F2 × 69F	
15	8H × 69J	
16	8E × 69J	
17	8Y × 69J	
18	68F8 × 69F	
19	68S4 × 69F	

and autoradiography were as described by Helentjaris et al. (5, 6).

Colony hybridizations were performed by using labeled total DNA isolated from strain 70 (11). Clones containing highly repetitive sequences were identified by their characteristic high intensity of hybridization. Clones containing DNA inserts with low repeated sequences were selected from the genomic library for RFLP analysis.

RFLP genotype and data analysis. The genotype of each strain was determined by taking into account the Southern hybridization banding patterns. Each strain was digested separately with two restriction enzymes, *Eco*RI and *Hin*dIII, and probed with a total of 18 different clones. The RFLP genotype for each strain was defined by scoring for the presence (scored as 1) or absence (scored as 0) of every band (of a specific DNA fragment length) hybridizing to a total of 18 cloned DNA fragments. The binary data set was analyzed by using a computer program (20). A correlation matrix was generated and expressed as coefficients of genetic similarity. In pairwise comparisons, if the coefficient of similarity has a value of 1 the two strains are identical, whereas a value of 0 indicates that the strains are completely dissimilar with respect to the RFLP data.

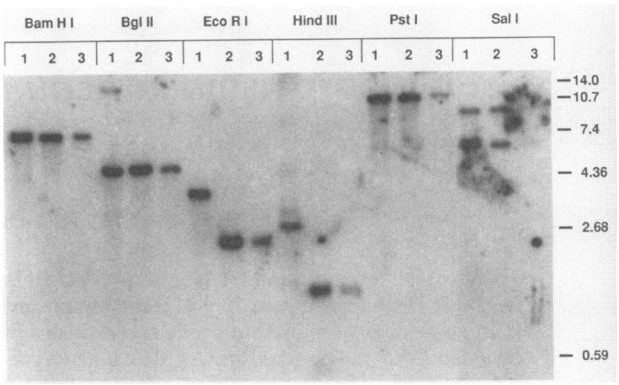

FIG. 1. Southern hybridization of genomic DNA of three shiitake strains (40, 68, and 69; designated 1, 2, and 3) digested separately with six different restriction enzymes and probed with clone 1A1. Size markers are shown to the right in kilobase pairs.

RESULTS AND DISCUSSION

Selection of probes and restriction enzymes. The genomic library containing 617 clones was screened by performing colony hybridizations with total genomic DNA isolated from strain 70. Approximately 90% of clones contained low-copy DNA sequences. These clones were selected to probe shiitake DNA. Initially, several restriction enzymes, including *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I, were used to detect DNA polymorphisms in three *L. edodes* strains (Fig. 1). When DNA from strains 40, 68, and 69 (designated 1, 2, and 3, respectively, in Fig. 1) was probed with probe 1A1 in Southern hybridizations, two enzyme-probe combinations (*Bam*HI-1A1 and *Pst*I-1A1) did not reveal polymorphisms. When additional probes were screened, however, polymorphisms were detected by using any one of the enzymes listed above; the less expensive enzymes (*Eco*RI and *Hin*dIII) were subsequently used to digest genomic DNA.

DNA polymorphism. It was of interest to determine the minimum number of clones that had to be screened in order to detect polymorphisms. Seven shiitake strains (strains 8, 31, 40, 68, 69, 70, and 77) were evaluated for DNA polymorphisms. Total DNA was digested either with *Eco*RI or *Hin*dIII and probed with 18 random genomic clones, containing low copy numbers of repetitive sequence, that were chosen as a result of colony hybridizations with total DNA from strain 70. Each of the 18 clones revealed polymorphisms among the seven shiitake strains. Hence, it was possible to distinguish at least one strain from the rest (Table 2). An example of Southern hybridization is depicted in Fig. 2, which shows *Eco*RI- or *Hin*dIII-digested DNA of seven strains probed with clone 8A5. Southern hybridization patterns were similar for strains 31, 40, and 69 and also for strains 70 and 77. Strain 8 was readily distinguished from the remaining six strains. For DNA digested with *Hin*dIII and probed with clone 8A5, the banding patterns were similar only for strains 31 and 40; strains 8, 68, 69, 70, and 77 had dissimilar hybridization patterns.

By comparing the Southern hybridization patterns, the utility of DNA probes to point out genetic differences among strains was assessed. When *Eco*RI-digested DNA was

TABLE 2. Utility of 18 random probes in differentiating seven shiitake strains by using total DNA that was digested with either *Eco*RI or *Hin*dIII[a]

No. of strains distinguished (out of seven)	No. of probes	
	*Eco*RI	*Hin*dIII
1	1	0
2	6	4
3	1	4
4	4	5
5	4	1
6	2	2
7	0	2

[a] Genomic DNA from seven strains was digested with either *Eco*RI or *Hin*dIII and probed with cloned DNA from strain 70.

probed with 18 random probes, 15 of 18 probes distinguished strain 8 from the rest. Two pairs of strains, however, were indistinguishable; identical banding patterns were observed for strains 31 and 40 and also for strains 70 and 77. However, 13 of 18 probes could distinguish between the two pairs. Only 8 of 18 probes distinguished strain 68 from 69.

When *Hin*dIII-digested DNA was probed with the same 18 probes, 11 of 18 probes distinguished strain 8 from the rest. Interestingly, 9 of 18 probes differentiated strain 31 from 40 and 3 of 18 probes differentiated strain 70 from 77. Strain 68 was differentiated from strain 69 by 11 of 18 probes.

Hence, by using different restriction enzyme-probe combinations, DNA polymorphisms were apparent in the seven shiitake strains. Polymorphisms were reported in the commercial and wild white button mushroom strains of *Agaricus brunnescens* (3, 10), but fewer genotypes were observed in the commercial strains.

Genotype and genetic relatedness. The coefficients of genetic similarity (CS) for the seven *L. edodes* strains ranged from 0.43 to 0.90 (Table 3). Strain pairs 31 and 40 and 70 and 77 appeared closely related (CS = 0.87 and 0.90, respectively). Strain 8, however, was most distantly related to the remaining six strains examined (CS values ranged from 0.43 to 0.54).

By using isozyme analysis to examine the genetic relatedness of 45 shiitake isolates, 24 genotypic classes were

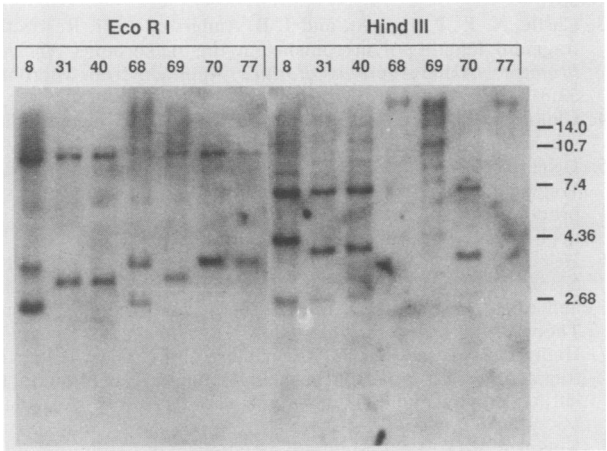

FIG. 2. Southern hybridization of genomic DNA from seven shiitake dikaryons digested with *Eco*RI or *Hin*dIII and probed with clone 8A5. Size markers are shown to the right in kilobase pairs.

TABLE 3. Genetic similarity among seven shiitake strains

Strain	Coefficient of similarity of strain:						
	8	31	40	68	69	70	77
8	1.00	0.54	0.53	0.43	0.46	0.50	0.54
31		1.00	0.87	0.65	0.63	0.80	0.78
40			1.00	0.56	0.66	0.70	0.70
68				1.00	0.65	0.64	0.69
69					1.00	0.61	0.67
70						1.00	0.90
77							1.00

reported (16). The CS values ranged between 0.63 and 0.97 when pairs of genotypic classes were compared. The difference in the observed CS ranges between the two studies (isozyme versus RFLP) may be due to a difference in the isolates used or to the potentially larger number of RFLP markers available in this study.

In this study, the RFLP analysis revealed CS values of 0.65, 0.61, and 0.64 for strain pairs 69 and 68, 69 and 70, and 68 and 70, respectively, whereas isozyme analysis based on 11 genetic loci for the same three strain pairs (16) revealed CS values of 0.87, 0.85, and 0.88, respectively. The lower coefficients of similarity observed by the RFLP method is probably a more accurate estimate, since a larger number of DNA markers were used to probe genetic variation at the genomic level.

Utility of RFLPs as genetic markers in monitoring inheritance. To examine the inheritance of RFLP markers, total DNA from parent dikaryons and their respective homokaryons, each derived from a single basidiospore isolate from the parent, was digested by *Hin*dIII and probed with selected clones (Table 4). In general, if a parent dikaryon was polymorphic, the haploid homokaryon displayed fewer bands. For example, probe 1A4 hybridized to two DNA fragments (4.4 and 5.3 kbp) in the parent dikaryon strains 8, 68, and 69, whereas the haploid homokaryons derived from

TABLE 4. Segregation of DNA RFLP markers in *L. edodes* strains by using total DNA digested with *Hin*dIII

Strain[a]	Fragment size (kbp) detected by:	
	Probe 1A4	Probe 1A5
8	4.4, 5.3	1.4, 4.0
8A	5.3	1.4, 4.0
8H	5.3	1.4, 4.0
8G	4.4	1.4, 4.0
8Y	5.3	1.4, 4.0
68	4.4, 5.3	1.4, 2.2, 4.0, 5.2, 5.8
68F1	4.4	1.4, 4.0, 5.8
68F2	5.3	1.4, 4.0, 5.8
68F8	4.4	2.2, 4.0, 5.2
68S4	4.4	2.2, 4.0, 5.2
68S10	5.3	2.2, 4.0, 5.2
69	4.4, 5.3	4.0, 5.2
69A	5.3	4.0, 5.2
69F	5.3	4.0, 5.2
69H	5.3	4.0, 5.2

[a] Parent heterokaryons are designated by numerals and each derived homokaryon is designated by the number of the respective parent followed by a letter or a letter and a number. Probes were prepared with DNA from strain 70.

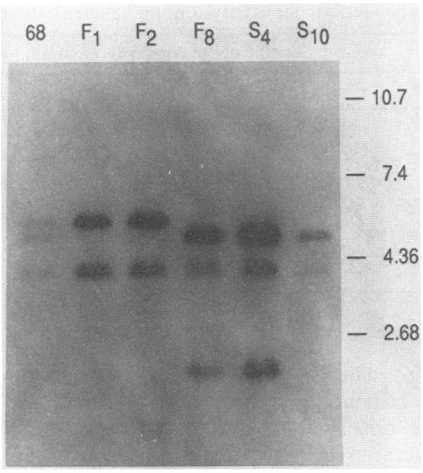

FIG. 3. Southern hybridization of genomic DNA of dikaryotic strain 68 and five homokaryons derived from strain 68 digested with *Hin*dIII and probed with clone 1A5. Size markers are shown to the right in kilobase pairs.

each parent showed only one band of either 4.4 or 5.3 kbp. Loss of polymorphic bands in putative homokaryons was also observed in *A. brunnescens* (3).

Probe 1A5 hybridized to five DNA fragments (1.4, 2.2, 4.0, 5.2, and 5.8 kbp) in dikaryotic strain 68 (Fig. 3), but the same probe hybridized to only three DNA fragments (either 1.4, 4.0, and 5.8 or 2.2, 4.0, and 5.2) in homokaryons which were derived from strain 68 (Table 4), indicative of three genetic loci. This probe detected two monomorphic loci in both strains 8 and 69, as the Southern hybridization patterns were identical in the parents and their respective homokaryons.

The utility of RFLP markers in confirming hybrids constructed by crossing compatible homokaryons was examined. The results of Southern hybridizations using two probes are reported in Table 5. Probe 1C5 hybridized to two DNA fragments (6.1 and 7.9 kbp) in strain 8 and to one DNA fragment each in strain 68 (9.1 kb) and strain 69 (13.6 kb).

TABLE 5. *Hin*dIII DNA fragments of *L. edodes* DNA homologous to probes containing strain 70 DNA

Strain	Fragment size (kbp) detected by:	
	Probe 1C5	Probe 1C6
Dikaryons		
8	6.1, 7.9	10.2, 10.7
68	9.1	7.9
69	13.6	10.2
Hybrids		
6	9.1, 13.6	7.9, 10.2
7	9.1, 13.6	7.9, 10.2
10	9.1, 13.6	7.9, 10.2
11	9.1, 13.6	7.9, 10.2
12	6.1, 13.6	10.2
14	9.1, 13.6	7.9, 10.2
15	7.9, 13.6	7.9, 10.2
16	6.1, 13.6	10.2
17	6.1, 13.6	10.2
18	9.1, 13.6	7.9, 10.2
19	9.1, 13.6	7.9, 10.2

When homokaryotic DNA was probed with clone 1C5 in Southern hybridizations, all homokaryons of strains 8, 68, and 69 showed one hybridization band, either 6.1 or 7.9 kbp for homokaryons of strain 8 and 9.1 and 13.6 kbp for homokaryons of strains 68 and 69, respectively. Hybrids produced by crossing homokaryons from strain 8 with homokaryons from either strain 68 or 69 showed one band (either a 6.1-kbp or a 7.9-kbp band) inherited from strain 8, and in addition there was one band inherited from the other homokaryon, strain 68 (9.1-kbp band) or strain 69 (13.6-kbp band). Hybrids produced by crossing homokaryons from strains 68 and 69 showed two fragments hybridizing to probe 1C5, a 9.1-kbp band inherited from strain 68 and a 13.6-kbp band inherited from strain 69.

Probe 1C6 (Table 5) hybridized to two DNA fragments in strain 8 (10.2 and 10.7 kbp) and one DNA fragment in both strain 68 (7.9 kbp) and strain 69 (10.2 kbp). Interstrain hybrids (designated 12, 16, and 17) constructed by mating homokaryons from strains 8 and 69 showed only one hybridization band (10.2 kbp). Hybrids derived by crossing homokaryons from strain 68 and homokaryons of either strain 8 or strain 69 showed two fragments (7.9 and 10.2 kbp) hybridizing to probe 1C6, the 7.9-kbp sequence presumably inherited from strain 68 and the 10.2-kbp sequence presumably inherited from either strain 8 or 69.

In conclusion, this study revealed RFLP polymorphisms in *L. edodes*. Clones containing low-copy DNA sequences were useful in detecting genetic relatedness among several lines. Informative RFLP markers could be used to examine genetic inheritance and also to fingerprint important commercial strains.

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