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ORIGINAL ARTICLE

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Screening of heterozygous DNA markers in shiitake (*Lentinula edodes*) using de-dikaryotization via preparation of protoplasts and isolation of four meiotic monokaryons from one basidium

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Abstract A suitable screening method for heterozygous DNA markers in shiitake, *Lentinula edodes* (Berk.) Pegler, is reported. Monokaryons were derived from a dikaryon by de-dikaryotization via protoplast formation. Compatibility of the monokaryons was determined by pairwise culture on agar plates. We selected the primers to amplify polymorphic fragments among the original strain (Hokken600:H600) and two monokaryons (H600PP-39 and H600PP-67) showing compatibility. A total of 135 fragments were selected as specific random amplified polymorphic DNAs (RAPDs) resulting from 56 primers of the 147 primers tested. Furthermore, we tested whether the polymorphic fragments segregated into 2:2 among four strains isolated from a basidium. Most of the polymorphic fragments (about 97.8%) showed 2:2 segregation among the four strains. We concluded that the polymorphic fragments were heterozygous if they were detected in either of the monokaryons (H600PP-39 and H600PP-67) and segregated to 2:2 among four meiotic strains (H600B-1, -2, -3, and -4). A total of 132 heterozygous DNA markers were therefore selected from a dikaryon of shiitake (Hokken600:H600).

Key words *Lentinula edodes* · Heterozygous DNA marker · RAPD · de-dikaryotization · Protoplast

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Introduction

Lentinula edodes (shiitake) is cultivated as an important edible mushroom in Japan. Although its cultivation originated in China and Japan, shiitake is now cultivated in most parts of the world using various cultivation techniques under different environmental conditions. As a result of the variations in cultivation techniques and environmental requirements, many varieties of shiitake with suitable qualities are needed. In general, fruit-body formation is necessary to assess the cultivation characters of edible mushrooms. Because fruit-body formation takes a long time, breeding and genetic analysis of shiitake also take much time. Therefore, it is important to develop a molecular biological method for use in breeding and genetic analysis of strains with desired characters in a shorter period. For this purpose, we are constructing a genetic map with molecular markers to relate important characters with the molecular markers and to assess the potential of shiitake strains without cultivation. The genetic maps of *Coprinus cinereus* and *Schizophillum commune* have already been constructed.[1,2] Among the edible mushrooms, a genetic map of *Agaricus bisporus* have been constructed with molecular markers such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD).[3,4]

Monokaryons are useful for genetic analysis as they are haploid and it is possible to culture in an artificial medium in the laboratory. For mushrooms, back-cross is not needed to detect the characters expressed by monokaryons (e.g., detection of carrier of DNA markers).[5] It is reported also that monokaryons could be obtained by preparation and regeneration of protoplasts from dikaryon of edible mushrooms.[6,7] The RAPD marker is used in many organisms and for many purposes, for example, as a probe for RFLP analysis of strains and for detection of linkage markers.[3,8–10] It has been suggested that de-dikaryotization would be useful for screening genetic markers with RAPD.[5] We therefore designed the methods for selection of DNA markers by (1) observing segregation of RAPD markers

among the parent strain and its component monokaryons derived from preparation and regeneration of protoplasts; (2) confirming 2:2 segregation among strains isolated from four haploid basidiospores of one basidium for screening heterozygous DNA markers in *L. edodes*.

Materials and methods

Strains

A cultivated strain (Hokken600:H600) of *L. edodes* was used as the original dikaryon (Table 1). Protoplasts were obtained by digesting cultured mycelia with a mycolytic enzyme solution (1% Novozym234, 0.1% chitinase, 0.5M mannitol, 0.05M MES buffer pH 5.5) at 30°C for 2h.[11] The suspension of protoplasts was diluted to a concentration of 10^5/ml and plated on a medium containing 0.5M sucrose, 0.05M MES buffer pH 7.0, 1% malt extract, 0.4% yeast extract, and 1.5% agar for regeneration. After culturing for 3–7 days, regenerated colonies were isolated and maintained on PDA slants. Altogether 67 strains were obtained by preparation of protoplasts. Regenerated mycelia from protoplasts were examined by microscopy (×400) to select the ones without clamp-connection, which are the monokaryotic cultures. Five strains (H600PP-1, -39, -40, -66, and -67) of the 67 strains obtained were used for mating tests and RAPD analysis (Table 1). H600PP-1 was a dikaryon, and the rest were monokaryons (Table 1).

To obtain the monokaryotic strains isolated from one basidium, pieces of gill taken from a fresh fruit-body were used. The picked gills were softly touched on the PDA plate. Four basidiospores close to each other were considered to be derived from a single basidium and were isolated by a micromanipulator and cultured. The obtained strains were confirmed for the absence of clamp-connections by microscopy (×400), and mating types were determined by a pairwise culture method on PDA agar plates. Four strains (H600B-1, -2, -3, and -4) showing four mating type were used for the analysis (Table 1).

Table 1. Strains, strain names, and distinctions between dikaryon and monokaryon strains

Strains	Dikaryon or monokaryon
Original strain	
H600	Di
Regenerated strains from protoplasts	
H600PP-1	Di
H600PP-39	Mono
H600PP-40	Mono
H600PP-66	Mono
H600PP-67	Mono
Isolated strains from a basidium	
H600B-1	Mono
H600B-2	Mono
H600B-3	Mono
H600B-4	Mono

Mating test

Petri dishes (ϕ 60mm) containing PDA medium (7ml) were inoculated with two pieces of each monokaryon mycelium and incubated at 24°C. The pieces of agar from the two monokaryons in the pairwise test in a petri dish were allowed to grow until two fronts of the advancing mycelia from the agar pieces met and developed a conspicuous contact zone. A piece of tissue was taken from such a contact zone on each plate and examined by miscroscopy (×400) for clamp-connections. If clamp-connections were observed in the contact zone, mycelia in the outer edges of paired colonies were also examined. When clamp-connections were also seen on the edge of paired colonies, sexual compatibility of the mated pair was scored positively.[12]

Preparation of DNA

Mycelia cultured in SMY (1% saccharose, 1% malt extract, 0.4% yeast extract) liquid medium were filtered through miracloth (pore size 10μm). Mycelia were washed (minimum three times each) with distilled water. After mycelia were squeezed with a dried paper towel, a piece of mycelium (about 100mg) was cut. The piece of mycelium was used to prepare DNA.

Crude genomic DNA was prepared by the modified cetyltrimethyl ammonium bromide (CTAB) procedure of Murray and Thompson.[13] A piece of mycelium prepared by the previous method was ground in liquid N_2. Mycelial powder was gently dispersed in an extraction buffer containing 2% CTAB, 0.1M Tris-HCl pH 9.0, 10mM EDTA, and 1.4M NaCl. The mixture was incubated for 60min at 65°C with occasional gentle mixing. The extract was emulsified by gentle inversion with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) for 20min; and after centrifugation (12000*g*, 20min) the aqueous phase was transferred to a new tube. The aqueous phase was then mixed with an equal volume of 2-propanol, and precipitated nucleic acids were observed. The precipitate was centrifuged (15000*g*, 20min). After the supernatant was removed, the pellet was rinsed with 70% ethanol and dried in a vacuum dryer for 15min or until completely dry. The pellet was resuspended in 45μl TE buffer (10mM Tris-HCl, 1mM EDTA). The DNA solution was treated with RNase by adding 5μl RNase solution (1mg/ml) (Wako) to 45μl TE buffer containg the DNA. After incubation at 37°C for 1h, the solution was purified with ELU-QUIK KIT (Schleicher & Shuell) and the purified solution was used as template genomic DNA for the polymerase chain reaction (PCR).

RAPD analysis

The amplification reaction was in volumes of 15μl containing 50mM Tris-HCl pH 8.5, 5mM $MgCl_2$, bovine serum albumin (BSA) 500μg/ml, 0.5mM each dNTP, 2.0% Ficoll 400, 4mM tartrazine, 0.01mM EDTA, 200nM

primer, 1.5 ng genomic DNA, and 0.6 unit Tth DNA polymerase. After predenaturation at 94°C for 1 min, the mixture was subjected to 60 cycles of the following thermal cycle: 10 s at 94°C, 30 s at 36°C, and 60 s at 72°C. Reaction products were analyzed by electrophoresis in 1.0% agarose gels and observed by staining with ethidium bromide. A total of 147 random primers of 10 nucleotides each, obtained from Operon Technologies, were used for RAPD analysis.

Results

Preparation of protoplasts

The number of protoplasts obtained from about 100 mg of *L. edodes* mycelia was 10^4–10^5 cells. This number was low compared with those of other edible mushrooms (e.g., *Pleurotus ostreatus* and others), which are known to yield 10^6–10^7 cells from mycelia of the same weight.[7] Altogether 67 strains from the regenerated colonies were isolated, of which 38 (57%) had clamp-connections. The rest of the strains (29 strains, 43%) did not have clamp-connections. The isolate H600PP-1 (which turned out to be a dikaryon) and four others (H600PP-39, -40, -66, and -67, which turned out to be monokaryons) were then used for the mating tests.

Mating test

Among the crosses tested, the pairs H600PP-39 × H600PP-67, H600PP-40 × H600PP-67, and H600PP-66 × H600PP-67 scored positive compatibility (Fig. 1, Table 2). When the four strains isolated from one basidium (H600B-1, -2, -3, and -4) were mated in all combinations, compatibility was detected in pairs of H600B-1 × H600B-2 and H600B-3 × H600B-4 (Table 3). Furthermore, when regenerated strains from protoplasts and isolated strains from one basidium were mated, compatibility was observed in pairs of H600PP-39 × H600B-3 and H600PP-67 × H600B-4 (Table 3). It was concluded that the mating types of H600PP-39 and H600B-4 and of H600PP-67 and H600B-3 were the same.

Distinction of the nuclear type of strains regenerated from protoplasts by RAPD analysis

Three primers (A02, B10, B11) were used for the analysis to distinguish nuclear types in the four protoplast strains (H600PP-39, -40, -66, and -67). The results of amplification using B10 is presented in Fig. 2. The amplification pattern of H600PP-1 (dikaryon) was the same as the pattern of the original strain, H600, with all primers. (Data for primer B10 are presented in Fig. 2; data for primers A02 and B11 are not presented.)

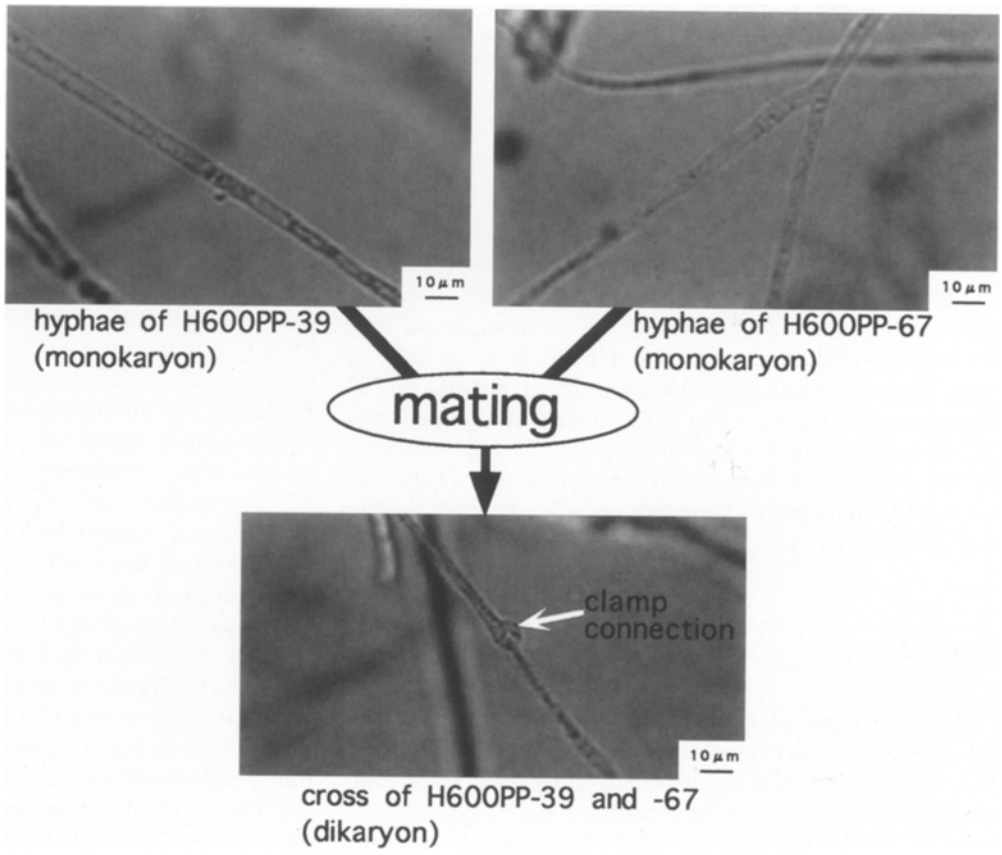

Fig. 1. Detection of compatibility by mating test

Table 2. Results of mating test among strains regenerated from protoplasts (H600PP-)

	−39	−40	−66	−67
−39	−	−	−	+
−40	−	−	−	+
−66	−	−	−	+
−67	+	+	+	−

+, −; presence and absence of clamps, respectively

Table 3. Results of mating tests among strains isolated from a basidium and strains regenerated from protoplasts

	PP-39	PP-67	B-1	B-2	B-3	B-4
PP-39	−	+	−	−	+	−
PP-67	+	−	−	−	−	+
B-1	−	−	−	+	−	−
B-2	−	−	+	−	−	−
B-3	+	−	−	−	−	+
B-4	−	+	−	−	+	−

+, −; presence and absence of clamps, respectively

Four polymorphic fragments (A02-750, B10-1200, B10-1000, B11-1500: primer name- fragment size) were found with these primers. Three monokaryons (H600PP-39, -40, -66) were considered the same nuclear type; and H600PP-67 was considered another nuclear type because H600PP-39, -40, and -66 showed the same amplification pattern (arbitrarily assigned H600PP-39 type), and H600PP-67 showed a different amplification pattern (arbitrarily assigned H600PP-67 type). (Data for primer B10 are presented in Fig. 2; data for primers A02 and B11 are not presented.) A RAPD (B10-1200) was specific in H600PP-67; and the rest of the RAPDs (A02-750, B10-1000, B11-1500) were specific fragments in others.

Screening of heterozygous markers

Primers that could amplify polymorphic markers between H600PP-39 and H600PP-67 were screened by using 147 primers. Altogether 135 fragments were selected as specific RAPDs in strains from 56 of the 147 primers tested. The six strains (H600PP-39 and -67; H600B-1, 2, -3, and -4) were analyzed using the selected primers. A total of 132 heterogeneous markers that were confirmed segregating to 2:2 among H600B-1, -2, -3 and -4 (Fig. 3) were detected by all primers selected by previous screening (Table 4). Of the 132 markers, 61 were H600PP-39 type and 71 were H600PP-67 type.

Discussion

Monokaryons of shiitake could be obtained by dedikaryotization via protoplast preparations, as Peberdy and Fox and Ohmasa et al. have already described.[6,7] However, we needed to investigate the conditions to make protoplasts from *L. edodes* because the yield of protoplasts obtained

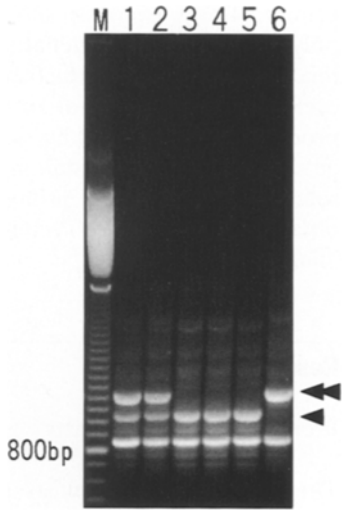

Fig. 2. Distinction of nuclear types of the regenerated strains from protoplasts. Polymerase chain reaction (PCR) products were separated on 1.0% agarose gel with ethidium bromide. Primer used was B10. Lanes 1–6 represent strains H600, H600PP-1, H600PP-39, H600PP-40, H600PP-66, and H600PP-67. *M*, size marker (100 bp ladder marker, Pharmacia Biotech). *Single arrowhead* shows specific fragments to H600PP-39, -40 and -66 (H600PP-39 type). *Double arrowhead* shows the specific fragment to H600PP-67 (H600PP-67 type)

from *L. edodes* mycelia was low compared to that with other edible mushrooms.[7]

The specific RAPD markers in each the derived monokaryons were detected, and the results correspond with the results of the mating tests carried out on the derived monokaryons (H600PP-67, H600PP-39) (see Fig. 2). The RAPDs were useful for identifying nuclear types of the strains obtained from preparation of protoplasts of shiitake, and they compares favorably with the results of Chiu et al.[5] It may be possible to distinguish strains of different nuclear types by their morphological characters (e.g., growth rate). However, distinction depending only on morphological characters could be wrong and misleading. A typical example is the fact that the growth rate of H600PP-66 that was used for this study was slower than that for H600PP-39 and H600PP-40, which are of the same mating type, but it was as fast as H600PP-67, which is of a different mating type (data not shown). However, the different growth rates among the strains of similar mating types are probably due to the mutagenic effects of the process of protoplast preparation. At determining mating type by pair-wise culture, there is a possibility of misapplication; an example is if there is a mutation that does not have the ability to form clamp-connections in one or more of the monokaryons being used for the pair-wise culture tests. We therefore suggest that it is essential to confirm nuclear type using molecular markers, as supported by the results we present here.

It has been shown that shiitake is a typical tetrapolar fungus with two mating factors that are not linked.[14] The four basidiospore strains (H600B -1, -2, -3 and -4) used for this study were distinguished into four mating types by the mating test (Table 3). Based on this result, one crossing-

Table 4. Segregation of heterozygous loci

No.	Locus[a]	H600PP-[b]		H600B-[c]				No.	Locus[a]	H600PP-[b]		H600B-[c]			
		39	67	1	2	3	4			39	67	1	2	3	4
1	A01-1030	−	+	−	−	+	+	68	S16-800	−	+	−	+	+	−
2	A01-600	+	−	+	−	+	−	69	S17-900	−	+	+	−	−	+
3	A02-800	+	−	−	+	−	+	70	S170-650	+	−	+	−	−	+
4	A04-1100	+	−	−	+	+	−	71	T01-1800	+	−	+	+	−	−
5	A04-800	−	+	−	+	−	+	72	T01-950	+	−	+	−	+	−
6	A04-580	+	−	+	−	−	+	73	T01-700	+	−	+	−	−	+
7	A08-1020	−	+	−	+	+	−	74	T01-600	−	+	−	+	−	+
8	A08-970	+	−	+	−	−	+	75	T02-1000	+	−	+	−	−	+
9	A09-850	−	+	+	−	−	+	76	T03-1100	−	+	+	−	−	+
10	A11-1200	−	+	+	−	−	+	77	T07-700	+	−	−	+	−	+
11	A11-1020	−	+	+	−	−	+	78	T11-1200	+	−	−	+	+	−
12	A16-950	−	+	+	−	+	−	79	T11-800	−	+	+	−	−	+
13	A19-1300	−	+	−	+	+	−	80	T16-1100	−	+	+	−	+	−
14	A19-1080	+	−	+	−	−	+	81	T16-900	−	+	−	−	+	+
15	A19-700	+	−	−	+	+	−	82	T17-1200	+	−	+	−	−	+
16	B07-1900	−	+	+	−	+	−	83	T18-1000	−	+	+	−	+	−
17	B10-1200	−	+	−	+	+	−	84	T18-650	+	−	+	−	−	+
18	B10-1080	+	−	+	−	−	+	85	T19-700	+	−	−	+	−	+
19	B11-1500	+	−	−	+	+	−	86	X06-1500	+	−	−	+	+	−
20	B13-1100	−	+	+	−	−	+	87	X06-1050	+	−	−	+	−	+
21	B15-1250	−	+	−	+	+	−	88	X06-1000	−	+	−	+	+	−
22	B15-1200	+	−	+	−	−	+	89	X09-1400	+	−	+	−	−	+
23	C01-1100	−	+	+	−	+	−	90	X09-1100	+	−	−	+	+	−
24	C01-600	+	−	−	+	−	+	91	X09-400	−	+	+	+	−	−
25	C01-550	−	+	−	+	+	−	92	X14-900	+	−	−	+	−	+
26	C15-1900	+	−	+	−	−	+	93	X14-500	+	−	+	−	+	−
27	C15-1500	+	−	+	−	−	+	94	X17-1100	−	+	−	−	+	+
28	C15-1000	−	+	+	−	−	+	95	X17-800	+	−	+	+	−	−
29	C15-700	−	+	−	+	+	−	96	X17-650	−	+	−	+	−	+
30	D02-1200	−	+	−	+	+	−	97	X18-1600	+	−	+	+	−	−
31	D02-1000	−	+	−	+	+	−	98	X18-1300	−	+	−	+	+	−
32	D02-800	+	−	−	+	+	−	99	X18-700	−	+	+	−	+	−
33	F08-1500	+	−	+	−	−	+	100	X18-500	−	+	−	+	+	−
34	F08-950	+	−	+	+	−	−	101	Y04-1500	−	+	−	+	+	−
35	F08-700	+	−	+	+	−	−	102	Y04-1400	−	+	+	−	+	−
36	H05-1200	−	+	−	+	+	−	103	Y04-1250	−	+	+	−	+	−
37	H05-1000	−	+	−	−	+	+	104	Y04-1200	+	−	−	+	−	+
38	I02-600	+	−	+	−	−	+	105	Y04-1150	−	+	+	−	+	−
39	I13-650	+	−	−	+	−	+	106	Y04-700	−	+	+	−	+	−
40	I13-450	+	−	+	−	−	+	107	Y04-660	−	+	+	−	+	−
41	L01-1000	−	+	−	+	+	−	108	Y04-450	+	−	+	−	−	+
42	L01-480	+	−	+	−	−	+	109	Y07-1300	−	+	−	+	+	−
43	L02-800	+	−	−	+	−	+	110	Y07-900	+	−	−	+	−	+
44	L03-680	−	+	−	+	+	−	111	Y07-750	+	−	+	−	−	+
45	L07-820	+	−	−	+	+	−	112	Y10-1400	−	+	+	−	+	−
46	N07-2000	+	−	−	+	−	+	113	Y10-900	+	−	+	−	−	+
47	N07-1700	+	−	−	+	−	+	114	Y14-1000	−	+	−	+	+	−
48	N07-1350	−	+	+	−	+	−	115	Y14-900	+	−	+	−	−	+
49	N07-530	−	+	−	+	+	−	116	Y14-800	−	+	+	−	−	+
50	N09-1200	−	+	−	+	+	−	117	Y14-650	−	+	+	−	−	+
51	N09-900	−	+	−	+	+	−	118	Y15-2200	+	−	−	+	+	−
52	N09-500	+	−	−	+	+	−	119	Y15-2100	−	+	+	−	−	+
53	O03-1600	−	+	+	−	−	+	120	Y15-1200	+	−	−	+	−	+
54	O03-1500	+	−	−	+	−	+	121	Y15-800	−	+	−	−	+	+
55	O07-750	−	+	−	+	+	−	122	Y16-2000	+	−	−	+	+	−
56	Q01-550	−	+	−	−	+	+	123	Y16-1600	−	+	+	−	−	+
57	Q17-1500	−	+	+	−	+	−	124	Y16-1400	−	+	+	−	−	+
58	Q17-1400	−	+	−	+	−	+	125	Y16-950	+	−	−	+	−	+
59	Q17-1300	−	+	−	+	−	+	126	Y16-400	−	+	−	+	+	−
60	Q17-1000	+	−	−	+	+	−	127	Y18-900	−	+	−	+	+	−
61	Q17-800	+	−	−	+	−	+	128	Y18-550	−	+	+	+	−	−
62	S09-1200	−	+	−	+	+	−	129	Y18-500	−	+	−	+	+	−
63	S09-900	−	+	+	−	+	−	130	Y19-1600	−	+	+	−	+	−
64	S09-800	+	−	+	−	−	+	131	Y19-900	+	−	−	+	+	−
65	S14-650	+	−	−	+	−	+	132	Y19-400	+	−	+	+	−	−
66	S14-400	−	+	−	−	+	+	*Total*		61	71	64	69	65	66
67	S16-1500	−	+	+	−	−	+								

+, −; presence and absence of fragments, respectively
[a]Locus, primer name – fragment size (bp)
[b]H600PP-, regenerated strains from protoplasts of H600
[c]H600B-, isolated strains from basidium of H600

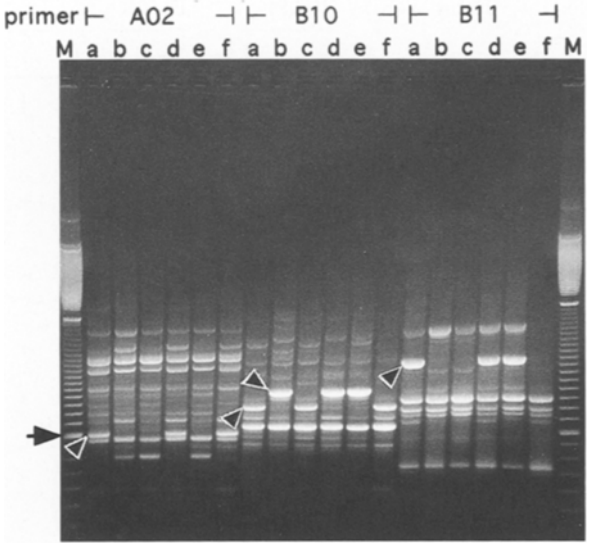

Fig. 3. Screening of heterozygous RAPD markers in shiitake. *Lanes a–f* represent strains H600PP-39, H600PP-67, H600B-1, H600B-2, H600B-3, and H600B-4. *M*, size marker (100 bp ladder marker; *arrow*, 800 bp). *Arrowheads* show fragments regarded as heterozygous marker. Fragments were considered heterozygous when they were detected in either H600PP-39 and or H600PP-67 and segregated to 2:2 among the four meiotic strains from one basidium

over was expected between the centromere and the A factor or B factor during the meiotic phase before the nuclear chromosomes had been distributed to the four basidiospores.[14] We believe these four strains represent normal recombinations and decided to use them for the later experiments.

From the primary screening, 56 of the 147 primers tested were selected by analysis of three strains (i.e., the original strain and two monokaryons regenerated from protoplasts). Altogether 135 fragments were specific RAPDs in either monokaryon strain. Most of the specific RAPDs (132/135, 97.8%) showed a 2:2 segregation among the four strains from one basidium (Fig. 3). The 132 markers that showed normal segregation can be regarded as heterozygous loci existing in the H600 dikaryon. Three fragments, however, did not show normal segregation. It is suspected that amplification of a nonspecific region, recombination within the locus, overlap of two loci, or repeatability of RAPD, among other factors, could be the reasons for abnormal segregation. Our results imply that markers can be screened by analyzing only three strains (original dikaryon strain and two monokaryon strains from the same parent but with a different mating type). This is because most RAPDs showed polymorphism between H600PP-39 and H600PP-67, segregated normally among the four strains from one basidium and were regarded as heterozygous markers. However, we suggest that confirmation is needed to show normal segregation among progenies for the selection of more reliable markers. In addition, we suggest that using sets of haploid strains derived from one basidium are important to improve the precision of genetic mapping. This is because if the analysis is done using sets isolated from one basidium it means that all markers segregate correctly (1:1) in the progenies. Distortions of the segregation ratio that may be caused by the difference in growth rate and viability of spores can be avoided by analyzing sets of haploid strains derived from one basidium.

In conclusion, it is sugested that the preparation of protoplasts leading to de-dikaryotization and confirming the segregation among the four strains derived from one basidium are useful for screening for highly reliable heterozygous RAPD markers in secondary hyphae. This procedure is considered useful for screening heterozygous markers from wild strains and strains obtained by dikaryon-monokaryon mating.[12]

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