Mycoscience (2007) 48:297–304
DOI 10.1007/s10267-007-0372-6
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FULL PAPER

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AFLP analysis for examining genetic differences in cultivated strains and their single-spore isolates and for confirming successful crosses in *Agaricus blazei*

Received: February 19, 2007 / Accepted: June 6, 2007

Abstract Amplified fragment length polymorphism (AFLP) analysis was used to examine genetic differences in *Agaricus blazei* cultivated strains and their single-spore isolates (SSIs). AFLP analysis with five primer combinations identified a total of 267 AFLP bands from nine cultivated strains (one from Brazil and eight from Japan), of which 165 were polymorphic between the nine strains. An AFLP data dendrogram grouped the eight Japanese strains, with the Brazilian strain acting as an outlier, suggesting that the Brazilian and Japanese strains are genetically quite different. Twelve SSIs derived from each of four cultivated strains were subjected to AFLP analysis. All the AFLP bands detected in the cultivated strains were also found in at least one SSI, but some unique bands were detected in SSIs. The total number of AFLP bands from individual SSIs was clearly less than those from their parental strains, and many of polymorphic AFLP bands from the parental strains segregated in SSIs at a ratio of 1:1, suggesting that the SSIs are homokaryotic. Distance values based on presence or absence of individual AFLP bands among SSIs from different strains were clearly higher than those among SSIs from a single strain. In addition, AFLP analysis was shown to be useful in confirming hybrid formation in crosses between SSIs.

Key words *Agaricus blazei* · Amplified fragment length polymorphism (AFLP) · Cultivated strain · Hybrid formation · Single-spore isolate

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Introduction

Agaricus blazei Murrill (Agaricales, Agaricaceae), known as Himematsutake in Japan, is an edible mushroom distributed across North America from Florida to southern California and in South America in the Sao Paolo, Brazil, region (Imazeki and Hongo 1987). The pharmacological effects of *A. blazei*, such as antitumor activity (Mizuno et al. 1990), antihypertensive effects (Eguchi et al. 1999), and antimutagenic potential (Menoli et al. 2001) are of particular interest.

The development of *A. blazei* cultivars with superior properties such as high fruiting body productivity, disease resistance, and human health benefits will be very important for promoting commercial production. In Japan, *A. blazei* is cultivated by a procedure based on the method of *Agaricus bisporus* (Lange) Imbach (e.g., Sumiya 2000). There is, however, little information available about the development of *A. blazei* cultivars. Therefore, many of the spawn cultures used in *A. blazei* cultivation may be derived from wild strains. In this context, fundamental genetic research on *A. blazei* takes on additional importance.

Basic research on the development of superior cultivars, including studies on genetic relatedness between breeding materials (e.g., wild strains and cultivars), has been conducted on many other cultivated mushrooms such as *A. bisporus* (Royse and May 1982; Loftus et al. 1988), *Lentinula edodes* (Berk.) Pegler (Fukuda and Tokimoto 1991; Terashima et al. 2002), *Flammulina velutipes* (Curt.: Fr.) Sing. (Nishizawa et al. 2003), *Pleurotus ostreatus* (Jack: Fr.) Kummer (Matsumoto and Fukumasa-Nakai 1995; Matsumoto et al. 1995), and *Pholiota nameko* (T. Ito) S. Ito and Imai (Obatake et al. 2002). These studies have provided important information on mushroom breeding. Although genetic variations among cultivated strains of *A. blazei* have been examined by random amplified polymorphic DNA (RAPD) analysis (Colauto et al. 2002; Fukuda et al. 2003), additional information is necessary to understand the genetic variation and relatedness among breeding materials.

Mushroom breeding is commonly an empirical process that consists of crossing compatible parental strains (monokaryons or homokaryons). Because *A. blazei* seems to be a tetrasporic species (Wasser et al. 2002), single-spore isolates (SSIs) are estimated to be homokaryotic if each single spore receives only one haploid meiotic nucleus. However, there are no significant differences in cytological characteristics such as nuclear number (about seven on average) per hyphal cell between cultivated strains (probably heterokaryons) and their SSIs (Fukuda et al., unpublished data). Therefore, some other indicators are needed to distinguish between them. In *A. bisporus*, homokaryons have been distinguished from heterokaryons by molecular markers such as isozyme (May and Royse 1982) and restriction fragment length polymorphism (RFLP) markers (Castle et al. 1987). It may also be possible in *A. blazei* to discriminate between heterokaryons and SSIs (i.e., presumed homokaryons), and to recover authentic hybrids from paired colonies between homokaryons by using DNA markers.

Vos et al. (1995) developed amplified fragment length polymorphism (AFLP) analysis based on polymerase chain reaction (PCR) amplification of DNA restriction fragments. AFLP analysis has been utilized to examine genetic differences among strains for a few mushrooms such as *Armillaria* species (Terashima et al. 2001, 2006a), *L. edodes* (Terashima et al. 2002; Matsumoto et al. 2003) and *Tricholoma matsutake* (S. Ito & Imai) Sing. (Chapela and Garbelotto 2004). The main purpose of the present study is to examine genetic differences in *A. blazei* cultivated strains and their SSIs by AFLP analysis, and by extension, to confirm hybrid formation in crosses between SSIs based on AFLP profiles.

Materials and methods

Strains of *A. blazei*

Nine cultivated strains of *A. blazei* were used in this study, including one strain from Brazil (SA514) and eight from Japan (SA515-520, SA527, and SA555). All but SA555 also had been employed in a previous study (Fukuda et al. 2003). These strains were kindly provided by cultivators in Brazil (SA514) and in Japan [Gifu (SA515, 516, 518, and SA527), Aich (SA517 and SA520), and Nagano (SA519 and SA555) prefectures]. Regrettably, details of their origins are uncertain. *A. blazei* strains were maintained in our laboratory on bark-compost malt agar (BMA; hot water extract of 200 g bark compost, 20 g malt extract, and 20 g agar per liter of distilled water).

Recently, *A. blazei* was described as a new species, *Agaricus brasiliensis* Wasser, Didukh, de Amazonas & Stamets (Wasser et al. 2002). Subsequently, Kerrigan (2005) suggested that *A. blazei* is one of the synonyms of *Agaricus subrufescens* Peck based on DNA sequence analyses of internal transcribed spacer (ITS) regions and mating tests. A BLAST (Altschul et al. 1997) search using the DNA database of DNA Data Bank of Japan (DDBJ) revealed that the sequences of ITS regions for SA514 (accession number AB113576) and SA515 (AB113577) showed high levels of identities to those of *A. subrufescens* (identities with SA514 and SA515, 98.3%–100%) and its synonyms [*A. blazei* (97.9%–99.2%), *A. brasiliensis* (98.8%–99.3%), and *Agaricus rufotegulis* Nauta (98.9%–99.0%)] (Kerrigan 2005) in the database, suggesting that these *Agaricus* species are conspecific. It may be appropriate to treat the strains used in this study as *A. subrufescens* if Kerrigan's proposal (2005) is supported.

Isolation of SSIs

To produce fruiting bodies, five BMA plugs (8 mm in diameter) containing mycelia of a cultivated strain were inoculated into 250 g bark compost medium (bark compost : peat moss : wheat bran = 5 : 2 : 2 v : v : v, about 67% moisture content) in a 800-ml polypropylene pot (9 cm in diameter). After 4 weeks incubation at 25°C in the dark, 100 g peat moss (about 67% moisture content) with 2.5% calcium carbonate was used to cover the culture as casing material. After a further 2 weeks incubation in the dark at 25°C, the pot was transferred to 12 h light/12 h dark cycle growing conditions at 23°C to induce fruiting. Mature fruiting bodies were obtained after 40–50 days of incubation under these conditions.

Basidiospores were collected and allowed to germinate on bark-compost agar (BA; BMA without 2% malt extract). Up to 30 germinating basidiospores were randomly isolated, transferred individually to BMA slants, and incubated at 25°C in the dark.

SSI crosses

Crosses between SSIs were made by placing two mycelial plugs (about 25 mm^2) 5 mm apart on BMA plates. After 30 days incubation at 25°C in the dark, the morphology of paired colonies was observed, and small agar plugs (about 1 mm^2) containing mycelium were picked up from the junction zone between the paired SSIs and from a region about 5 mm away from each side of the junction zone and transferred to new BMA plates. Three subcultures at intervals of 15 days at 25°C were performed for some isolates from paired colony by transferring hyphal tips (fewer than five cells) from the colony periphery to new BMA plates.

DNA extraction

To prepare mycelia for total genomic DNA extraction, a small BMA plug (about 25 mm^2) containing mycelium was inoculated into 15 ml BM liquid medium in a 100-ml Erlenmeyer flask and incubated stationary in the dark at 25°C for 14 days. Cultures were fragmented with a Waring blender, and 10 ml homogenate was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml BM liquid medium. Cultures were incubated stationary in the dark at 25°C for

14 days, harvested, washed with distilled water, and lyophilized. Extraction of total genomic DNA was done by the method of Fukumasa-Nakai et al. (1992).

AFLP analysis

AFLP analysis was performed by a procedure based on the method of Vos et al. (1995) with minor modifications: the AFLP Core Reagent Kit (Invitrogen, Carlsbad, CA, USA) and the AFLP Microbial Fingerprinting Kit (Applied Biosystems, Foster City, CA, USA) were used. After DNA (25 ng) digestion and ligation with adaptors using the AFLP Core Reagent Kit, the reaction mixtures were diluted twofold with TE buffer [10 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0)] before preselective amplification.

Preselective amplification was performed using a thermal cycler (model PC-320; Astec, Fukuoka, Japan) in a 20-μl reaction mixture containing 4 μl diluted DNA from the digestion/ligation step, 0.5 μl each of 5 μm primers [*Eco*RI (E) primer (5′-GAC TGC GTA CCA ATT C-3′) and *Mse*I (M) primer (5′-GAT GAG TCC TGA GTA A-3′) (Vos et al. 1995)], and 15 μl AFLP amplification core mix (Applied Biosystems). The thermal cycling program was as follows: 2.5 min initial denaturation at 94°C; 30 cycles consisting of 0.5 min denaturation at 94°C, 1 min primer annealing at 56°C, and 1 min extension at 72°C; and a final 8.5 min extension at 72°C. Preselective amplification reaction mixtures were diluted fivefold with TE.

Selective amplification was performed using E and M primers having two additional nucleotides; four E + 2 (+AA, +AC, +AG, or +AT) primers and four M + 2 (+CA, +CC, +CG, or +CT) primers. The E + 2 primers were labeled with fluorescent dye (fluorescein isothocyanate, FITC). Then, 1.5 μl diluted preselective amplification reaction mixture was mixed with 0.5 μl dye-labeled 1 μm E + 2 primer, 0.5 μl 5 μm M + 2 primer, and 7.5 μl AFLP amplification core mix (Applied Biosystems). Selective PCR was performed following the supplier's AFLP Microbial Fingerprinting Kit specifications. To select primer combinations for selective amplification, 16 different primer combinations (four E + 2 primers × four M + 2 primers) were tested on two strains (SA514 and SA516). Five primer combinations, E + AC/M + CA, E + AC/M + CC, E + AC/M + CG, E + AG/M + CC, and E + AT/M + CA, were selected for selective amplification to detect many polymorphic AFLP bands between the two strains.

Electrophoresis and detection of amplified fragments were performed using a DNA sequencer (model 2000L; Shimadzu, Tokyo, Japan). A 100-bp fluorescein molecular ruler (Bio-Rad, Hercules, CA, USA) was used as a molecular size standard. PCR reactions were repeated at least twice to confirm the reproducibility of each AFLP band.

Data analysis

The presence or absence of individual AFLP bands was scored, and a distance value (D), based on the scoring data, was calculated between the strains as $D = 1 - 2(N_{XY})/(N_X + N_Y)$, in which N_{XY} is the number of AFLP bands shared between the strains, and N_X and N_Y are the total number of AFLP bands in strains X and Y (Nei and Li 1979). A dendrogram based on the distance values was constructed by an unweighted pair-group method with arithmetic clustering (UPGMA) analysis using the PHYLIP software package, version 3.57c (Felsenstein 1995). Because fragments smaller than 100 bases in length gave low reproducibility, and fragments greater than 1000 bases were not clearly determined, DNA fragments of 100–1000 bases were used for data analysis.

Results and discussion

AFLP analysis for cultivated strains

Part of an electrophoresis image of AFLP analysis is shown in Fig. 1. AFLP patterns by each of the five primer

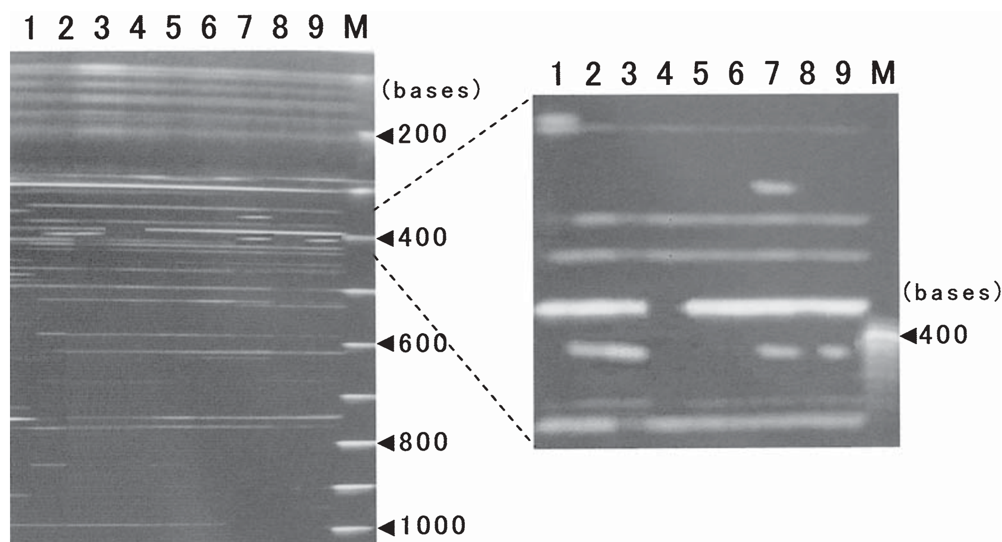

Fig. 1. Amplified fragment length polymorphism (AFLP) analysis electrophoresis gel using the *Eco*RI + AT / *Mse*I + CA primer combination for nine *Agaricus blazei* cultivated strains. *Lanes 1–9*, SA514, SA515, SA516, SA517, SA518, SA519, SA520, SA527, and SA555, respectively; *lane M*, fluorescein molecular ruler (100 bp)

Table 1. Number of amplified fragment length polymorphism (AFLP) bands from nine *Agaricus blazei* cultivated strains using five primer combinations

Primer combination	E + AC/ M + CA	E + AC/ M + CC	E + AC/ M + CG	E + AG/ M + CC	E + AT/ M + CA	Total
Total number of AFLP bands	58	52	42	50	65	267
Number of AFLP bands in each strain[a]	36–41 (38.7)	30–39 (33.8)	23–31 (25.4)	29–41 (35.7)	42–55 (48.3)	170–197 (181.9)
Total number of polymorphic AFLP bands	41	37	27	23	37	165
Percentage of polymorphic AFLP bands	70.7	71.2	64.3	46.0	56.9	61.8

[a] Number in parentheses indicates the average value

Table 2. Distance matrix based on data from AFLP analysis of *Agaricus blazei* cultivated strains

Strain no.	SA514	SA515	SA516	SA517	SA518	SA519	SA520	SA527	SA555
SA514	0.000								
SA515	0.328	0.000							
SA516	0.320	0.165	0.000						
SA517	0.306	0.155	0.150	0.000					
SA518	0.301	0.155	0.041	0.151	0.000				
SA519	0.333	0.170	0.048	0.161	0.033	0.000			
SA520	0.303	0.140	0.125	0.124	0.141	0.145	0.000		
SA527	0.305	0.157	0.042	0.136	0.027	0.023	0.127	0.000	
SA555	0.309	0.165	0.056	0.139	0.052	0.048	0.135	0.025	0.000

AFLP, amplified fragment length polymorphism

combinations varied in the nine *A. blazei* cultivated strains. Each of the nine strains had a distinct AFLP profile, and a single primer pair was adequate to distinguish between the nine strains.

The total number of DNA fragments produced from the nine strains by an individual primer combination ranged from 42 (for the primer combination E + AC/M + CG) to 65 (for E + AT/M + CA) (Table 1). The polymorphic DNA fragment number for each primer combination ranged from 23 (E + AG/M + CC) to 41 (E + AC/M + CA), with a mean of 33.0 polymorphic AFLP bands per primer combination (see Table 1). Thus, AFLP analysis with the five primer combinations identified a total of 267 AFLP bands, of which 165 (61.8%) were polymorphic between the nine strains.

Previous RAPD analysis with eight of the nine strains used in this study and with eight primers detected four to ten DNA fragments per primer (Fukuda et al. 2003), about one-sixth the number of DNA fragments per reaction as the present AFLP analysis. In comparison, AFLP analysis of 15 Japanese cultivated *L. edodes* strains of various fruiting season types produced a total of 190 AFLP bands and 113 polymorphic AFLP bands (59.5%, polymorphic/total bands) using four common primer combinations (E + AC/M + CA, E + AC/M + CC, E + AC/M + CG, and E + AT/M + CA) (Terashima et al. 2002), which are also employed in this study. The degree of genetic variation among the nine *A. blazei* strains used in this study may be similar to that among the 15 various cultivated strains of *L. edodes*, because we observed a total of 217 AFLP bands and 142 polymorphic AFLP bands (65.4%) among them using the four primer combinations.

To infer the genetic relationships among the nine cultivated strains, distance values were calculated using all

Fig. 2. Unweighted pair-group method with arithmetic clustering (UPGMA) dendrogram based on data from AFLP analysis of *Agaricus blazei* cultivated strains

267 AFLP bands. Distance values ranged from 0.023 with the least dissimilarity between SA519 and SA527 to 0.333 with the greatest dissimilarity between SA514 and SA519 (Table 2). A dendrogram was constructed by UPGMA analysis based on the distance matrix (Fig. 2). All strains, except SA514, grouped together with distance values below 0.158, and this group was linked to SA514 with a value of 0.313. Dendrogram based on RAPD data have also been

Table 3. Number of AFLP bands detected by AFLP analysis using five primer combinations and distance values among the isolates within intrastrain combinations

	Single-spore origin strains			
	SA514	SA515	SA517	SA520
Total number of AFLP bands	181	212	183	224
Number of AFLP bands in each isolate[a]	117–127 (122.6)	136–166 (152.1)	92–137 (114.2)	109–156 (131.1)
Number of AFLP bands in heterokaryotic strain	169	195	173	202
Total number of polymorphic AFLP bands[b]	108	137	129	154
Distance value[a]	0.131–0.258 (0.192)	0.088–0.308 (0.176)	0.112–0.333 (0.232)	0.112–0.280 (0.195)

[a] Number in parentheses indicates the average value
[b] Polymorphic bands that are also detected for the original strain

constructed (Fukuda et al. 2003). The dendrograms from both the analyses show that there are clear genetic differences between the Brazilian strain (SA514) and the Japanese cultivated strains. However, different relationships within the Japanese cultivated strains were observed between the two dendrograms. The AFLP dendrogram may more accurately describe the relationship among the Japanese cultivated strains because of the quantity of analyzed bands (56 for RAPD vs. 267 for AFLP), although further studies are required to clarify their genetic relationships, including mating type analysis (although even the mating system of *A. blazei* is as yet uncertain). It will also be important to examine the relationship between the genotype of each strain and phenotypes, such as fruiting body productivity and pharmacological effects, to efficiently develop new and superior cultivars of *A. blazei*.

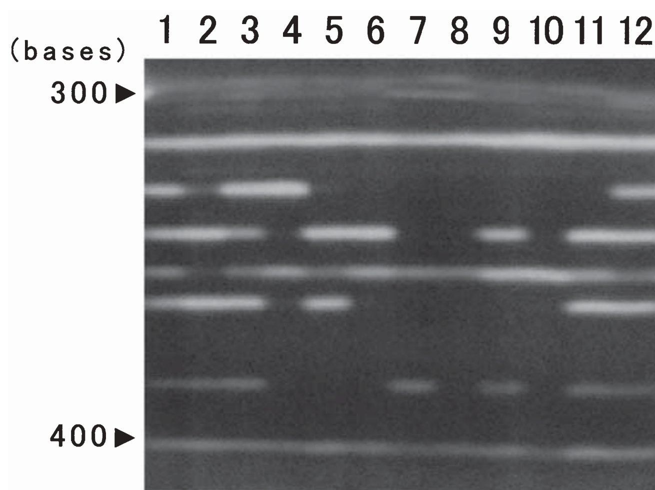

Fig. 3. AFLP analysis electrophoresis gel using the *Eco*RI + AC / *Mse*I + CC primer combination for 12 single-spore isolates (SSIs) of *Agaricus blazei* cultivated strain SA515. *Lanes 1–12*, SSIs 1–12, respectively. *Numbers on the left* indicate fragment sizes in fluorescein molecular ruler (100 bp)

AFLP analysis of SSIs

Up to 30 SSIs were obtained from each of four cultivated strains (SA514, SA515, SA517, and SA520). Of these, 12 SSIs from each strain were randomly selected and subjected to AFLP analysis. Part of an electrophoresis image is shown in Fig. 3, and the results from this analysis are summarized in Table 3.

Use of the five primer combinations produced a total of 181, 212, 183, and 224 AFLP bands from SSIs derived from strains SA514, SA515, SA517, and SA520, respectively. All the AFLP bands detected in the parental strains were also detected in at least one SSI. In addition, SSIs had unique bands that were not found in the parental strains. SA514 SSIs had 12 unique AFLP bands, SA515 SSIs had 17, SA517 SSIs had 10, and SA520 SSIs had 22. In *A. bisporus*, a novel RFLP band (different in length from a parental heterokaryotic strain) in a progeny has been observed, as probably a result of crossing over of chromosomes in the parental strain (Summerbell et al. 1989). The unique bands detected in this study may also be a result of crossing over of chromosomes in the parental strains.

The total number of AFLP bands from individual SSIs were clearly less than those from their parental strains: 69.2%–75.1% (mean, 72.5%), 69.7%–85.1% (78.0), 53.1%–79.2% (66.0%), and 54.0%–77.2% (64.9%) for SA514, SA515, SA517, and SA520, respectively. On the other hand, polymorphic AFLP bands that were also detected in the original parental strain are probably derived from heterogeneous regions on homologous chromosomes in the parental strain. Therefore, each progeny should have half the number of such polymorphic AFLP bands if meiotic recombination occurs randomly between the chromosomes. The mean values of polymorphic band totals for individual SSIs were 47.6 for SA514 (96 for the parental strain), 75.3 for SA515 (121), 61.2 for SA517 (119), and 60.8 for SA520 (130). Thus, about half the number of total polymorphic bands within parental strains were detected in their SSIs. In addition, each polymorphic band should be detected in progenies at a ratio of 1:1 if meiotic segregation is random. In fact, a chi-square distribution analysis of the segregation of many polymorphic AFLP bands supported a ratio of 1:1 ($\chi^2 < 3.84$); 85, 77, 81, and 85 bands for SA514, SA515, SA517, and SA520, respectively. These results indicate that the SSIs analyzed in this study are homokaryotic. Therefore, the SSIs of *A. blazei* will be useful in establishing hybrid (heterokaryotic) strains in breeding. Kerrigan (2005) reported that some SSIs of *A. subrufescens* were heterokaryotic whereas other SSIs were homokaryotic. Therefore, some heterokaryotic isolates may be found by analyzing more SSIs from the strains used in this study.

Further studies, such as cytological observation of nuclear behavior during basidiospore formation, are required to clarify the nuclear phases of the SSIs of *A. blazei*.

Means of distance values among the SSIs from each parental strain ranged from 0.176 for SA515 to 0.232 for SA517 (see Table 3). Distance values among the SSIs from different strains were higher than those from a single strain, ranging from 0.371 (mean value between SA517 and SA520) to 0.496 (between SA514 and SA515) (Table 4). Thus, distance values among the SSIs within interstrain combinations were clearly higher than those within intrastrain combinations. Although genetic similarities between SSIs may be predicted from those of their parental strains, the use of data obtained directly from SSIs reflects their actual genetic similarities. This information will be useful for planning effective breeding programs. For example, the selection of parental strain combinations can be used to avoid inbreeding depression.

Confirmation of crosses between SSIs by AFLP

The mating system and mating reactions of *A. blazei* are not well understood. Heterokaryotizations have been confirmed by comparing colony morphologies between isolates from paired colonies and uncrossed parental homokaryons for other *Agaricus* species (Raper 1976; Castle et al. 1988; Callac et al. 1993). Therefore, colony morphologies of isolates from paired colonies were used as the first indicators of successful crosses in this study. Eight SSIs were randomly selected for each of the four parental strains that were used for SSI isolation and were crossed in all possible intrastrain combinations (28 combinations per strain). Mycelial plugs from the paired colonies were cultured for every cross combination. Of 112 intrastrain cross combinations performed in this study, 107 produced no morphologically distinct isolates. Colony morphologies of isolates recovered from the junction zones of paired colonies were distinct from those of both parental SSIs in only five cross combinations: two for SA514, two for SA515, and one for SA517 (Fig. 4). This finding indicates that it is difficult

Table 4. Distance values among single-spore isolates within interstrain combinations

Interstrain combination between	Distance value[a]
SA514 and SA515	0.406–0.576 (0.496)
SA514 and SA517	0.395–0.553 (0.471)
SA514 and SA520	0.394–0.594 (0.465)
SA515 and SA517	0.342–0.504 (0.423)
SA515 and SA520	0.329–0.542 (0.413)
SA517 and SA520	0.260–0.510 (0.371)

[a] Number in parentheses indicates the mean

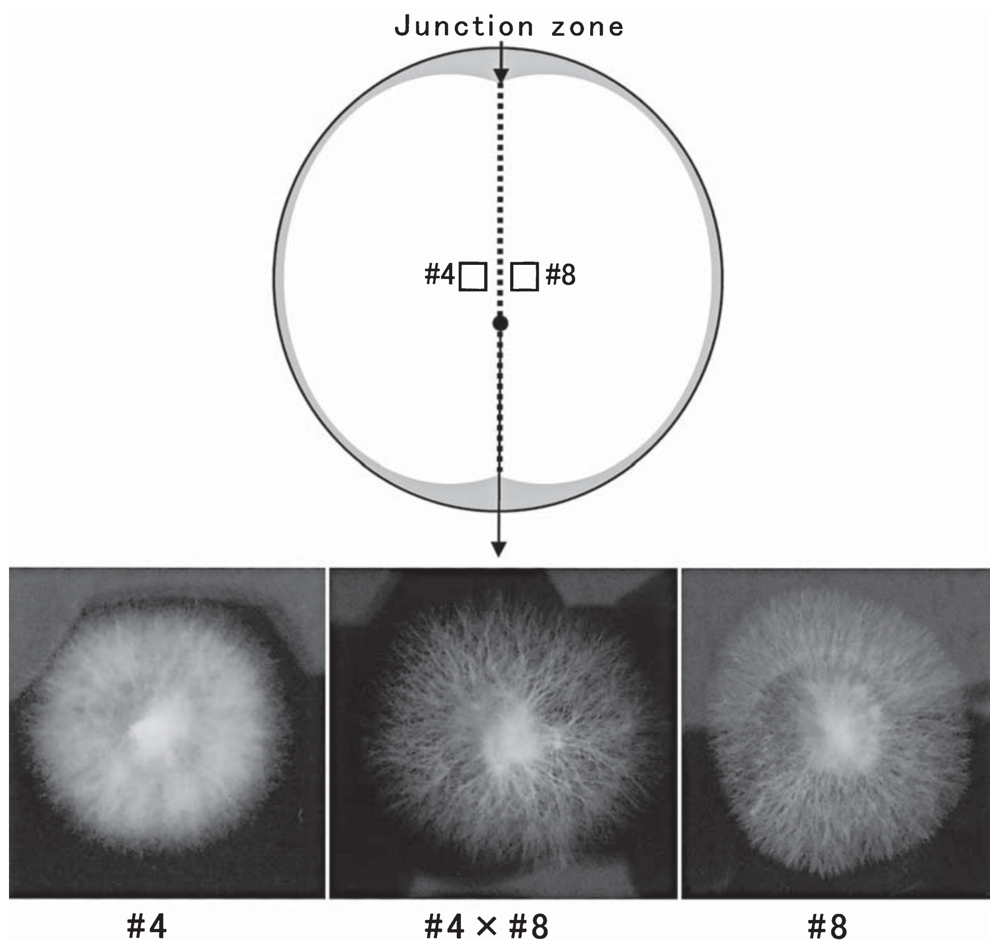

Fig. 4. Diagram of cross between single-spore isolates (#4 and #8) from SA517 and colony morphologies of the parents and an isolate #4 × #8 from the *junction zone* of the paired colony

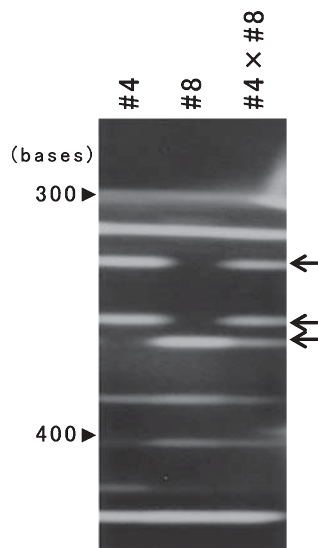

Fig. 5. AFLP analysis electrophoresis gel using the *Eco*RI + AC / *Mse*I + CC primer combination for an isolate #4 × #8 from the junction zone of paired single-spore isolates (#4 and #8 from SA517). *Arrows* indicate distinct AFLP bands between #4 and #8. *Numbers on the left* indicate fragment sizes in fluorescein molecular ruler (100 bp)

to see if crosses were successful on the basis of colony morphology. One factor that may have constrained the development of distinct morphologies is that crosses were limited to intrastrain combinations.

Morphological changes resulting from paired colonies suggest that the isolates are newly established heterokaryons. The five putative heterokaryotic isolates were thus subjected to AFLP analysis to determine whether they were the result of successful crosses. To avoid contamination by hyphal cells derived from parental SSIs, these isolates were subcultured three times before DNA extraction.

As shown in Fig. 5, the AFLP pattern of the morphologically distinct isolate from the cross of SA517 SSI #4 × #8 was different from those of the parental SSIs, which were the mixed AFLP patterns consisting of AFLP bands from both the parents. Mixed AFLP patterns were also detected in another isolate from the cross of SA514 SSI #2 × #3 (data not shown). The AFLP results and the fact that repeated subculturing had no apparent effect on colony morphology indicate that these two isolates are hybrid (heterokaryotic) strains. In addition, nuclear migration may be limited in *A. blazei* sexual crosses, judging from the fact that hybrid strains were obtained only from the junction zone between the paired colonies. However, AFLP profiles of the remaining three isolates were identical to one or other parental SSI (data not shown), suggesting that the crosses were unsuccessful. Using an RFLP marker, Castle et al. (1988) found that about half of morphologically distinct *A. bisporus* isolates from paired colonies were not hybrids. It may be also difficult for *A. blazei* to confirm hybrid formation by only colony morphologies of the isolates from paired colony.

Confirmation of hybrid formation is not easy for species having no significant cytological differences between heterokaryotic and homokaryotic hypha, as has been described for *A. bisporus* (Castle et al. 1988). Similar to *A. bisporus,* there is no significant difference in *A. blazei* hyphal cytological characters between cultivated strains (probably heterokaryons) and their SSIs (putative homokaryons) such as nuclear number per hyphal cell (Fukuda et al., unpublished data). Therefore, the use of unambiguous genetic makers is very important for detecting hybrid (heterokaryotic) strains of *A. blazei*. This study indicates that AFLP bands are suitable genetic makers for the confirmation of successful crosses and can be used for studying the mating system and mating reactions of *A. blazei*. A better understanding of *A. blazei* mating should prove to be very useful for efficient breeding. These and other AFLP markers will be useful for generating genetic maps, as has been done for *L. edodes* (Terashima et al. 2006b). In addition, we are presently constructing *A. blazei* hybrid strains from different combinations of SSIs using AFLP makers to confirm successful crosses. Some of these strains will likely surpass the existing cultivated strains for important traits such as fruiting body productivity.

Acknowledgments This work was supported by a Grant-in Aid for Scientific Research (No. 165800002, 2004–2006) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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