Mycoscience (2003) 44:431–436
DOI 10.1007/s10267-003-0136-x

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FULL PAPER

Masaki Fukuda · Shotaro Ohno · Masako Kato

Genetic variation in cultivated strains of *Agaricus blazei*

Received: April 28, 2003 / Accepted: August 7, 2003

Abstract Genetic differences among *Agaricus blazei* strains were investigated using somatic incompatibility testing, isozyme analysis, restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA), and random amplified polymorphic DNA (RAPD) analysis. Eight strains, one cultivated strain from Brazil and seven from Japan, were used in this study. Somatic incompatibility interactions were observed between the Brazilian cultivated strain and the Japanese strains. The Brazilian cultivated strain had its own distinct patterns of esterase isozyme and mtDNA RFLP, but all seven Japanese cultivated strains showed identical patterns. When the RAPD patterns, obtained using eight primers, were compared the eight strains had their own distinct RAPD profiles. Distance values were calculated between all pairs of the strains based on presence or absence of individual RAPD bands, and a dendrogram was constructed by unweighted pair-group method with arithmetic clustering (UPGMA) analysis. Seven Japanese cultivated strains were grouped to each other, and this group was finally linked to the Brazilian cultivated strain. Based on these results, the degree of genetic variation among the *A. blazei* strains used is discussed.

Key words *Agaricus blazei* · Somatic incompatibility · Isozyme · Mitochondrial DNA · RAPD

Introduction

Agaricus blazei Murill (Agaricales, Agaricaceae), called Himematsutake in Japan, is an edible mushroom distributed in North America (from Florida to southern California) and Brazil (around Sao Paulo) (Imazeki and Hongo 1987). Recently, this mushroom has been noticed especially for its pharmacological effects, such as antitumor activity (Mizuno et al. 1990) and inhibitory effect on hypertension (Eguchi et al. 1999). The use of *A. blazei* as a functional food has increased in recent years, although an accurate evaluation of production is not yet known. In Japan, artificial cultivation of *A. blazei* has been generally performed by a procedure based on the method of *A. bisporus* (Lange) Imbach (e.g., Sumiya 2000). However, spawn cultures used in *A. blazei* cultivation have probably been derived from wild strains, because little is known about the development of *A. blazei* cultivars. There has been basic research on the development of superior cultivars of many other cultivated mushrooms, i.e., studies on the genetic relatedness between breeding materials (e.g., wild strains and cultivars) of cultivated mushrooms such as *A. bisporus* (Royse and May 1982), *Lentinula edodes* (Berk.) Pegler (Fukuda and Tokimoto 1991), *Pleurotus ostreatus* (Jacq: Fr.) Kummer (Matsumoto and Fukumasa-Nakai 1995), and *Pholiota nameko* (T. Ito) S. Ito and Imai (Obatake et al. 2002). These studies provide important information on mushroom breeding. For *A. blazei*, it is also important to clarify the genetic relatedness among breeding materials to efficiently develop superior cultivars with higher productivity and greater pharmacological effects.

The purpose of the present study was to examine the genetic differences among strains of *A. blazei* by somatic incompatibility testing, isozyme analysis, restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA), and random amplified polymorphic DNA (RAPD) analysis.

M. Fukuda (✉)
Graduate School of Agriculture, Shinshu University, Minamiminowa, Nagano 399-4598, Japan
Tel. +81-265-77-1621; Fax +81-265-77-1700
e-mail: mf0130y@gipmc.shinshu-u.ac.jp

S. Ohno · M. Kato
Faculty of Agriculture, Shinshu University, Nagano, Japan

Materials and methods

Strains of *A. blazei*

Eight heterokaryotic strains of *A. blazei*, one cultivated strain from Brazil (SA514) and seven from Japan

(SA515–520 and SA527), were examined in this study. Respective strains were kindly provided from different cultivators of *A. blazei* in Brazil (SA514) and in Japan [Gifu (SA515, 516–518, and 527), Aich (SA517 and 520), and Nagano (SA519) Prefectures]. Regrettably, details of their origins are uncertain. They were maintained 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).

The sequences of the 5.8 S ribosomal RNA gene and the internal transcribed spacer regions 1 and 2 (approximately 700 bp) for two strains, SA514 from Brazil and SA515 from Japan, have been analyzed for phylogenetic system of *A. blazei* (Fukuda et al., unpublished data). These sequences were deposited at DNA Data Bank of Japan (DDBJ) under the accession numbers AB113576 (SA514) and AB113577 (SA515). A BLAST (Altschul et al. 1997) search using the DNA database of DDBJ revealed that the sequences of the two strains showed higher levels of identities, 98.6%–99.1% (99.3% between SA514 and SA515), to those of all *A. blazei* strains in the database (accession numbers AF161013, AJ131126, AJ131128, AJ131129, AJ133376, and AJ244543) than the other fungal species including *Agaricus* L.: Fr., indicating that the two strains, SA514 from Brazil and SA515 from Japan, are conspecific (*A. blazei*).

Somatic incompatibility test

Somatic incompatibility interactions, i.e., interactions between two strains when paired together in culture, were tested among the strains by inoculating three mycelial plugs (about 25 mm^2) onto a BMA plate (90 mm in diameter) separated by approximately 50 mm. Eight strains were paired against each other in every possible combination, including self-pairing. After 15 days incubation at 25°C, the morphology of paired colonies was examined. Each of the pairings was repeated at least twice.

Isozyme analysis

To prepare the mycelium for preparation of cell-free extracts, a BMA disk (approximately 5 mm in diameter) from the mycelial culture was inoculated into 15 ml BM liquid medium in a 100-ml Erlenmeyer flask. Cultures were incubated in a stationary state in the dark at 25°C for 14 days, harvested, washed with distilled water, and lyophilized. The lyophilized mycelium of each strain was ground to a fine powder in a mortar and pestle. Ten volumes of distilled water was added to the mycelium powder, allowed to stand at 5°C for 1 h with occasional stirring, and then centrifuged at 5°C and 20000 *g* for 20 min. The supernatant was subjected to isoelectric focusing analysis. Isoelectric focusing and esterase staining were performed according to the method of Fukuda and Tokimoto (1991) using the carrier ampholyte Phalmalyte 3.0–10 (Amersham, Piscataway, NJ, USA).

DNA isolation

To prepare mycelium for DNA isolation, cultures were grown in BA liquid medium at 25°C for 14 days and fragmented with a Waring blender; 10 ml was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the BA liquid medium. The flask cultures were incubated in a stationary state in the dark at 25°C for 14 days, harvested, washed with distilled water, and lyophilized. Isolation of mtDNAs and extraction of total DNAs from the lyophilized mycelia were both done by the procedure of Fukumasa-Nakai et al. (1992).

mtDNA restriction analysis

mtDNA isolated from each of the eight strains was digested separately with two endonucleases, *Eco*RI and *Eco*RV (Nippon Gene, Tokyo, Japan), following the supplier's specifications. Electrophoresis of all digests was carried out on 0.8% agarose (Nippon Gene, Type S) at 5 v/cm for 4 h, and the gels were stained with ethidium bromide (0.5 µl/ml). RFLP patterns were recorded by photographing the gels on a UV transilluminator.

RAPD analysis

RAPD analysis was performed by a procedure based on the method of Williams et al. (1990) with minor modifications. To select RAPD primers, total DNAs from two strains (SA516 and SA517) were chosen to screen 20 primers: primer set A (Operon, Alameda, CA, USA). Eight primers, OPA-03, -04, -05, -07, -08, -09, -13, and -18, were selected to detect multiple RAPD bands. PCR was performed in a 25-µl reaction mixture containing 25 ng total DNA, 5 pM of a single primer, 0.1 mM of each dNTP, 2.5 mM $MgCl_2$, 0.5 unit of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), and 2.5 µl 10 × PCR buffer for HotStarTaq DNA polymerase (Qiagen). A thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Norwalk, CT, USA) was used at the following parameters: 14 min at 94°C; 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C for 45 cycles; 4 min at 72°C. Detection of PCR products was performed with 1.4% agarose using the method described above for mtDNA restriction analysis. RAPD reactions were repeated at least twice to confirm the reproducibility of each RAPD band.

Phenetic analysis

The presence or absence of individual RAPD 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 RAPD bands shared between the strains, and N_X and N_Y are the total number of RAPD bands in the strain X and Y. A dendrogram based on the distance values was constructed by unweighted pair-group method with arithmetic clustering (UPGMA) analysis using the computer package PHYLIP, version 3.57c (Felsenstein 1995).

Results

Somatic incompatibility test

Self-pairings (pairings of isolates with themselves) always resulted in complete intermingling without any evidence of antagonism (Fig. 1). Interactions similar to that seen for the self-pairings were observed in the interstrain combinations among all strains, except SA514, indicating that the paired strains are somatically compatible. In the pairings between SA514 and the other seven strains, somatic incompatibility interactions, similar to barrage formation, were observed (Fig. 1).

Isozyme analysis

Electrophoretic analysis of mycelial esterase from the eight strains produced very similar isozyme patterns (Fig. 2). All strains, except SA514, shared the same isozyme patterns. Strain SA514 had its own distinct pattern, although only the position of one isozyme band, marked with an arrowhead in Fig. 2, was slightly different from those of the other seven strains.

mtDNA RFLP analysis

*Eco*RI and *Eco*RV digests of mtDNAs from the eight strains produced two distinct RFLP patterns (Fig. 3). All strains, except SA514, showed identical RFLP patterns of mtDNA by digestion with the two endonucleases. SA514 showed distinct RFLP patterns. Based on the summation of restriction fragment sizes from the *Eco*RI and *Eco*RV digests, the estimated molecular sizes of mtDNAs were 96.0 kb for SA514 and 105.8 kb for the other seven strains; the average estimated molecular size of *A. blazei* mtDNA was 100.9 kb.

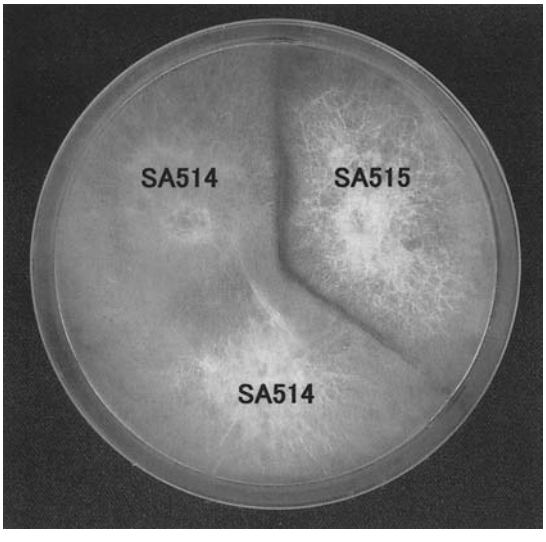


Fig. 1. Somatic incompatibility interactions between paired *Agaricus blazei* strains

RAPD analysis

Various RAPD patterns were detected with each of the eight primers on the eight strains (Fig. 4). When the RAPD patterns were compared, the eight strains had their own distinct RAPD profiles.

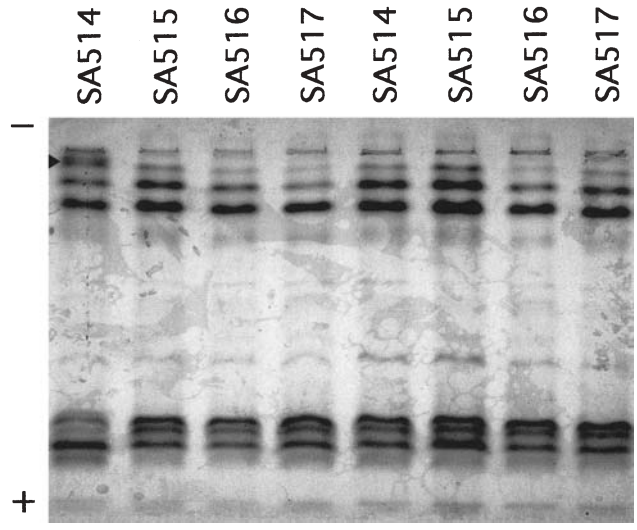


Fig. 2. Isozyme patterns of esterase from the mycelium of *Agaricus blazei*

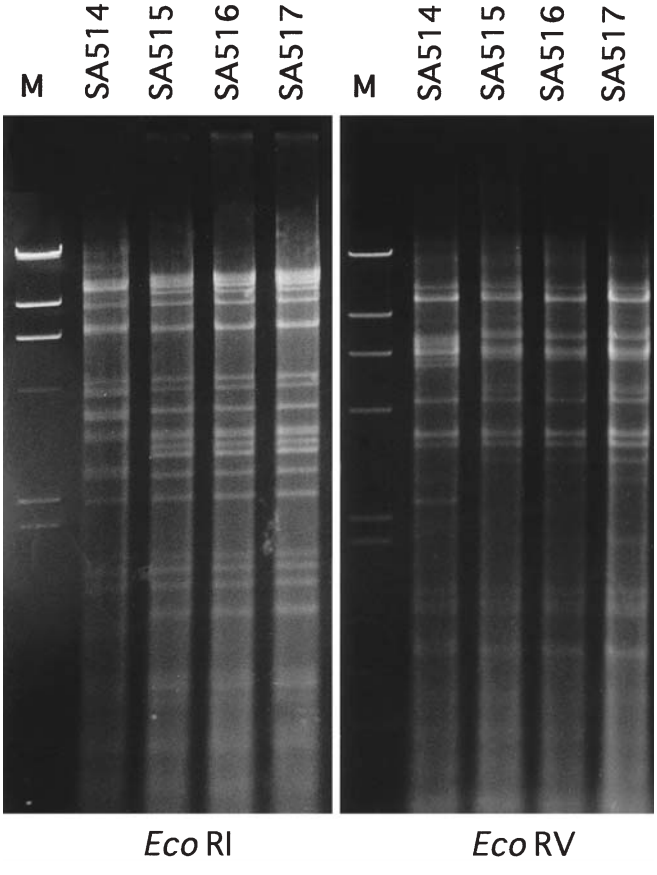
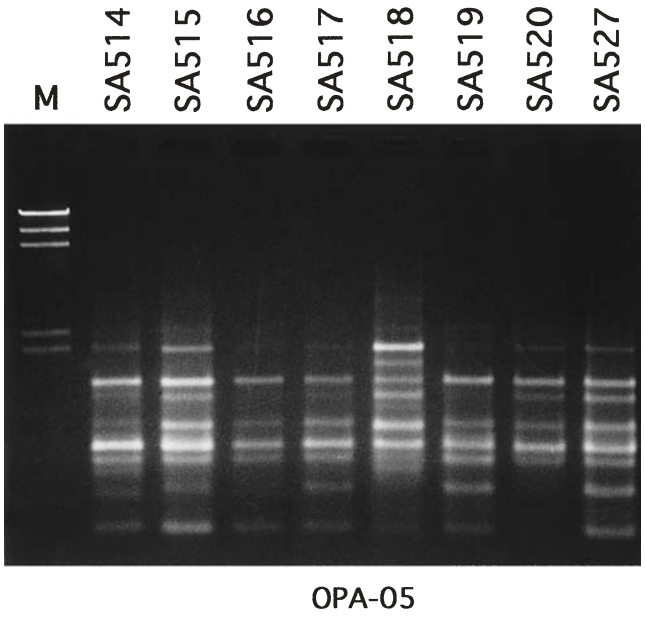


Fig. 3. Restriction fragment length polymorphism (RFLP) patterns of mtDNA produced from four strains of *Agaricus blazei* with *Eco*RI and *Eco*RV. *Lane M*, *Hin*dIII-digested lambda phage DNA



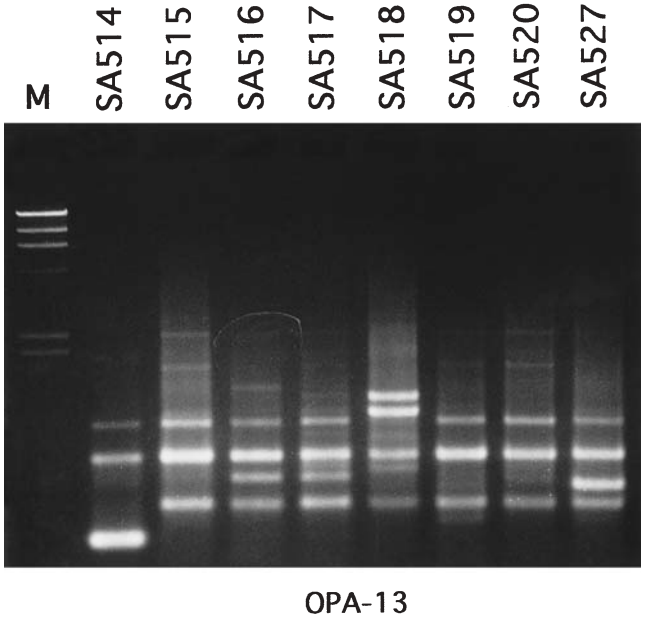


Fig. 4. Random amplified polymorphic DNA (RAPD) patterns produced from *Agaricus blazei* strains using primers OPA-05 (*top*) and OPA-13 (*bottom*). *Lane M*, *Hin*dIII-digested lambda phage DNA

Distance values among the strains were calculated using a total of 56 reproducible RAPD bands [OPA-03 (9), -04 (6), -05 (10), -07 (4), -08 (4), -09 (6), -13 (9), and -18 (8)] (Table 1). Based on the distance matrix, a dendrogram was constructed by UPGMA analysis (Fig. 5). All strains, except SA514, were grouped to each other with a distance value below 0.24, and this group was finally linked to SA514 with the value of 0.35.

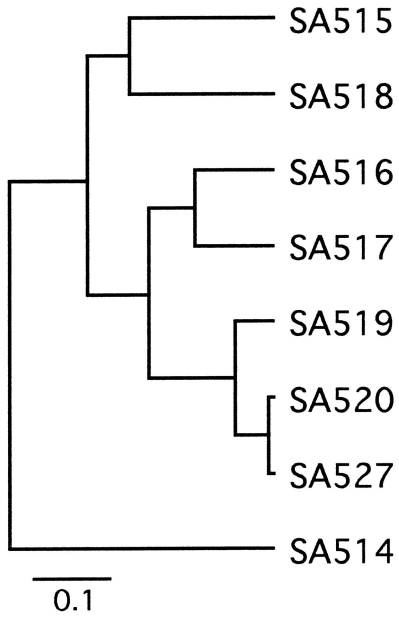


Fig. 5. Unweighted pair-group method with arithmetic clustering (UPGMA) dendrogram based on data from RAPD analysis of *Agaricus blazei* strains

Discussion

In the present study, four experiments, somatic incompatibility testing, isozyme analysis, mtDNA RFLP analysis, and RAPD analysis, were performed to examine the genetic differences among cultivated strains of *A. blazei*, and useful information was obtained about the genetic variation among them.

Within the seven Japanese cultivated strains used (SA515–520 and SA527), no somatic incompatibility reaction was observed in all interstrain combinations. On the other hand, we expected various patterns of esterase isozyme and mtDNA RFLP in the strains of *A. blazei* used, because the analysis of esterase isozyme using isoelectric focusing is one useful method used to examine genetic differences between mushroom strains (Ohmasa and Furukawa 1986; Itävaara 1988; Fukuda and Tokimoto 1991; Matsumoto et al. 1995) and mtDNA is high polymorphic from strain to strain, as described for *Schizophyllum commune* Fr. (Specht et al. 1983), *A. bitorquis* (Quél.) Sacc. (Hintz et al. 1985), *L. edodes* (Fukuda et al. 1994), *P. ostreatus* (Matsumoto and Fukumasa-Nakai 1995), and *P. nameko* (Obatake et al. 2002). However, no variation in the migration of the isozyme bands and the restriction fragments by digestion with *Eco*RI and *Eco*RV was detected among the seven strains. These findings suggest that the seven Japanese cultivated strains used in this study were derived from cultures with little genetic variation. mtDNA homogeneity in *A. bisporus* cultivated strains has been reported, indicating the narrowness of the genetic basis between the strains (Hintz et al. 1985). The genetic basis of the Japanese cultivated strains of *A. blazei* may also be narrow. However, one factor in this finding may be that the culti-

Table 1. Distance matrix based on data from RAPD analysis of *Agaricus blazei* strains

Strain no.	SA514	SA515	SA516	SA517	SA518	SA519	SA520	SA527
SA514	0.000							
SA515	0.370	0.000						
SA516	0.365	0.219	0.000					
SA517	0.313	0.235	0.103	0.000				
SA518	0.447	0.190	0.313	0.296	0.000			
SA519	0.313	0.206	0.138	0.129	0.296	0.000		
SA520	0.333	0.200	0.133	0.125	0.260	0.094	0.000	
SA527	0.304	0.171	0.167	0.296	0.260	0.007	0.006	0.000

RAPD, random amplified polymorphic DNA

vated strains used for this study were gathered from a limited area (Aich, Gifu, and Nagano Prefectures) in Japan. Because *A. blazei* cultivation is probably performed everywhere in Japan, a cultivated strain used in another area may have a different genetic composition from that of the strains used in this study.

The Brazilian cultivated strain (SA514) was somatically incompatible against all the Japanese strains and had its own distinct patterns of esterase isozyme and mtDNA RFLP. This result indicates that the genetic composition of the Brazilian cultivated strain differs from that of the Japanese strains. However, the genetic variation may be small between the Brazilian strain and Japanese strains, judging from the slight difference in the isozyme patterns. This result suggests that (i) the strains used were selected from the same wild-type population of *A. blazei* and (ii) intraspecific variation of *A. blazei* was originally low in comparison with other mushrooms already mentioned.

The mtDNA size of *A. blazei* estimated in this study (100.2 kb) falls within the range of mtDNA sizes described previously in other basidiomycetes, from 36 kb for *Boletinus cavipes* (Opat.) Kalchbr. (Bruns et al. 1988) to *A. bitorquis* (Hintz et al. 1985), and similar to 98.3 kb for *A. bisporus* (Hintz et al. 1985). In addition, mtDNA RFLP patterns have been used as markers to monitor cytoplasmic inheritance in matings between sexually compatible strains, as studied in *A. bitorquis* (Hintz et al. 1988), *L. edodes* (Fukuda et al. 1995), and *P. ostreatus* (Matsumoto and Fukumasa-Nakai 1996). The distinct mtDNA RFLP patterns of *A. blazei* detected in this study allowed us to examine the mode of mitochondrial inheritance in this mushroom.

To examine genetic differences among the *A. blazei* strains, RAPD analysis was more effective than the other three methods used in this study, because every strain showed its own distinct RAPD profile. The dendrogram, based on data from RAPD analysis, provided useful information on the genetic relatedness among the *A. blazei* strains, which were found to have unique DNA sequences.

Distance values among the eight strains, based on the data from RAPD analysis, ranged from 0.006 (SA520 and SA527) to 0.447 (SA514 and SA518); a similar range (0.040–0.430) was calculated, using 53 RAPD bands from three primers, for 36 wild strains of *P. nameko* from a wide region in Japan (Obatake et al. 2002). In addition, the values among the seven Japanese strains varied between 0.006 and 0.313 (SA516 and SA518). Based on this, genetic variation among the *A. blazei* strains used may not be so low as suggested. In fact, the fruiting body productivity among the strains differs greatly between each other (Fukuda et al., unpublished data). It is necessary to examine more strains and to employ additional indicators, such as mating types (although even the mating system of *A. blazei* is yet uncertain), to clarify the degree of intraspecific variation of *A. blazei*. It is also important to examine the relationship between the genetic divergence of each strain and its characteristics, such as fruiting body productivity and pharmacological effects, to efficiently develop new superior cultivars of *A. blazei.*

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