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SHORT COMMUNICATION

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Strain typing of shiitake (*Lentinula edodes*) cultivars by AFLP analysis, focusing on a heat-dried fruiting body

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Abstract To validate strain typing by amplified fragment length polymorphism (AFLP) analysis in shiitake (*Lentinula edodes*) cultivars, the reproducibility of AFLP markers with DNA extracted from the heat-dried fruiting body was evaluated. DNAs were extracted from three different portions of the heat-dried fruiting body – the stipe, pileus, and gill – and AFLP analysis of all parts was carried out using two combinations of selected amplification primer pairs. AFLP profiles of DNA from the gill tissue of heat-dried fruiting body were almost identical to those of cultured mycelia in the same strains, although it was difficult to detect reproducible AFLP profiles from stipe and pileus DNA. These results indicated that AFLP analysis would be applicable for strain typing with heat-dried fruiting bodies of *L. edodes* by using the DNA extracted from gills.

Key words DNA fingerprinting · Hoshi Shiitake · Protecting breeder's right

The shiitake mushroom, *Lentinula edodes* (Berk.) Pegler, is the most important cultivated mushroom in Japan, accounting for about 30% of the total production of cultivated mushrooms in 2001. More than 100 cultivated shiitake strains have been developed, reflecting the large economic investment and enterprise of many breeders with considerable labor. A system for registering cultivated strains of mushroom commenced in Japan in 1978, to protect breeders' rights, and was revised in 1991 in accordance with the International Convention for the Protection of New Varieties of Plants (http://www.upov.int). In the revised registration system, breeders' rights were further strengthened, in which it was now applied not only to new cultivated strains, but also to strains derived from them. Therefore, an efficient method for strain typing is necessary to put this registration system into practice. Heat-dried fruiting bodies of *L. edodes*, commonly called "Hosi Shiitake," accounted for over 50% of the distribution volume of the Japanese shiitake market in 2001. Thus, it is particularly important that methods for strain typing with the heat-dried fruiting body should be validated to potentiate the universal applicability of the registration system.

The current system of strain typing in shiitake cultivars was carried out mainly based on morphological and physiological characters and somatic compatibility (Chiu et al. 1999). To support the system, various molecular techniques such as isozyme profiling (Ohmasa and Furukawa 1986; Royse et al. 1983), RAPD (random amplified polymorphic DNA) profiling (Chiu et al. 1996, 1999; Kwan et al. 1992; Sunagawa et al. 1995; Zhang and Molina 1995), and AP-PCR (arbitrary-primed polymerase chain reaction) profiling (Chiu et al. 1996, 1999) have been conducted. However, these molecular techniques could not detect many loci; thus, they failed to differentiate closely related strains of shiitake. Meanwhile, Vos et al. (1995) developed AFLP (amplified fragment length polymorphism) analysis based on selective PCR amplification of genomic DNA restriction fragments, and demonstrated that AFLP analysis could reproducibly detect a large number of polymorphic loci. Furthermore, we previously reported that AFLP analysis reproducibly identified more DNA markers than isozyme and RAPD profiling and that this technique could be used to differentiate major commercial strains of Japanese shiitake (Terashima et al. 2002) using freeze-dried mycelia.

In contrast, heat-dried fruiting bodies of *L. edodes* cannot be used in the present system for strain typing as described above. Furthermore, it has been generally considered that DNA fingerprinting also could not be applied to heat-dried biological specimens because heat-drying of biological specimens before DNA extraction causes decompo-

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sition of macromolecules, such as polysaccharides, proteins, and nucleic acids, and the generation of impurities that inhibit PCR analysis of DNA. To date, there have been no reports of DNA fingerprinting, including those obtained by AFLP analysis, analyzing the whole genome of heat-dried shiitake fruiting bodies.

In this study, we estimated the reproducibility of AFLP markers from heat-dried fruiting bodies of shiitake and discussed the possible application of this technique for strain typing of heat-dried shiitake as starting materials.

For AFLP analysis, mycelia and fruiting bodies of three cultivated strains of shiitake, *L. edodes*, Kinko-115, Kinko-241, and Kinko-697, were used. All strains are developed by the Japan Kinoko Research Center Foundation, Tottori, Japan. Cultured mycelia from each strain were harvested and freeze-dried as described previously (Terashima et al. 2002) and used for DNA extraction. Fruiting bodies from each strain were produced by log-wood cultivation under outdoor conditions (Japan Kinoko Research Center 1977). Samples of fruiting bodies for DNA extraction were prepared by freeze-drying immediately after harvesting, or were heat-dried at temperatures ranging from 40°C to 60°C for approximately 18–24 h using a C-45 oven (Kinko, Tottori, Japan). Freeze-dried samples of cultured mycelia and fruiting bodies were preserved at −20°C until required. Heat-dried fruiting bodies used in this study were stored at room temperature in low-moisture conditions for 2–3 years. Furthermore, each fruiting body was divided into stipe, pileus, and gill tissues for DNA extraction. Approximately 10 mg freeze-dried or heat-dried materials as already described were used for DNA extraction. This step was performed using a Genelute™ plant Genomic DNA Kit (Sigma, Toronto, Canada) and DNA was eluted from the binding column twice with 100 µl TE [10 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0]. AFLP analysis was carried out using a modification of the procedures described by Vos et al. (1995) and Terashima et al. (2002). Genomic DNA (1 µl from elution fluid in DNA extraction procedure, approximately 20 ng or less) was digested with endonucleases (*Eco*RI and *Mse*I). For preselective amplification, PCR reactions were carried out in a Takara PCR Thermal Cycler MP (Takara, Shiga, Japan) with Takara *Ex Taq* DNA polymerase (Takara), E + 0 primer, and M + 0 primer. Selective amplification was carried out in a Takara PCR Thermal Cycler MP with Takara *Ex Taq* DNA polymerase, E + AC primer, and M + 2 primer (+CA, or +CC). The size of nucleotide extensions of these selective primers was described by Majer et al. (1996) and Terashima et al. (2002). Electrophoresis and detection of amplified fragments was carried out using an ABI Prizm 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). In this study, DNA fragments of 100–500 bp were scored for strain typing because electrophoresis of DNA fragments below 100 bp or above 500 bp in length could not be accurately performed. Similarity value between DNA samples was calculated as the percentage of shared AFLP bands (number of shared peaks between two strains/total number of distinct peaks detected in two strains ×100). For each DNA sample, AFLP analysis was performed at least twice and nonreproducible fragment peaks, which may have constituted artifacts, were excluded.

In previous studies (Terashima et al. 2002), AFLP analysis was carried out with approximately 200 ng genomic DNA from cultured mycelia. However, in retrospect, this quantity of DNA did not result in efficient preamplification and gave rise to low reproducibility of AFLP markers (data not shown). In this study, smaller amounts of genomic DNA (less than 20 ng) were used for endonuclease digestion. As a result, we were able to increase the reproducibility of preamplification, and of AFLP markers, in comparison to previous studies (Terashima et al. 2002). In this study, AFLP analysis with the two primer pairs E + AC/M + CA and E + AC/M + CC for cultured mycelia detected 55 and 44 DNA fragments from Kinko-115; 53 and 42 DNA fragments from Kinko-241; and 58 and 41 DNA fragments from Kinko-697 (Tables 1, 2), and AFLP profiles between different mycelial cultures that originated from one strain were almost identical. Specifically, there were fewer than three unshared DNA fragments per two primer combinations, with weak signal peaks in the electrophoretogram, between different mycelial samples for each strain, and similarity values between samples of each strain ranged from 97% to 100% (data not shown). Under the condition of AFLP analysis with cultured mycelia in this study, the similarity values between Kinko-115 and Kinko-241, between Kinko-115 and Kinko-697, and between Kinko-241 and Kinko-697 were 81.4%, 65.0%, and 70.7%, respectively. Therefore in this study, with the two primer pairs E + AC/M + CA and E + AC/M + CC, we judged a similarity value of more than 97% as an identical strain profile.

To confirm whether the AFLP profile of genomic DNA from a fruiting body was identical with that from cultured mycelia, we extracted DNAs from freeze-dried stipe, pileus, and gill of Kinko-115 and carried out AFLP analysis. As shown in Table 1, every AFLP profile obtained from DNAs of these three regions of fruiting body using two selective primer pairs had high similarity values, greater than 99%, compared with mycelial profiles in which fewer than two unshared fragments were detected. These results indicated that strain typing of cultivated strains of shiitake was also possible using DNA from any region of the freeze-dried fruiting body.

Using DNA samples extracted from heat-dried stipe and pileus of Kinko-115 fruiting body, the AFLP profiles had 5 to 9 DNA fragments, which differed from the mycelial AFLP profile (see Table 1). Their AFLP profiles were not improved by increasing or decreasing the amount of DNA subjected to restriction enzyme treatment, the initial step in AFLP analysis (data not shown). Therefore, we concluded that DNA from the stipe and pileus regions of heat-dried fruiting bodies was not suitable for strain typing shiitake cultivars using AFLP analysis. On the other hand, almost identical AFLP profiles were obtained from cultured mycelia and from heat-dried gills of Kinko-115 fruiting body, in which the similarity values were greater than 97% (Fig. 1, Table 1). The reproducibility of AFLP profiles from heat-dried gills was examined by extending the study to the Kinko-241 and Kinko-697 strains. AFLP profiles from gills

Table 1. Comparison of amplified fragment length polymorphism (AFLP) profiles between DNAs from cultured mycelia and fruiting body of Kinko-115 of *Lentinula edodes*

Samples	Cultured mycelia		Freeze-dried fruiting body									
			Stipe		Pileus		Gills 1		Gills 2		Gills 3	
Selective primer pairs	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC
Total number of fragments	55	44	56	44	55	45	54	44	55	43	53	44
Unshared fragments for mycelial data			1	0	0	1	1	0	0	1	2	0
Similarity for mycelial data (%)			99.1	100	100	98.9	99.1	100	100	98.9	98.1	100
(average)			(99.6)		(99.5)		(99.6)		(99.5)		(99.1)	
Samples			Heat-dried fruiting body									
			Stipe		Pileus		Gills 1		Gills 2		Gills 3	
Selective primer pairs			M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC
Total number of fragments			48	43	55	47	57	44	56	45	54	44
Unshared fragments for mycelial data			7	5	9	5	2	0	1	1	3	0
Similarity for mycelial data (%)			93.2	94.3	91.8	94.5	98.2	100	99.1	98.9	97.2	100
(average)			(93.8)		(93.2)		(99.1)		(99.0)		(98.6)	

Table 2. Comparison of AFLP profiles between DNAs from cultured mycelia and gills of fruiting body of Kinko-241 and Kinko-697 of *Lentinula edodes*

Samples	Freeze-dried cultured mycelia		Heat-dried fruiting body							
			Gills 1		Gills 2		Gills 3		Gills 4	
Kinko-241										
Selective primer pairs	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC
Total number of fragments	53	42	53	43	53	43	53	43	53	43
Unshared fragments for mycelial data			0	1	0	1	0	1	0	1
Similarity for mycelial data (%)			100	98.8	100	98.8	100	98.8	100	98.8
(average)			(99.4)		(99.4)		(99.4)		(99.4)	
Kinko-697										
Selective primer pairs	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC	M + CA	M + CC
Total number of fragments	58	41	58	40	58	40	58	40	58	40
Unshared fragments for mycelial data			0	1	0	1	0	1	0	1
Similarity for mycelial data (%)			100	98.7	100	98.7	100	98.7	100	98.7
(average)			(99.4)		(99.4)		(99.4)		(99.4)	

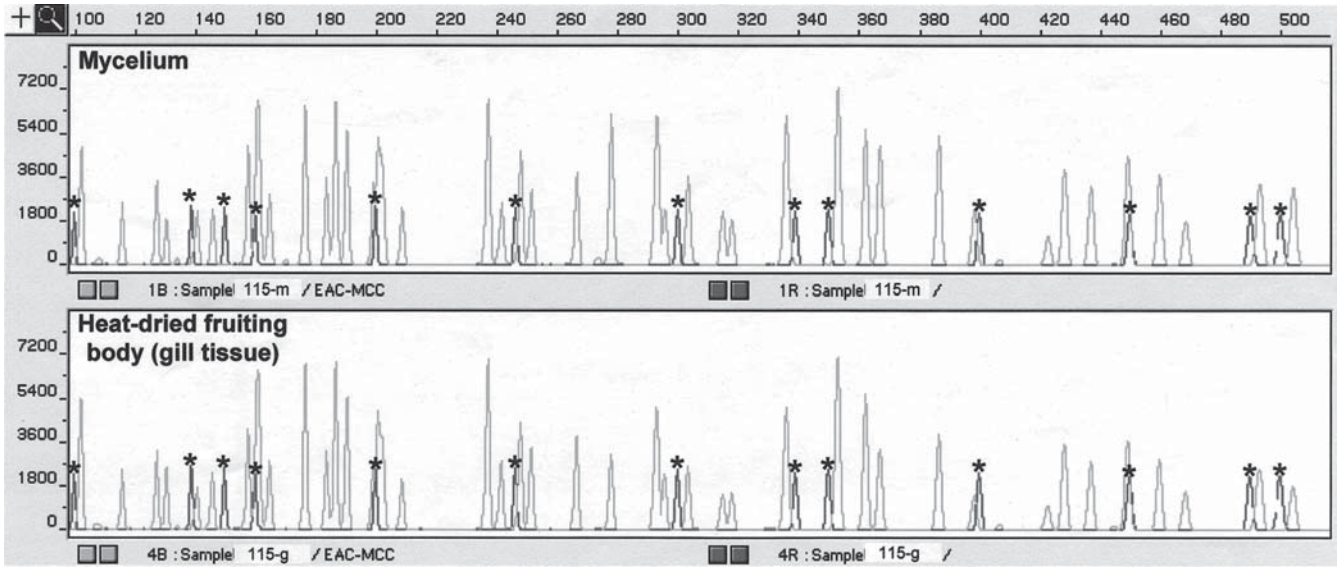

Fig. 1. Representative electrophoresis image of amplified fragment length polymorphism (AFLP) analysis in cultivated strain Kinko-115 of *Lentinula edodes*. The image shows DNA fragments between 100 bp and 500 bp in length. These DNA fragments were produced with E + AC/M + CC primer combination. *Asterisks* show fragments of size markers of 100, 130, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp, respectively

of both strains also closely agreed with those from their respective mycelial DNA (see Table 2). Additionally, AFLP analysis for heat-dried gills from other fruiting bodies of these strains and three other strains also revealed high similarity (greater than 97% identity) between the AFLP profile from mycelia and that from gills (data not shown). These results indicate that AFLP analysis enabled strain typing using DNA from gills of the heat-dried fruiting body.

It is noteworthy that only gill DNA of the heat-dried fruiting body produced reproducible AFLP profiles in this study. In general, the gills of a fruiting body are dried much faster than stipe and pileus by heat-drying, so that decomposition of DNA and generation of inhibitors for PCR of DNA by heat-drying in gills are depressed as compared with that in the stipe and pileus. This result may be a reason why AFLP profiles were reproducibly obtained with only heat-dried gills in this study. Then, why did the AFLP profile from heat-dried gills, where meiosis occurred, agree with that of cultured mycelia in this study? We have as yet no persuasive explanation and data for our results. Accordingly, it is necessary to carry out detailed research to explain the mechanism of our results in this study.

In this study, we demonstrated that AFLP analysis was applicable to strain typing with heat-dried shiitake. This is the first report of strain typing with heat-dried fruiting bodies of the shiitake mushroom. The technique for strain typing with heat-dried shiitake is of significance because more than 50% of shiitake production volume was distributed as the heat-dried fruiting body. Hereafter, an accurate database of registered strains with a number of specimens consisting of both cultured mycelia and heat-dried fruiting bodies should be constructed to apply this method in practical identification of shiitake cultivars.

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