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Classification of Korean *Lentinula edodes* Strains by Random Amplified Polymorphic DNA (RAPD) Markers

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RAPD(Random Amplified Polymorphic DNA) 검정을 이용한 한국 표고균주의 계통분류

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ABSTRACT: Random Amplified Polymorphic DNA (RAPD) assay was used to identify seven typical *Lentinula edodes* (Berk.) Pegler strains isolated in Korea. Twenty primers from OPA-01 to OPA-20 were applied to generate the recognition of *L. edodes* strains. Out of 20 primers, nine primers showed efficient RAPD patterns to classify the 7 strains tested, but the rest eleven primers were not useful to be used. Even though there was no single primer that could classify all of the strains, any combination of two primers among the nine primers could identify the strains tested. Thus, RAPD assay turned out to be very precise method for classifying *L. edodes* strains.

KEYWORDS: Random Amplivied Polymorphic DNA (RAPD), *Lentinula edodes*, strains, primers

Oak mushroom, *Lentinula edodes* (Berk.) Pegler [=*Lentinus edodes* (Berk.) Singer], is the second in amount of mushroom production after button-mushroom in the world, but it is the first in mushroom production in Asian countries (Chang and Miles, 1989). In recent years, new cultivars or strains have been hybridized in Korea, Japan, China, and some other countries, and classification methods of *L. edodes* have been proceeded in these countries.

Generally, morphological classification or classification by the antithetic line formation on PDA was used for the differentiation of different strains. To analyse the genetic information of each strain, electrophoretic profiles of proteins was used (Itavaara, 1988; Lee *et al.*, 1996a), but the method did not provide good differentiation of each strain because of the large number of protein bands. Zymograms of esterase isozymes was also used to analyse the genetic differences of strains (Itavaara, 1988; Lee *et al.*, 1996b). In recent days, the analysis of DNA such as RFLP (Restriction Fragment Length Polymorphisms) method and RAPD (Random Amplified Polymorphic DNA) method are frequently used for the identification of different strains or cultivars of mushrooms. RFLP method to identify the difference of DNA bands on mushroom had been reported by several researchers (Fukuda *et al.*, 1994; Molina *et al.*, 1992). RAPD using random primer and PCR (Polymerase Chain Reaction) was developed by Williams *et al.* (1990) and broadly used for the identification of DNA for the

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human, animals, plants, fungi and even lower microoganisms. RAPD method compared with the RFLP methods is easy to manipulate, saves the examining times and needs only small amount of test materials. Some papers related on RAPD assay of *L. edodes* had been reported in recent years (Kwan *et al.*, 1992; Zang and Molina, 1994; Snugawa *et al.*, 1995). In this study we tried to identify seven typical *L. edodes* strains isolated in Korea by use of the RAPD assay.

Materials and Methods

Strains and samples of *Lentinula edodes* tested

Seven strains of *Lentinula edodes* (=*Lentinus edodes*), which had been registered for commercial use in Korea were prepared. The strains are from Sanlim No. 1 through No. 6 and Imhyup No. 1. The characteristics of each strain are summarized in Table 1.

Mycelium of each strain of *L. edodes* was cultured on a liquid medium supplemented with 2% glucose, 2% malt extract, and 1% peptone. Polypropylene bottles (vol. 100 m*l*) with 40 m*l* of malt extract liquid medium were stoppered with cap and autoclaved for 20 minutes at 121°C. A piece (5 mm diameter) of mycelium from each stock agar plate culture on PDA medium was transferred to liquid medium. The mycelium was cultured by shaking incubator (70 rpm) in the light condition for a month at 22.5°C.

Table 1. Characteristics of *Lentinula edodes* strains tested

Strains	Patentee of strain	Temperature type	Suitablity of cultivation
Sanlim No. 1	FRI[a]	Low	Bed-log
Sanlim No. 2	FRI	High	Bed-log
Sanlim No. 3	FRI	Low	Bed-log
Sanlim No. 4	FRI	High	Bed-log
Sanlim No. 5	FRI	High	Bag-culture
Sanlim No. 6	FRI	High	Bag-culture
Imhyup No. 1	NFCF[b]	High	Bed-log

[a]: Forestry Research Institute (FRI) in Korea
[b]: National Forestry Cooperatives Federation (NFCF) in Korea

In case of *L. edodes* strains suitable for bed-log cultivation, the spawn was inoculated to oak (*Quercus mongolica*) logs in spring. In addition, the spawn was inoculated to autoclaved sawdust-based medium in polypropylene bag for bag-cultivation. After 14 months of bed-log and 3 months of bag-culture, fruit-bodies were shown from the bed-logs and sawdust media. The pileus of the fruit-bodies produced from both cultivating methods were used for the preparation of DNA extraction.

DNA Extraction

Mycelia were separated from the liquid media by filtering on Miracloth (CalBiotech., U.S.A.) and washed with sterilized water. Before extraction of DNA, mycelia and fruit-body of *L. edodes* were reserved in deep-freezer at − 75°C.

DNA was extracted by modified Ahrens method (Ahrens and Seemuller, 1992). One gram of collected mycelia or fruit-bodies were homogenized with liquid nitrogen in a mortar. Three mililiters of CTAB extraction buffer [2.5 M NaCl, 0.5%(w/v) PVP-10(polyvinylpyrolidone-10) (Sigma, USA), 1%(w/v) Cetavlon (hexadecyltrimethyl-ammonium bromide), 0.5 M Tris-HCl (pH 8.0), 0.25 M EDTA (pH 8.0)] and 6 μ*l* of β-mercaptoethanol were transferred and mixed into homogenized powder of mycelia or fruit bodies. The mixture was put into the tube and incubated for 40 minutes in water-bath at 65°C and followed by centrifugation for 5 minutes at 12,000 rpm.

After centrifugation, supernatant suspension was transferred into the other tube, and the same volume of chloroform isoamylalchol (24 : 1) were mixed carefully, and centrifuged for 5 minutes at 12,000 rpm again. The same process was continued 3 times to purify the

supernatant suspension.

Isopropanol reserved at −20°C was mixed into the supernatant suspension (0.7 : 1 volume ratio). After shaking them gently, they were kept for 5 minutes at room temperature (20~25°C) to precipitate DNA. After that, the mixture was kept for 24 hours at −20°C. After the appearence of white DNA precipitate, mixture was centrifuged for 5 minutes at 12,000 rpm and supernatant suspension was poured out. Remained pellet were washed by 70% ethanol reserved at −20°C and dried for 1 hour by freeze-dryer to extract DNA. Extracted DNA was melted into 150 μl of distilled water and reserved at −20°C. Before the amplification of DNA by PCR (Polymerase Chain Reaction), concentration of DNA was adjusted to 5 ng/ml by the mixing of distilled water. DNA concentration was measured by DNA Fluorometer (Hoefer TKO 100).

PCR and Electrophoresis

For the amplification of DNA by PCR, 25 μl of reaction solution was put into the tube. The reaction solution was composed with 0.5 μl of each 10 mM dNTP (dATP, dTTP, dCTP and dGTP, respectively; Perkin Elemer Cetus), 2.3 μl of 25 mM $MgCl_2$ (Perkin Elmer Cetus), 2.5 μl of 10×PCR buffer II (Perkin Elmer Cetus), 0.125 μl of Taq polymerase (10 U/μl, Perkin Elemer Cetus), 12.575 μl of distilled water, 0.5 μl of primer and 5 μl of template DNA (concentration of template DNA of *L. edodes* was 5 ng/μl).

Primers used in this experiment were 20 sorts of arbitrary 10-base primers (Operon Technologies Inc.). The sequences of primers are listed in Table 2.

Thus, DNA reaction solution was amplified by DNA Thermal Cycler 9600 (Perkin Elmer Cetus). At first, they were denaturated for 5 minutes at 94°C, and then continued 1 minute of denaturation at 94°C, 1 minute of annealing at 36°C and 2 minutes of extention at 72°C. This cycle was repeated 45 times.

Table 2. Sequences of primers used in this experiment

Primers	Sequences of primers	Primers	Sequences of primers
OPA-01	CAGGCCCTTC	OPA-11	CAATCGCCGT
OPA-02	TGCCGAGCTG	OPA-12	TGCGCGATAG
OPA-03	AGTCAGCCAC	OPA-13	CAGCACCCAC
OPA-04	AATCGGGCTG	OPA-14	TCTGTGCTGG
OPA-05	AGGGGTCTTG	OPA-15	TTCCGAACCC
OPA-06	GGTCCCTGAC	OPA-16	AGCCAGCGAA
OPA-07	GAAACGGGTG	OPA-17	GACCGCTTGT
OPA-08	GTGACGTAGG	OPA-18	AGGTGACCGT
OPA-09	GGGTAACGCC	OPA-19	CAAACGTCGG
OPA-10	GTGATCGCAG	OPA-20	GTTGCGATCC

For the electrophoresis, 1.0% agarose gel which added 0.005%(v/v) ethidium bromide was used. Amplified DNA solution was added with 2 μl of bromophenol blue for stain, and 20 μl of them were loaded in each well (comb hole). 10 μl of molecular weight marker (100-base pair DNA ladder, Pharmacia Biotech) diluted 10 times by distilled water was also loaded.

1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) was used as an electrode buffer, and the electrophoresis was proceeded for 4 hours under the 80V of voltage. After electrophoresis, DNA bands stained in gel were observed under the UV light and a photograph was taken to make record.

DNA bands were different by each primer and every strain, respectively. Therefore, the dendrogram of 7 strains was made by the average linkage cluster analysis which was taken statistics from the presence or absence of bands by each strain and primer.

Results and Discussion

Among 20 kinds of primers, 9 primers such

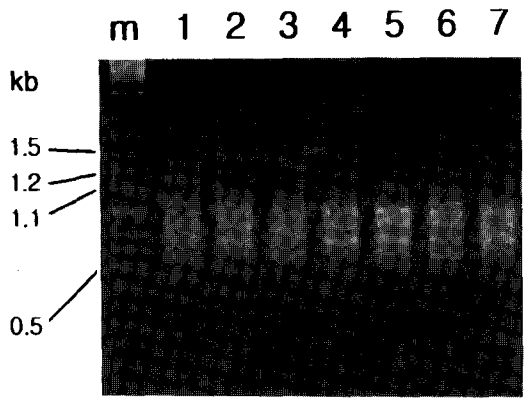

Fig. 1. RAPD profiles in fruiting bodies of *Lentinula edodes* strains with primer OPA-05.
m: Molecular size marker (100 base-Pair Ladder, Parmacia Biotec).
Lane 1 to 6: Sanlim No. 1 to No. 6. Lane 7: Imhyup No. 1.

as OPA-02, OPA-03, OPA-04, OPA-05, OPA-07, OPA-08, OPA-09, OPA-10 and OPA-12 were efficient to applying amplification of the DNA. These 9 primers showed significant DNA band profiles on the tested 7 strains, and showed high possibilities to identify each strains (Fig. 1, 2 and 3).

When DNA patterns of mycelium were compared with DNA patterns of fruiting body in the same strain, the patterns were almost the same (Fig. 4).

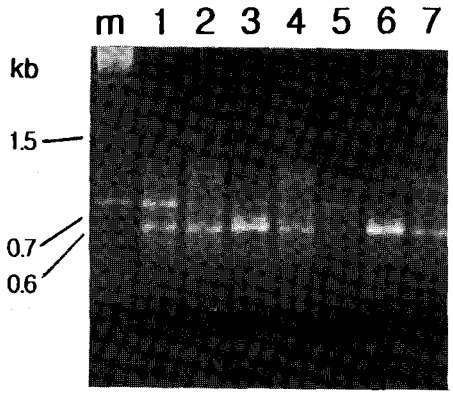

Fig. 2. RAPD profiles in fruiting bodies of *Lentinula edodes* strains with primer OPA-07.
m: Molecular size marker (100 base-Pair Ladder, Parmacia Biotec).
Lane 1 to 6: Sanlim No. 1 to No. 6. Lane 7: Imhyup No. 1.

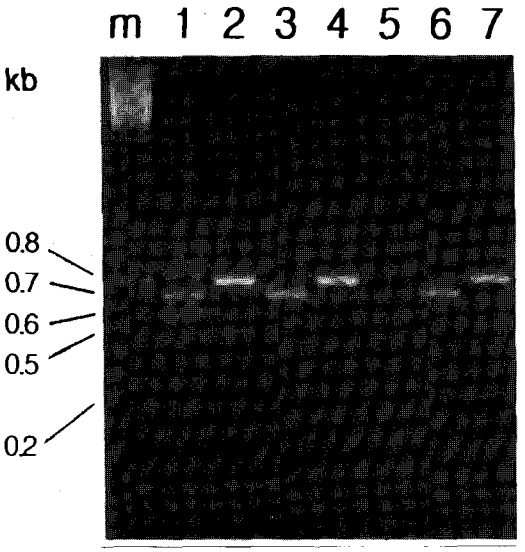

Fig. 3. RAPD profiles in fruiting bodies of *Lentinula edodes* strains with primer OPA-08.
m: Molecular size marker (100 base-Pair Ladder, Parmacia Biotec).
Lane 1 to 6: Sanlim No. 1 to No. 6. Lane 7: Imhyup No. 1.

Comparison of DNA Specificity and Classification of Diversity on the Strains

DNA bands appeared in 9 efficient primers

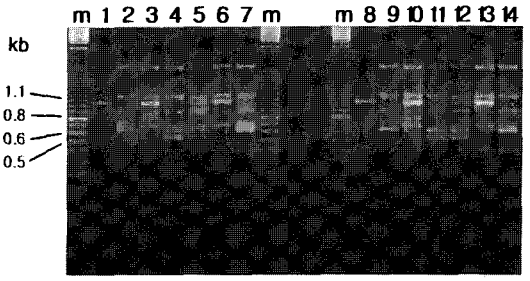

Fig. 4. RAPD profiles in fruiting bodies and mycelia of *Lentinula edodes* with primer OPA-04.
m: Molecular size marker (100 base-Pair Ladder, Parmacia Biotec).
Lane 1 to 7: Fruit-bodies of Sanlim No. 1 to No. 6. and Imhyup No. 1.
Lane 8 to 14: Mycelia of Sanlim No. 1 to No. 6 and Imhyup No. 1.

were listed in Table 3. Common DNA-bands indicated that all of tested strains showed presence of band in the same site of kilobase (Kb), and specific DNA-bands indicated that some of tested strains showed presence of band, but others showed absence of band in the same site of kilobase.

As shown on Table 3, there was no single primer that could classify all of strains by specific DNA-band. If we combine and

Table 3. Specific and common DNA-bands of fruit-body in *Lentinula edodes* strains by RAPD assay on 10-base primers

Primers	Size of specific DNA bands (Kb)	Strains							Common DNA bands (Kb)
		Sanlim No. 1	Sanlim No. 2	Sanlim No. 3	Sanlim No. 4	Sanlim No. 5	Sanlim No. 6	Imhyup No. 1	
OPA-02	1.1	–[a]	–	+	+	–	–	–	1.9, 1.7,
	0.7	–	+	–	+	+	–	+	1.0, 0.6
	0.5	+	–	–	–	–	–	–	0.4
OPA-03	2.4	–	+	+	+	–	+	+	1.8, 1.6
	1.3	–	–	+	–	–	–	–	1.2, 0.9
	0.8	–	–	–	+	–	–	–	0.5
	0.6	–	+	+	+	–	–	–	
OPA-04	1.1	+	–	+	–	+	+	–	1.9, 1.2
	0.8	–	+	–	+	+	–	+	
	0.6	–	+	–	+	+	–	+	
	0.5	–	–	–	+	+	+	–	
OPA-05	1.5	–	–	–	+	+	+	–	0.8, 0.7
	1.2	–	–	+	–	–	–	–	0.6
	1.1	+	–	–	–	–	–	–	
	0.7	+	+	–	–	+	+	+	
	0.6	–	–	+	–	–	+	–	
OPA-07	1.5	+	+	–	+	+	–	+	1.0, 0.8
	1.1	+	+	–	+	–	–	+	
	0.7	–	–	+	–	–	+	–	
	0.6	+	+	+	+	–	+	+	
OPA-08	0.8	–	+	–	+	+	–	+	
	0.7	+	–	+	–	–	+	–	
	0.6	+	–	+	–	+	–	–	
	0.5	–	+	–	–	–	–	+	
	0.2	+	–	+	–	–	+	–	
OPA-09	1.1	+	+	+	–	–	+	+	1.9, 1.5
	1.0	–	–	+	–	–	–	–	0.9, 0.7
	0.8	–	–	–	+	–	–	–	0.6,
	0.5	+	+	+	+	+	–	+	
OPA-10	1.4	–	+	–	–	+	–	+	1.7, 1.0
	1.1	–	+	–	–	+	–	+	0.5
	0.9	+	+	–	+	+	–	+	
	0.7	+	+	–	+	+	–	+	
OPA-12	1.5	+	–	+	–	–	+	–	
	1.3	–	+	–	+	+	–	–	
	1.0	+	+	–	+	–	–	–	
	0.8	–	+	–	–	+	–	+	

[a]: –, means absence of DNA band, and +, means presence of DNA band

analyse optional 2 primers, all of different strains can be classified by different specfic DNA-bands.

Polymorphisms of DNA bands showed the same characteristics in the replication tests. Thus, if a certain strain is tested as DNA polymorphisms by the same primers afterwards, it could be identified whether the strain is the same strain or not by consulting with the Table 3.

Meanwhile, the dendrogram was made by average linkage cluster analysis with the statistics on the presence or absence of bands by strains in Table 3. The dendrogram based on RAPD markers of *L. edodes* strains is shown on Fig. 5.

As showed on Fig. 5, the strains devided into 2 clusters in a large view point, but they devided into 6 clusters in a narrow view point. In a large view point, Sanlim No. 1, No. 3 and No. 6 belonged to the same group, and Sanlim No. 2, No. 4, No. 5 and Imhyup No. 1 belonged to another group.

In the first cluster, Sanlim No. 3 and No. 6 showed close relation, but Sanlim No. 1 was far from them. In the second cluster, Sanlim No. 2 and Imhyup No. 1 showed very close relation, whereas Sanlim No. 5 and Sanlim No. 6 showed further relation.

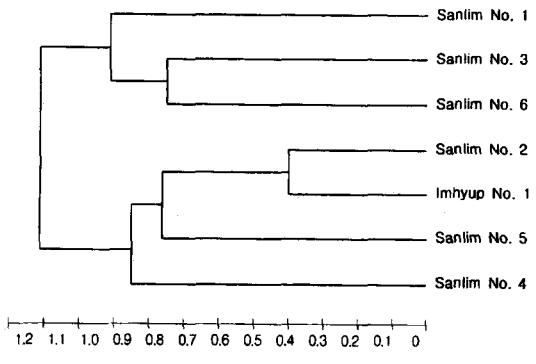

Fig. 5. A dendrogram based on RAPD markers of *Lentinula edodes* strains determined by average linkage cluster analysis.

적 요

한국의 7가지 대표적인 표고품종에 대하여 RAPD(Random Amplified Polymorpic DNA) 검정을 실시하여 품종간의 구분이 가능한 지를 시도하였다. 표고 품종간의 계통분류에 적합한 RAPD marker를 생성시키기 위하여 OPA-01에서 OPA-20까지 20개의 primer를 사용한 결과, 9가지의 primer는 품종식별에 유용한 RAPD pattern을 보였으나, 나머지 11가지의 primer는 품종 식별에 사용하기 어려운 것으로 나타났다. 9가지 primer중 7개 품종을 모두 구분할 수 있는 단일 primer는 없었지만, 9가지중 2개의 조합을 취하면 어떤 경우도 7개의 표고 품종을 구분지울 수 있음으로써 RAPD 검정법이 표고 계통의 분류에 매우 정밀한 방법임을 알 수 있다.

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