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Genotyping of the Wild Mushroom *Lentinula edodes* from Mt. Jungwang and Mt. Gariwang in Korea

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ABSTRACT : *Lentinula edodes* is an edible wild mushroom that can be found in mountainous regions of the Korean peninsula. Wild oak mushrooms were collected from *Quercus mongolica* at an elevation of more than 1,000 m on Mt. Jungwang and Mt. Gariwang in Gangwon province. We examined 10 oak mushroom strains to evaluate the genetic similarity among strains. Genetic similarity was determined based on the analysis of microsatellite markers (Led A2, Led A8, Led B2, Led B6, and Led D6) registered in the National Center for Biotechnology Information. We also performed dual culture tests on potato dextrose agar for 2 months at 25 °C. The observed heterozygosity across all microsatellites ranged from 0.00 and 0.60 among 5 microsatellite markers, and the polymorphism information content values of Led A2, Led A8, Led B2, Led B6, and Led D6 were 0.0000, 0.8144, 0.6194, 0.4892, and 0.5702, respectively (mean value = 0.4987). Confrontation lines between strains were formed for almost all combinations. In conclusion, the oak mushroom populations of Mt. Jungwang and Mt. Gariwang have mixed gene pools. However, further studies are needed to identify genetic similarities and variations among these populations.

KEYWORDS : Gene diversity, *Lentinula edodes*, Microsatellite markers, Population

Introduction

Lentinula edodes (shiitake) is one of the most important edible mushrooms in the world. The fungus is distributed in Asia and some subtropical regions. Republic of Korea, China, Taiwan, and Japan cultivate and consume shiitake mushrooms [1]. In the majority of cases, cultivated shiitake mushrooms have different cultivar names; therefore, several names can exist for describing the same species. This makes it difficult to accurately match names and species. For the last decade, several molecular marker techniques have been utilized to classify different strains of shiitake. Restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and sequence characterized amplified region (SCAR) have been used. However, these methods have limitations regarding economic efficiency, reliability, and analysis time. In order to overcome these limitations, amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) are being used as promising alternative methods [2-4]. SSR also referred to as microsatellites consist of short DNA sequence motifs that are abundant in the genomes of prokaryotic and eukaryotic organisms [5]. They are useful for tracking the country of origin and for distinguishing between individual strains. They have several alleles, high heterozygosity, and many polymorphous nucleotides. In addition, a number of repeated sequences can be easily calculated, and these short DNA sequence motifs exhibit a low rate of mutations [1]. Compared to the other techniques, co-dominant SSR was shown to be highly polymorphic, reproducible, stable, reliable, easy to score, fast, and accurate [6, 7]. Using *Suillus bovinus*, *Pleurotus ostreatus*, and *Agaricus bisporus*, it has been reported that microsatellite markers are useful for genetic studies on mushrooms [2]. Moreover, this analysis can reveal genetic linkage maps, genetic diversity analysis, molecular ecology, and phylogenetics.

In this study, we identified genotypes of wild *L. edodes* strains collected in Mt. Jungwang and Mt. Gariwang using

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Table 1. Sampling site of the shiitake mushrooms

NIFoS No.	Sampling site (compartment)[a]	North latitude	East longitude	Height (m)
1520	Mt. Jungwang (123)	37° 27′ 27.39″	128° 32′ 18.59″	1,050
1521	Mt. Jungwang (125)	37° 28′ 26.63″	128° 30′ 55.37″	1,229
1522	Mt. Jungwang (125)	37° 28′ 28.88″	128° 30′ 47.52″	1,224
1561	Mt. Gariwang (122)	37° 28′ 29.03″	128° 30′ 46.91″	1,105
2290	Mt. Gariwang (122)			
2497	Mt. Jungwang (125)	37° 28′ 29.27″	128° 30′ 29.27″	1,081
2498	Mt. Jungwang (125)		Similar NIFoS 2497	
2499	Mt. Jungwang (124)	37° 28′ 33.37″	128° 31′ 31.08″	1,202
2500	Mt. Jungwang (127)	37° 30′ 09.21″	128° 30′ 39.83″	1,081
2783	Mt. Jungwang (126)			

NIFoS, National Institute of Forest Science.
[a]One division of separated mountains in Korea.

microsatellite markers to understand the population structure of this mushroom.

Materials and Methods

Sampling and cultivation

We used 10 *Lentinula edodes* strains registered in the National Institute of Forest Science (NIFoS, Table 1). Each strain was isolated from mushrooms collected from Mt. Jungwang and Mt. Gariwang in Korea (Fig. 1). They were maintained at 4°C on slants of potato dextrose agar (PDA; Difco, Detroit, MI, USA).

DNA extraction, amplification, and genotyping

We isolated the genomic DNAs of 10 shiitake strains using the DNeasy Plant mini kit (Qiagen, Germantown, MD, USA). We made microsatellite markers using sequence information from GenBank (DQ231475-DQ-23479) and the Primer3 v0.4 program. One of each primer pair was labeled with 6 FAM fluorescent dyes (Table 2). PCR was performed using a Thermocycler (Bio-Rad, Hercules, CA, USA). Each 25 mL reaction mixture contained 30 ng of DNA, 1X PCR buffer, 2.5 mM of dNTPs, 200 nM of each primer, and 1 U of Taq polymerase. A first denaturation step was performed at 95°C for 3 minutes, followed by 30 cycles at 94°C for 30 seconds, 54°C or 56°C for 30

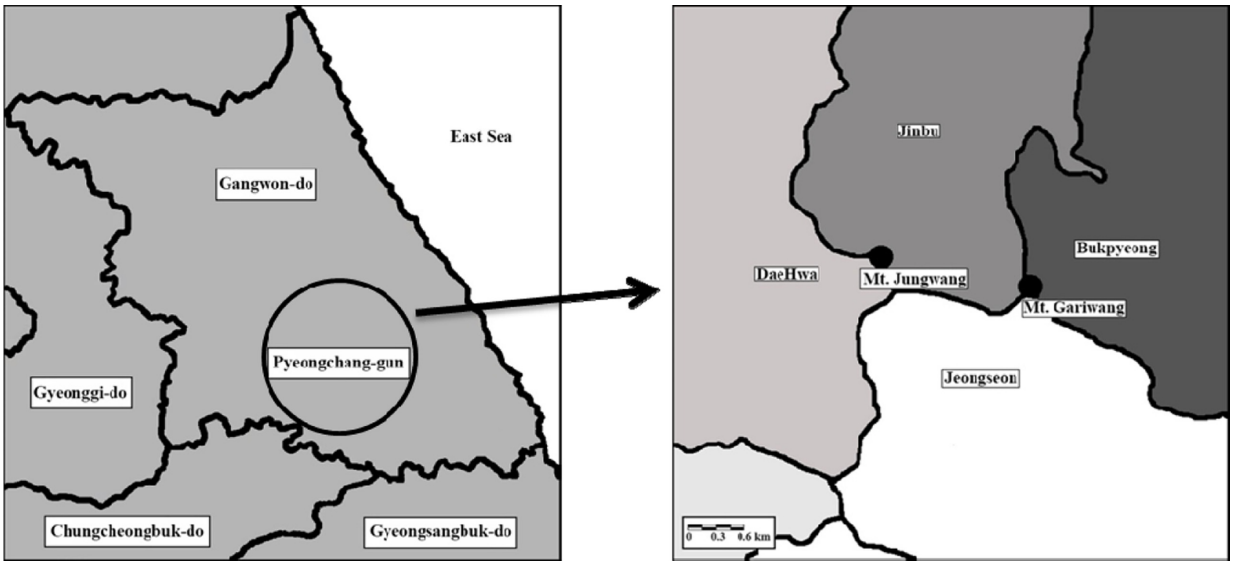

Fig. 1. Location of the wild Lentinula edodes strains collected in Mt. Jungwang and Mt. Gariwang, Pyeongchang-gun, Gangwon province in Korea.

Table 2. Summary data of the five microsatellite markers used in this study

Locus Genbank No.	Primer sequence	Size range (bp)	Motifs[a]	T_m (°C)
LedA2 DQ231475	5'-FAM ACT GGT GCT TTA GTG GTC G-3' 5'-TTC GGA TCC CTT TGC CTC-3'	78~121	Ramdom	54
LedA8-F DQ231476	5'-FAM TCA TCT CCT TC CAT GTT CC-3 5'-CAA TAC CGG TAA CAC GTC C-3'	247~286	Ramdom	54
LedB2-F DQ231477	5'-GAM ACC ACC TTC CTT GAT CTC C-3' 5'-CTA AAC ACC AAC ATC CGC C-3'	185~213	$(GGT)_n$	54
LedB6-F DQ231478	5'-GAM GGT GAG AAA GAG ATC GAG G-3' 5'-GTG GCC GTG ATG TTC CTT-3'	175~239	$(GGT)_n$	54
LedD6-F DQ231479	5'-FAM GCT CCT TCA CCT CGA CTT TGA-3' 5'-AGT GAA GGA ACA CCA CGG TCA-3'	182~257	$(CCA)_n$	56

[a]Repeated sequence indicates the motif of the strain allele.

seconds, and 72°C for 1 minute. A final extension step was performed at 72°C for 5 minutes. PCR products were separated and analyzed by an ABI 3730xl DNA analyzer (Applied Biosystems, Foster city, CA, USA). We analyzed allele sizes using GENE MAPPER version 4.0 (Applied Biosystems), and calculated allele numbers and heterozygosity [1].

Analysis of variation and population relationship

We calculated the polymorphism information content (PIC) using the PowerMarker program [8]. The allelic and genotypic frequencies were calculated using the analyzed samples. The genetic variability of the sample as a whole was estimated based on the number of alleles per locus (total numbers of alleles/numbers of loci).

The percentage of polymorphic loci = numbers of polymorphic loci/total number of loci analyzed × 100, and PIC contents = PIC = $1 - \Sigma_{i=1} Pi^2 - \Sigma_{i=1}\Sigma_{i=i+1} Pi^2 Pj^2$

Phylogenetic analysis

The dataset was converted to frequency data using the PowerMarker v3.0 software. Calculation of genetic distance among samples indicated the population structure. Various distance measures used for frequency data have been described [9, 10]. For evolutionary studies, the clustering of operational taxonomic units (OTUs) naturally lead to a phylogenetic tree. The neighbor joining method was used to reconstruct the phylogeny from a distance matrix [9].

Confrontation test

The confrontation test was applied to assess the homogeneity among the 10 strains. The strains were inoculated on PDA agar plates and incubated at 25°C for 21 days. Mycelium disks (ca. 8 mm) from different strains were inoculated on a PDA agar plate on both sides and incubated at 25°C. Among the 10 strains, 45 combinations were performed. After 2 months, we assessed samples for the presence of confrontation lines.

Results and Discussion

The allele sizes of all 10 *L. edodes* strains was analyzed for the 5 microsatellite markers evaluated (Table 3). Led A8 is the allele for 8 types, specifically 256, 258, 268, 270, 274, 280, 282, and 286 bp (Fig. 2). Led B2 is the allele for 5 types, specifically 200, 209, 212, 215, and 224 bp. Led B6 is the allele for 5 types, specifically 177, 180, 183, 186, and 195 bp. Led D6 is the allele for 6 types, specifically 188, 209, 215, 218, 221, and 227 bp. Led A2 is the allele for 114 bp only (Table 3). Table 4 shows the statistical analysis of the genotypic characteristics from the 10 shiitake analyzed in this study. The number of alleles ranged from 1 to 8 with an average value of 5/locus, and the observed allele frequency was the highest in Led A2 (1.00) followed by Led B6, Led D6 (0.60), Led B2 (0.50), and Led A8 (0.25). The genotype number ranged from 1 to 8 and the average value was 5.40/locus. Led A8 had the highest gene diversity (H_e) among the 5 markers, followed by Led B2 (0.6650), Led D6 (0.6000), and Led B6 (0.5300). Led A2 did not demonstrate gene diversity in this study. The observed heterozygosity (H_o) ranged from 0.4000 to 0.6000, except for Led A2. In addition, Led B2 had the highest heterozygosity among the 5 markers. PIC values ranged from 0.0000 to 0.8144 with an average of 0.4987/locus, and a relatively high polymor-

Table 3. Genotypes of the 10 shiitake strains analyzed using the 5 markers

NIFoS No.	Genotype				
	LedA2	LedA8	LedB2	LedB6	LedD6
1520	114/114	286/286	200/224	177/180	188/221
1521	114/114	258/258	215/215	183/183	215/221
1522	114/114	258/258	200/224	183/183	215/215
1561	114/114	258/280	200/224	183/195	215/227
2290	114/114	268/268	200/224	177/183	209/215
2497	114/114	256/282	200/200	183/183	215/215
2498	114/114	256/274	200/200	183/183	215/215
2499	114/114	268/268	200/212	183/186	215/218
2500	114/114	274/274	200/224	183/183	215/215
2783	114/114	270/286	209/209	177/177	188/188

NIFoS, National Institute of Forest Science.

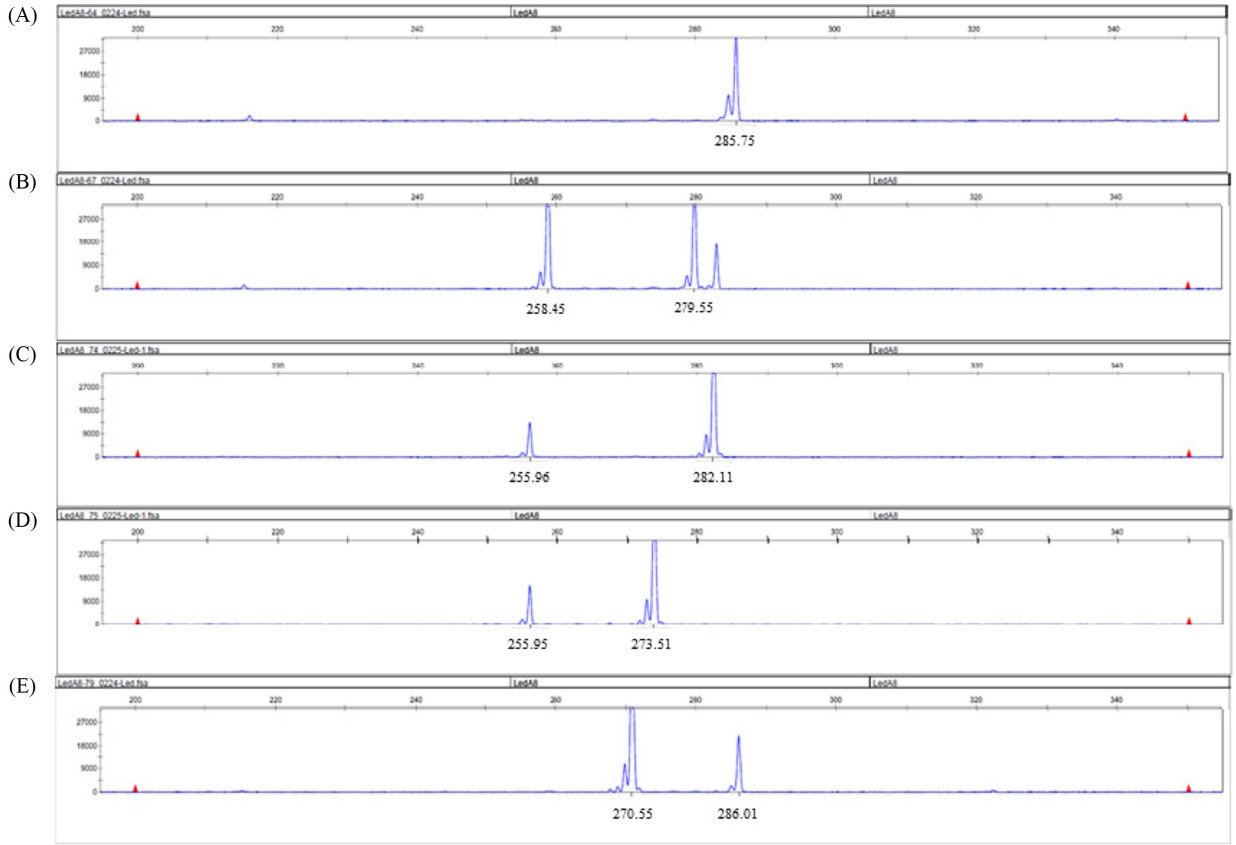

Fig. 2. Electropherogram of Led A8 microsatellite marker in the wild *Lentinula edodes*. Results of genotype analysis performed using the GENEMAPPER v4.0 software on raw data obtained from analysis using a genetic analyzer on NIFoS 1520 (A), NIFoS 1561 (B), NIFoS 2497 (C), NIFoS 2498 (D), and NIFoS 2783 (E). X-axis, DNA size; Y-axis, peak signal height; NIFoS, National Institute of Forest Science.

phism was found in Led A8 (0.8144) > Led B2 (0.6194) > Led D6 (0.5702) > Led B6 (0.4892) > Led A2 (0.0000). The average PIC value among the 5 markers was 0.4987.

We also examined samples for the presence of confrontation lines in order to identify the homozygosity and heterozygosity of the 10 shiitake strains (Table 5). Five

Table 4. Statistic analysis of the genotypic characteristics of the 10 shiitake strains

Locus	Major allele frequency	Genotype numbers	Sample size	Allele numbers	Gene Diversity[a] (H_e)	Observed Heterozygosity[b] (H_o)	PIC[c]
LedA2	1.00	1	10	1	0.0000	0.0000	0.0000
LedA8	0.25	8	10	8	0.8350	0.4000	0.8144
LedB2	0.50	5	10	5	0.6650	0.6000	0.6194
LedB6	0.65	6	10	5	0.5300	0.4000	0.4892
LedD6	0.60	7	10	6	0.6000	0.5000	0.5702
Mean	0.60	5.40	10	5	0.5260	0.3800	0.4987

[a]Gene diversity, often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different.
[b]Observed heterozygosity is the proportion of heterozygous individuals in the population.
[c]A closely related diversity measure is the polymorphism information content (PIC).

Table 5. Dual culture assay results using on the 10 shiitake strains

NIFoS	1520	1521	1522	1561	2290	2497	2498	2499	2500	2783
1520		O	O	X	O	O	O	O	O	O
1521			O	O	O	O	O	O	O	O
1522				X	O	O	O	O	O	O
1561					O	O	X	O	O	O
2290						O	O	O	O	O
2497							X	O	X	O
2498								O	O	O
2499									O	O
2500										O
2783										

O indicates formation of a confrontation line. X indicates no formation of the confrontation line.
NIFoS, National Institute of Forest Science.

cases among the 45 combinations (NIFoS 1520 × 1561, 1522 × 1651, 1561 × 2498, 2497 × 2498, 2497 × 2500) showed no confrontation lines. However, the other 40 combinations showed distinct confrontation lines (Table 5). Analysis of the population relationships using the expectation-maximization (EM) algorithm function, a neighbor joining method, showed genetic similarity based on frequency data (Fig. 3). Ten shiitake strains were grouped into 3 clades, and there was no correlation between geographical locations and genetic distance.

We used five microsatellite markers in this study. The marker Led A2 showed no genetic diversity of shiitake strains. All strains showed different genotypes regarding the other 4 markers. Led A8 had the highest allele number, genotype number, and PIC. The PIC was estimated as the probability that a particular strain is informative with respect to segregation of its inherited alleles. Statistical analysis of PIC is an indicator of the power of discrimination. PIC values greater than 0.5 indicate a high power of discrimination [1]. The average PIC value for the five markers was 0.4987. However, the PIC values of three markers (Led B2, Led D6, and Led B6) were larger than 0.5; therefore, they were used to distinguish shiitake diversity. When the 10 strains were cultivated on PDA agar plates for the dual culture assay, 40/45 combinations formed combination lines. Confrontation lines indicate that tested strains are not compatible with each other although they are the same species. The neighbor-joining tree with the 10 *L. edodes* strains showed genetic distance among them. Strains were grouped into 3 clades; strains in clade I and III were originated from Mt. Jungwang, and strains in clade II from Mt. Gariwang and Mt. Jungwang. When collected in the same compartment, genetic distances are closer than when collected in different compartments. Strains in clade II were from 4 compartments in 2 mountains. NIFoS 2497 and 2498 were collected from the same site and the genotypic difference was small. Xiang et al. [11] reported that genetic diversity was closely

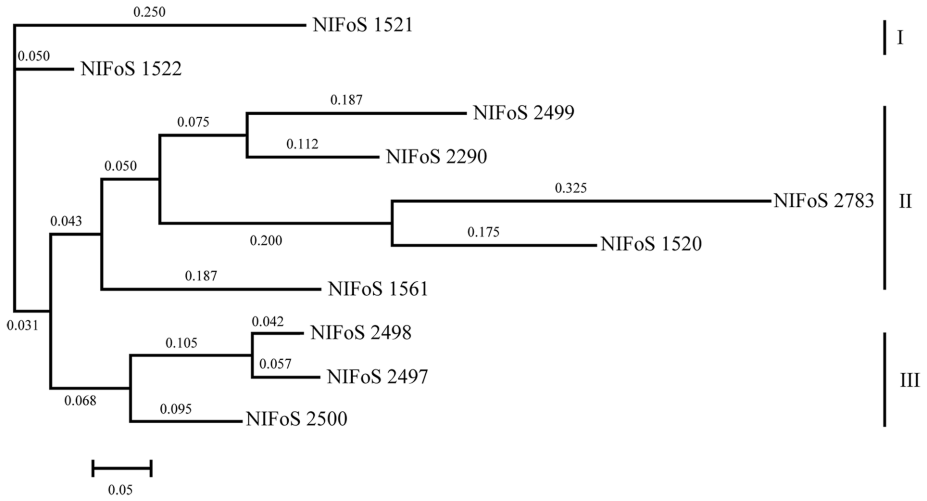

Fig. 3. Neighbor joining tree showing the genetic relationships among the 10 strains of *Lentinula edodes* using five microsatellite markers. NIFoS, National Institute of Forest Science.

related to geographical distribution. However, NIFoS 2499 and 2290 were collected from two distant locations, but their genotypic difference was small. Chiu et al. [12] reported that neighboring isolated shiitake mostly belong to different somatic compatibility groups, implying that the territory of *L. edodes* in the wild is very small. The smallest fruiting territory of *L. edodes* genes proved to be less than 20 cm in diameter. *L. edodes* possess a tetropolar incompatibility system, which allows mating between basidiospores from the same parent, at a rate of 25% [13]. Various genotypes in the same species are the consequence of adaptation as a result of environmental change and preservation. This property of *L. edodes* in the natural environment is useful for the development and improvement of new *L. edodes* cultivars. The genetic diversity indicates that the total genetic characteristics in the genome of a species play important roles in the species' survival and adaptability. Analysis of genetic diversity and population structure provide important information for surveying the origin and evolution of a species, and for identifying potential genetic resources for further utilization [14]. Ten shiitake mushrooms in the two mountains had various genotypic characteristics and they revealed no correlation with geographical distribution. This means that correlation between genetic diversity and geographical location is not absolute. *L. edodes* has high reproducibility, polymorphism, reliability, and stability when using the microsatellite technique [2]. Microsatellite marker data was able to contribute as the groundwork for selecting hybrid parent strains for producing superior strains of *L. edodes*. In addition, microsatellites can significantly increase the density of markers, and can be used to compare and integrate different genetic maps. They can also aid in the location of genes of interest that have been identified in various crosses. Based on these advantages of the SSR and microsatellite techniques, they can be beneficial in constructing a genetic map of *L. edodes* [2]. An increased number of markers could be used to determine the genotypic variety of shiitake, and for construction of linkage maps, cross breeding, mapping of quantitative trait loci, evolutional science. The markers can also aid in the protection of our mushroom under UPOV institution and varied illegal cloned strains.

In conclusion, the oak mushroom populations of Mt. Jungwang and Mt. Gariwang have a mixed gene pools. However, further studies are needed to identify genetic similarities and variations among them.

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