Development and Molecular Characterization of Novel Polymorphic Genomic DNA SSR Markers in *Lentinula edodes*

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Abstract  Sixteen genomic DNA simple sequence repeat (SSR) markers of *Lentinula edodes* were developed from 205 SSR motifs present in 46.1-Mb long *L. edodes* genome sequences. The number of alleles ranged from 3–14 and the major allele frequency was distributed from 0.17–0.96. The values of observed and expected heterozygosity ranged from 0.00–0.76 and 0.07–0.90, respectively. The polymorphic information content value ranged from 0.07–0.89. A dendrogram, based on 16 SSR markers clustered by the paired hierarchical clustering method, showed that 33 shiitake cultivars could be divided into three major groups and successfully identified. These SSR markers will contribute to the efficient breeding of this species by providing diversity in shiitake varieties. Furthermore, the genomic information covered by the markers can provide a valuable resource for genetic linkage map construction, molecular mapping, and marker-assisted selection in the shiitake mushroom.

Keywords  gDNA-SSR, genetic diversity, *Lentinula edodes*, UPGMA

As one of the most important edible mushrooms, *Lentinula edodes* (shiitake) is mainly cultivated in the East Asian and Oceania region. Its unique flavor and high nutritional value have attracted much attention as a highly valuable food [1, 2]. The medicinal value of shiitake mushroom has drawn particular interest since lentinan, a β-glucan component, has shown high immune-enhancing activity in cancer patients [3, 4].

An international convention to protect breeder rights was established in 1991; this convention was put in place for the protection of newly cultivated varieties of plants, including mushrooms (http://www.upov.int/). With this revision, the number of new shiitake mushroom varieties with advantageous traits have gradually increased. Therefore, identifying the distinctiveness of each variety has emerged as an important problem. Since it is difficult to accurately distinguish one cultivar amongst several shiitake mushroom cultivars [5, 6], it is important to develop molecular markers that can complement cultivar discrimination based on both internal genetic and external phenotypic traits.

To date, some molecular markers including inter-simple sequence repeat (ISSR), random amplification of polymorphic DNA (RAPD), sequence-related amplified polymorphism, and simple sequence repeat (SSR) have been developed for analyzing the genetic diversity of shiitake mushroom [6-8]. Amongst these, studies of SSR markers have revealed insights into important genetic characteristics including reproducibility, multi-allelic nature, and co-dominant inheritance [9]. Although genomic DNA SSRs are highly polymorphic and widely distributed in the genome [10], development of genomic DNA based SSR markers have rarely been studied.
because of the absence of detailed genomic data for *L. edodes*.

Next-generation sequencing (NGS) is a powerful tool that can detect large numbers of molecular markers within a short time [11]. NGS is also very useful for the validation and evaluation of molecular markers in a given population [12]. Recently, the whole genome of the *L. edodes* monokaryon strain B17 was published by our group [13]. In this study, using whole genome data, we developed 16 SSR markers to analyze genetic diversity and discriminate between shiitake mushroom varieties.

In this study, genomic DNA was extracted using shiitake mycelia cultured in potato dextrose broth at 25°C, 110 rpm for approximately 2 wk in the dark. The cultured mycelium was filtered through Miracloth and washed with phosphate buffered saline buffer (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.4 mM KH2PO4), and water was removed with an absorbent towel. Dried hyphae (100 mg) was frozen in liquid nitrogen and ground in a mortar. Genomic DNA was then extracted using a GenEx Plant Kit (GeneAll Biotechnol Co., Shanghai, China). The extracted DNA was quantified using a K5600 Micro-spectrophotometer (Shanghai Biotechnol Co., Shanghai, China).

To design reliable SSR markers from reference whole genome sequencing data for shiitake mushroom [13], we produced 2 GB of read data by re-sequencing genomic DNA extracted from 33 tested strains developed in East Asian countries (15 accessions originating from Korea, 12 accessions originating from Japan, and 6 accessions originating from China) (Table 1). Sequencing reads were then mapped to selected SSR motif regions of the reference genome. We selected 205 motifs that showed the largest conservation rate and diversity amongst all SSR motifs and selected a total of 16 SSR markers (Table 2). Primer design parameters were set as follows: length, 18–23-bp with 21-bp as the optimum; PCR product size range, 150–200-bp; optimum annealing temperature, 58°C; GC content, 50–61%, with 51% as the optimum.

Table 1. Strains of *Lentinula edodes* used in this study

| Strain No. | Strain name | Origin |
|-----------|-------------|--------|
| 1         | SI 713      | Korea  |
| 2         | SI 302      | Korea  |
| 3         | SI 502      | Korea  |
| 4         | SI 701      | Korea  |
| 5         | KFRI 407    | Korea  |
| 6         | KFRI 299    | Korea  |
| 7         | KFRI 169    | Korea  |
| 8         | KFRI 53     | Korea  |
| 9         | KFRI 542    | Korea  |
| 10        | KFRI 2778   | Korea  |
| 11        | KFRI 354    | Korea  |
| 12        | KFRI 554    | Korea  |
| 13        | KFRI 549    | Korea  |
| 14        | KFRI 547    | Korea  |
| 15        | KFRI 619    | Korea  |
| 16        | KFRI 1068   | China  |
| 17        | KFRI 261    | China  |
| 18        | KFRI 496    | China  |
| 19        | KFRI 495    | China  |
| 20        | KFRI 491    | China  |
| 21        | KFRI 2695   | China  |
| 22        | KFRI 1255   | Japan  |
| 23        | KFRI 1058   | Japan  |
| 24        | KFRI 31     | Japan  |
| 25        | KFRI 804    | Japan  |
| 26        | KFRI 755    | Japan  |
| 27        | KFRI 1514   | Japan  |
| 28        | KFRI 33     | Japan  |
| 29        | KFRI 1046   | Japan  |
| 30        | KFRI 761    | Japan  |
| 31        | KFRI 22     | Japan  |
| 32        | KFRI 812    | Japan  |
| 33        | KFRI 813    | Japan  |

After allele scoring, the number of alleles (N_a), major allele frequency (M_a), observed heterozygosity (H_o), expected heterozygosity (H_e), number of genotypes (N_g), and polymorphic information content (PIC) were calculated using PowerMarker v3.25 [14]. The distance between each sample was calculated by using the Shared Allele method and the 33 tested strains were clustered by the unweighted pair group method with arithmetic mean (UPGMA). In the sample we analyzed (n = 34), N_a amongst the markers ranged from 3–14, with an average of 6.8. M_a ranged from 0.17–0.96, with an average of 0.482. The H_o value ranged from 0.00–0.76, and H_e indicating gene diversity, ranged from 0.07–0.90. The average of H_e and H_o was 0.322 and 0.643, respectively, and overall the PIC value ranged from 0.07–0.89, with an average of 0.612 (Table 3). SSR markers of the *Auricularia auricula-judae* and *Flammulina velutipes* have been developed, and the average PIC value indicating the diversity of the markers was 0.47 and 0.42, respectively [15, 16]. The estimated PIC value of SSR markers developed in our study revealed a higher score, suggesting that our markers are more efficient than those of previous reports.

UPGMA clustering and the subsequent dendrogram showed that 33 shiitake strains could be divided into three clusters when analyzed with our 16 SSR markers (Fig. 1).
Cluster 1 contained six Japanese, five Chinese, and two Korean strains. Cluster 2 contained six Korean, three Japanese, and one Chinese strain, and cluster 3 contained seven Korean and three Japanese strains. The 33 strains of shiitake mushrooms showed a tendency to be grouped to their origin; however, clustering was not completely reflected by geographical location. Dangi et al. [17] analyzed *Trigonella foenum-graecum* using ISSR and RAPD, and reported that the association between genetic similarity and geographical distance was less significant. This contradiction could not exclude the possibility that various genetic traits from different geographic regions may be mixed during the phenotype-assisted selection during the breeding process. Therefore, it may be necessary to use a greater number of strains from each geographical location to confirm the observed patterns. Whilst geographical clustering was not observed with our SSRs, they could discriminate between varieties of shiitake mushroom that had a narrow gene pool through selective breeding. Therefore, using these markers for discrimination of accessions with greater diversity, significant results could be more efficiently derived.

### Table 3. Characteristics of 16 genomic DNA-SSR markers of Lentinula edodes

| Marker       | Primer sequence (5'-3') | Product size (bp) | Motifs | Accession No.   |
|--------------|-------------------------|-------------------|--------|-----------------|
| RL-LE-001    | F:GTGTCACAAATCAGCAGGATC R:AATCAGTGACGTCCGTCGAGTCCGTCGAGTC | 154    | (TA)5  | NM0434-000001   |
| RL-LE-002    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 194    | (AT)7  | NM0434-000002   |
| RL-LE-003    | F:GTGACCAATGAGACGTCCGACTTAGTC | 179    | (AC)9  | NM0434-000003   |
| RL-LE-004    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 192    | (CCA)5 | NM0434-000004   |
| RL-LE-005    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 194    | (GGA)6 | NM0434-000005   |
| RL-LE-006    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 177    | (TC)7  | NM0434-000006   |
| RL-LE-007    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 194    | (CT)12 | NM0434-000007   |
| RL-LE-008    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 150    | (AT)5  | NM0434-000008   |
| RL-LE-009    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 157    | (AT)5  | NM0434-000009   |
| RL-LE-010    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 194    | (AT)6  | NM0434-000010   |
| RL-LE-011    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 191    | (AT)5  | NM0434-000011   |
| RL-LE-012    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 194    | (CA)5  | NM0434-000012   |
| RL-LE-013    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 196    | (CG)5  | NM0434-000013   |
| RL-LE-014    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 178    | (TT)5  | NM0434-000014   |
| RL-LE-015    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 185    | (CA)7  | NM0434-000015   |
| RL-LE-016    | F:GTGACAAATGACCGGGTATAAAG R:GCTACTTGCTGTCCGACTTAGTC | 167    | (CG)6  | NM0434-000016   |

Table 3. Diversity statistics from primer screening in 33 strains of *Lentinula edodes*

| Marker      | M<sub>oa</sub> | N<sub>o</sub> | N<sub>g</sub> | H<sub>o</sub> | H<sub>e</sub> | PIC       |
|-------------|----------------|-------------|-------------|-------------|-------------|-----------|
| RL-LE-001   | 0.34           | 11          | 7           | 0.16        | 0.78        | 0.75      |
| RL-LE-002   | 0.17           | 19          | 14          | 0.45        | 0.90        | 0.89      |
| RL-LE-003   | 0.68           | 4           | 4           | 0.05        | 0.48        | 0.42      |
| RL-LE-004   | 0.19           | 15          | 11          | 0.29        | 0.85        | 0.84      |
| RL-LE-005   | 0.45           | 9           | 6           | 0.61        | 0.72        | 0.69      |
| RL-LE-006   | 0.38           | 15          | 9           | 0.76        | 0.78        | 0.75      |
| RL-LE-007   | 0.52           | 9           | 8           | 0.38        | 0.66        | 0.62      |
| RL-LE-008   | 0.39           | 8           | 7           | 0.33        | 0.72        | 0.73      |
| RL-LE-009   | 0.25           | 9           | 9           | 0.41        | 0.82        | 0.80      |
| RL-LE-010   | 0.27           | 13          | 7           | 0.63        | 0.83        | 0.81      |
| RL-LE-011   | 0.73           | 3           | 3           | 0.12        | 0.42        | 0.37      |
| RL-LE-012   | 0.38           | 10          | 7           | 0.30        | 0.76        | 0.73      |
| RL-LE-013   | 0.47           | 7           | 4           | 0.36        | 0.68        | 0.62      |
| RL-LE-014   | 0.71           | 9           | 6           | 0.24        | 0.47        | 0.44      |
| RL-LE-015   | 0.82           | 3           | 3           | 0.00        | 0.31        | 0.27      |
| RL-LE-016   | 0.96           | 3           | 3           | 0.07        | 0.07        | 0.07      |

M<sub>oa</sub>, major allele frequency; N<sub>o</sub>, number of genotypes; N<sub>g</sub>, number of alleles; H<sub>o</sub>, observed heterozygosity; H<sub>e</sub>, expected heterozygosity; PIC, polymorphic information content.
plant species [18-20]. The development of SSR markers and further investigation of the genetic relationships between varieties is of great importance for germplasm management, parent selection, and cross breeding [21]. The novel markers developed in this study will contribute to the efficient breeding of shiitake mushrooms by giving the diversity with the varieties of narrow gene pools. Additionally, genomic information covered by these markers can provide valuable resources utilized for genetic linkage map construction, molecular mapping, and marker-assisted selection.

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Fig. 1. Dendrogram generated using unweighted pair group method with arithmetic mean cluster analysis based on genetic diversity of 33 *Lentinula edodes* strains. The simple sequence repeat markers developed in this study were not grouped by strain development country. This result considered for grouping by qualitative and quantitative traits. KOR, Korea; CHN, China; JPN, Japan.

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