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Research Note

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

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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 Na_2HPO_4, and 1.4 mM KH_2PO_4), 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., Seoul, Korea). The extracted DNA was quantified using a K5600 Micro-spectrophotometer (Shanghai Biotechnol Co., Shanghai, China).

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

Strain No.	Strain name	Origin
1	SJ 713	Korea
2	SJ 302	Korea
3	SJ 502	Korea
4	SJ 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

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.

PCR was performed using 20 ng of DNA template using 2× PCR Master Mix Solution (i-Taq; iNtRON Biotechnol Inc., Seongnam, Korea) according to the manufacturer's instructions. The final volume for all reactions was fixed at 20 μL and the final concentration of primers was 10 μM. Samples were amplified using the following thermal cycling profile: initial denaturation step at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 20 sec. The final extension was 72°C for 20 min. The size of the PCR reaction product was analyzed using a Fragment Analyzer (Advanced Analytical Technologies Inc., Ankeny, IA, USA) and allele size was scored using PROSize 2.0 software (Advanced Analytical Technologies Inc.).

After allele scoring, the number of alleles (N_A), major allele frequency (M_{AF}), observed heterozygosity (H_O), expected heterozygosity (H_E), number of genotypes (N_G), and polymorphic information content (*PIC*) were calculated using a 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_{AF} 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_O and H_E 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).

Table 2. 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:GTGTGACAGATTACACGGGTC R:ACTCAAGTAGGTCCAAGTCGC	154	(TA)5	NM0434-000001
RL-LE-002	F:GTAGAGGGATACGCGAAGTAAG R:ACAATGGTCGTTCACAGTCG	194	(AT)7	NM0434-000002
RL-LE-003	F:CGTTTGCTTGCCCTTTTC R:GGAGATACCAACACCACTACTAC	179	(AC)9	NM0434-000003
RL-LE-004	F:TGAGCTATGGTGCTACTCCTTC R:GCGCCTATATCCGATGGT	192	(CCA)5	NM0434-000004
RL-LE-005	F:GGCAGAACAGAACCTAGCTCAT R:AGTCCATATGGCTTCCACCT	194	(GGA)6	NM0434-000005
RL-LE-006	F:CTCTGGATAAGAACGCAAGC R:CAGTGTTCTCCAGTCCGAGT	177	(TC)7	NM0434-000006
RL-LE-007	F:CTACCACTCGTCACTCCTTAGGT R:GAAGGAGTGTGAAGCTGAAACC	194	(CT)12	NM0434-000007
RL-LE-008	F:AAGCTCTCATATGTTGGGGG R:AACGACGAGTGGGTTAGGTAG	150	(TC)5	NM0434-000008
RL-LE-009	F:GTCAGTGAGGAGTTGGATGTTC R:AGTGCTCTGGTCAAACCAAC	157	(AT)5	NM0434-000009
RL-LE-010	F:GTAAGCTCATGGTAACAGTGCC R:AACTTGGAAGCCTTCTCCC	194	(AT)6	NM0434-000010
RL-LE-011	F:GTGCTACACCAGGGATAAGACT R:GTCGTCTTCGATTTCTAGCG	191	(ATG)5	NM0434-000011
RL-LE-012	F:CTGAAGACGTGGAAAAACGC R:GCGTCAGCTTCTTACTCCTTAC	194	(CA)5	NM0434-000012
RL-LE-013	F:CGTTGTGACTCGTGACCTCT R:CAGTCGTAGGATACCGCATAAG	196	(CG)5	NM0434-000013
RL-LE-014	F:ACTGGCTCTCAGACTGTGTTCT R:CAACACCTGTACTTGAGCTGC	178	(TTC)5	NM0434-000014
RL-LE-015	F:GAACCCCAGAAGGACTTGTT R:GAAAGAGAAGGTGACGCTAAGG	185	(CAA)7	NM0434-000015
RL-LE-016	F:GATGTTGAGACCTGGGACAGT R:CTGTGATAGGTGACCCGTAGAT	167	(CG)6	NM0434-000016

SSR, simple sequence repeat.

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

Marker	M_{AF}	N_G	N_A	H_O	H_E	*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	0.76	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
Mean	0.482	9.2	6.8	0.322	0.643	0.612

M_{AF}, major allele frequency; N_G, number of genotypes; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosit; *PIC*, polymorphic information content.

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 foenumgraecum* 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.

Identification and development of genomic SSR loci using whole genomes have been successfully applied in several

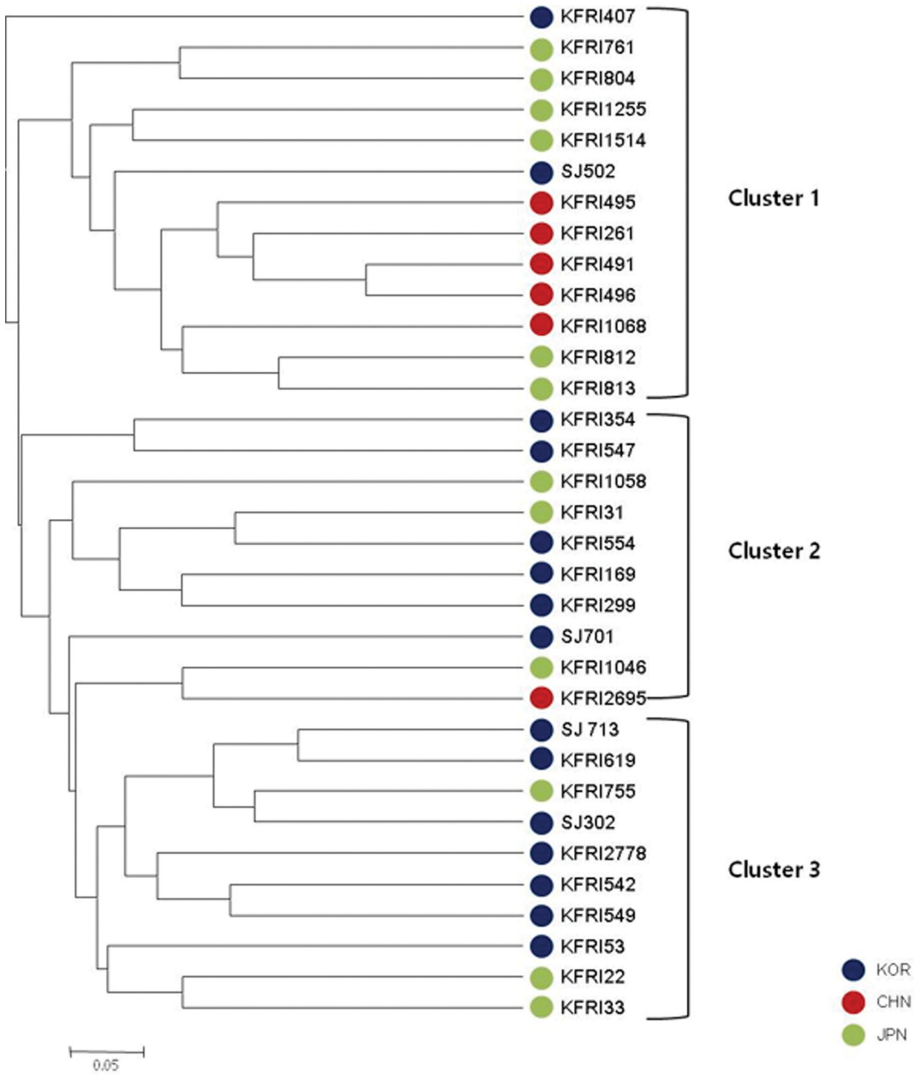

Fig. 1. Dendrogram generated using unweighted pair group method with arithmetic mean cluster analysis based on genetic diversity of 33 *Lentinula edodes* strains. The simple sequence repeat markers developed in this study were not grouped by strain development country. This result considered for grouping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

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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REFERENCES

1. Wasser SP. Shiitake (*Lentinus edodes*). In: Coates PM, Blackman MR, Cragg GM, Levine M, Moss J, White JD, editors. Encyclopedia of dietary supplements. New York: Marcel Dekker; 2005. p. 653-64.
2. Finimundy TC, Dillon AJ, Henriques JA, Ely MR. A review on general nutritional compounds and pharmacological properties of the *Lentinula edodes* mushroom. Food Nutr Sci 2014;5:1095-105.
3. Ng ML, Yap AT. Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes*). J Altern Complement Med 2002;8:581-9.
4. Rop O, Mlcek J, Jurikova T. Beta-glucans in higher fungi and their health effects. Nutr Rev 2009;67:624-31.
5. Qin LH, Tan Q, Chen MJ, Pan YJ. Use of intersimple sequence repeats markers to develop strain-specific SCAR markers for

Lentinula edodes. FEMS Microbiol Lett 2006;257:112-6.
6. Zhang R, Huang C, Zheng S, Zhang J, Ng TB, Jiang R, Zuo X, Wang H. Strain-typing of *Lentinula edodes* in China with inter simple sequence repeat markers. Appl Microbiol Biotechnol 2007;74:140-5.
7. Fu LZ, Zhang HY, Wu XQ, Li HB, Wei HL, Wu QQ, Wang LA. Evaluation of genetic diversity in *Lentinula edodes* strains using RAPD, ISSR and SRAP markers. World J Microbiol Biotechnol 2010;26:709-16.
8. Xiao Y, Liu W, Dai Y, Fu C, Bian Y. Using SSR markers to evaluate the genetic diversity of *Lentinula edodes*' natural germplasm in China. World J Microbiol Biotechnol 2010; 26:527-36.
9. Parida SK, Kalia SK, Kaul S, Dalal V, Hemaprabha G, Selvi A, Pandit A, Singh A, Gaikwad K, Sharma TR, et al. Informative genomic microsatellite markers for efficient genotyping applications in sugarcane. Theor Appl Genet 2009;118:327-38.
10. Saha MC, Cooper JD, Mian MR, Chekhovskiy K, May GD. Tall fescue genomic SSR markers: development and transferability across multiple grass species. Theor Appl Genet 2006;113: 1449-58.
11. Yang H, Tao Y, Zheng Z, Li C, Sweetingham MW, Howieson JG. Application of next-generation sequencing for rapid marker development in molecular plant breeding: a case study on anthracnose disease resistance in *Lupinus angustifolius* L. BMC Genomics 2012;13:318.
12. Davey JW, Hohenlohe PA, Etter PD, Boone JQ, Catchen JM, Blaxter ML. Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat Rev Genet 2011;12:499-510.
13. Shim D, Park SG, Kim K, Bae W, Lee GW, Ha BS, Ro HS, Kim M, Ryoo R, Rhee SK, et al. Whole genome *de novo* sequencing and genome annotation of the world popular cultivated edible mushroom, *Lentinula edodes*. J Biotechnol 2016;223:24-5.
14. Liu K, Muse SV. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 2005;21:2128-9.
15. Zhang RY, Hu DD, Gu JG, Hu QX, Zuo XM, Wang HX. Development of SSR markers for typing cultivars in the mushroom *Auricularia auricula-judae*. Mycol Prog 2012;11: 587-92.
16. Zhang R, Hu D, Zhang J, Zuo X, Jiang R, Wang H, Ng TB. Development and characterization of simple sequence repeat (SSR) markers for the mushroom *Flammulina velutipes*. J Biosci Bioeng 2010;110:273-5.
17. Dangi RS, Lagu MD, Choudhary LB, Ranjekar PK, Gupta VS. Assessment of genetic diversity in *Trigonella foenum-graecum* and *Trigonella caerulea* using ISSR and RAPD markers. BMC Plant Biol 2004;4:13.
18. Cavagnaro PF, Senalik DA, Yang L, Simon PW, Harkins TT, Kodira CD, Huang S, Weng Y. Genome-wide characterization of simple sequence repeats in cucumber (*Cucumis sativus* L.). BMC Genomics 2010;11:569.
19. Shi J, Huang, S, Zhan J, Yu J, Wang X, Hua W, Liu S, Liu G, Wang H. Genome-wide microsatellite characterization and marker development in the sequenced *Brassica* crop species. DNA Res 2014;21:53-68.
20. Wang Q, Fang L, Chen J, Hu Y, Si Z, Wang S, Chang L, Guo W, Zhang T. Genome-wide mining, characterization, and development of microsatellite markers in *Gossypium* species. Sci Rep 2015;5:10638.
21. Zhou R, Wu Z, Jiang FL, Liang M. Comparison of gSSR and EST-SSR markers for analyzing genetic variability among tomato cultivars (*Solanum lycopersicum* L.). Genet Mol Res 2015;14:13184-94.