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RESEARCH NOTE

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Molecular Characterization of 170 New gDNA-SSR Markers for Genetic Diversity in Button Mushroom (*Agaricus bisporus*)

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ABSTRACT

We designed 170 new simple sequence repeat (SSR) markers based on the whole-genome sequence data of button mushroom (*Agaricus bisporus*), and selected 121 polymorphic markers. A total of 121 polymorphic markers, the average major allele frequency (M_{AF}) and the average number of alleles (N_A) were 0.50 and 5.47, respectively. The average number of genotypes (N_G), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphic information content (PIC) were 6.177, 0.227, 0.619, and 0.569, respectively. Pearson's correlation coefficient showed that M_{AF} was negatively correlated with N_G (−0.683), N_A (−0.600), H_O (−0.584), and PIC (−0.941). N_G, N_A, H_O, and PIC were positively correlated with other polymorphic parameters except for M_{AF}. UPGMA clustering showed that 26 *A. bisporus* accessions were classified into 3 groups, and each accession was differentiated. The 121 SSR markers should facilitate the use of molecular markers in button mushroom breeding and genetic studies.

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The button mushroom (*Agaricus bisporus*) is an edible mushroom that belongs to the Basidiomycota, and it is one of the most popular mushrooms consumed worldwide [1]. *A. bisporus* primarily contains dietary fibers, polysaccharide cell walls, beta-glucans, homoglucans, and heteroglucans, that are known to have antioxidant and anticancer effects and are effective for treating cardiovascular disease [2–4]. The market for *A. bisporus* has been steadily growing since it was first cultivated as a commercial species in the Netherlands in the seventeenth century, and, recently, its production has greatly increased in China [5,6]. However, it is difficult to breed *A. bisporus* owing to its unique life cycle of secondary homothallism [7,8]. The hybrid variety of *A. bisporus,* Horst U1, was developed in 1981, and several varieties were subsequently developed from Horst U1. The varieties of *A. bisporus* developed to date are genetically similar to Horst U1. The similarities have resulted in a genepool of *A. bisporus* that is much more restricted compared to that of other mushrooms (*Pleurotus ostreatus*, *Lentinus edodes*) [9,10]. To address such difficulties, a molecular marker system is needed to efficiently classify *A. bisporus* accessions. Since the Nagoya Protocol came into effect in 2010, the importance of reinforcing protection and sovereignty of national genetic resources has been highlighted, and the development of molecular markers in various crops has been actively promoted [11–13]. Simple sequence repeats (SSR) or microsatellites are highly polymorphic markers based on 1–6 bp repeated sequence motifs distributed throughout the genome. Owing to their high reproducibility, multi-allelic nature, and codominance, SSR markers have been widely used in studies of population structure, genetic diversity, and breed classification [12–15].

Traditionally, SSR markers have been developed by selecting microsatellites from sequences of enriched genomic libraries [16,17]. Recent NGS technology has led to more efficient processes for developing DNA markers, as it facilitates SSR mining based on the whole-genome sequence. To date, SSR markers have been developed from various biological resources including plants, such as rice and peas, and fungi [11,18–20]. SSR markers have previously been developed for the mushrooms *L. edodes* [21,22], *Tricholoma matsutake* [23,24], and *A.*

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bisporus [25–28]. However, the majority of existing SSR markers are based on enriched sequencing [26,27], and further development of markers based on the entire *A. bisporus* genome is necessary.

Therefore, in this study, we developed SSR markers based on whole-genome re-sequencing [28] data from 6 *A. bisporus* accessions and evaluated these markers for use in genetic diversity and population structure analyses of *A. bisporus*, cultivar discrimination, development of QTL markers, and core-collection selection.

A total of 26 *A. bisporus* accessions, obtained from the strain collection of the Ginseng Research Division at the National Institute of Horticultural and Herbal Science (NIHHS) in South Korea, were used in this study (Supplemental Table S1). The mycelia of each strain were cultured on a CDA medium (4% dried compost, 0.7% malt extract, 1% sucrose, 2% agar) at 25 °C without light for 2 months. The cultured strains were harvested, lyophilized for 4 days, and pulverized, and DNA was extracted using the Tissue SV mini kit (GeneAll Biotechnology Co. Ltd., Seoul, Korea) according to the manufacturer's protocol. The final concentration of the extracted DNA used for the experiment was 20 ng/μL.

Based on the genome sequencing results of 6 *A. bisporus* strains, which were reported by Lee et al. (2018) [28], 1,835 SSR markers that were designed (Supplemental Table S2). Among the 170 SSR markers with di-, tri-, and tetra-nucleotide repeat motifs with high frequency were selected after filtering motifs with at least four repeats and having an annealing temperature of 55 °C. The 170 selected SSR markers were applied to 26 *A. bisporus* accessions to confirm their polymorphism (Supplemental Table S3). The PCR mixture contained 20 ng genomic DNA, 10 pmol primer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, and 0.5 U Taq polymerase (Inclone, Deajeon, Korea), in a 40 μL reaction volume. The CFX96 PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) was used for DNA amplification, with 30 cycles of: 2 min predenaturation at 95 °C, 20 s denaturation at 95 °C, 40 s annealing at 55 °C, and 45 s extension at 72 °C. The size of each DNA fragment was measured using the Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA), and genotyping was performed using Pro Size® 2.0 software.

PowerMarker (ver 3.25) [29] was used to analyze the frequency of alleles amplified by the marker (major allele frequency, M_{AF}), the number of alleles originating from markers confirmed polymorphism (number of alleles, N_A), the number of DNA bands that could be genotyped (number of genotypes, N_G), the ratio of heterozygosity in the analyzed samples (observed heterozygosity, H_O), the ratio of individuals expected to show heterozygosity from all individuals (Expected heterozygosity, H_E), and elements of polymorphism (polymorphism information content, PIC). Phylogenetic analysis was performed using the C.S. chord 1967 distance method in PowerMarker (ver. 3.25); phylogenetic trees were constructed with MEGA 7 [30] using the unweighted pair group method with arithmetic mean (UPGMA).

For the molecular discrimination of 26 *A. bisporus* accessions, we considered N_A and PIC and selected four out of the 121 SSR markers (AB-gSSR-0959, AB-gSSR-1142, AB-gSSR-1180, and AB-gSSR-1184). To begin with, we attempted the 1st stage of discrimination after constructing a phylogenetic tree using the AB-gSSR-1184 marker. For the remaining unidentified resources, we generated a phylogenetic tree by adding AB-gSSR-1142, AB-gSSR-1180, and AB-gSSR-1184 one by one and conducted the molecular discrimination of 26 A. *bisporus* accessions over the course of four stages.

Analysis of variance was performed for statistical significance among repeated motif types using SAS 9.2 (SAS Institute, Cary, NC, USA). Pearson's bivariate correlation coefficient among polymorphic parameters such as M_{AF}, N_G, N_A, H_O, and PIC value was obtained by SAS 9.2 using a 2-tailed test of significance.

PCR analysis of 26 *A. bisporus* accessions with 170 SSR markers indicated that 22 of the markers did not amplify, and 27 markers produced monomorphic bands. Polymorphism was confirmed in the remaining 121 markers. The M_{AF} of the 121 polymorphic SSR markers in 26 *A. bisporus* accessions ranged from 0.19 (AB-gSSR-1142) to 0.94 (AB-gSSR-1364), with a mean of 0.5. The N_G ranged from 3 (AB-gSSR-0049, -0262, 0350, -0435, -0708, -0945, -0972, -1364, -1619, -1628, -1629) to 13 (AB-gSSR-0900 and AB-gSSR-1064), and the mean was 6.177. The N_A per marker ranged from 2 (AB-gSSR-1364) to 13 (AB-gSSR-1208), with a mean of 5.47 alleles. The H_O ranged from 0 (AB-gSSR-0049, -0233, -0262, -0350, -0435, -0588, -0795, -0844, -0972, -1084, -1111, -1169, -1271, -1531, -1619, -1623, -1628, -1629) to 0.96 (AB-gSSR-0140 and AB-gSSR-1180), and the mean was 0.227. The H_E ranged from 0.11 (AB-gSSR-1364) to 0.88 (AB-gSSR-1142), and the mean was 0.619. The PIC ranged from 0.1 (AB-gSSR-1364) to 0.86 (AB-gSSR-1142), and the mean was 0.569 (Supplemental Table S3).

The diversity of 121 SSR markers developed in this study was evaluated in comparison with those of previous studies. The mean PIC value of the SSR markers developed in this study (0.569) was higher than that (0.34) of 43 SSR markers analyzed in 6 *A.*

bisporus accessions by Lee et al. [28]. For more detailed comparison, 20 SSR markers with high PIC from the previously developed markers [28] were selected and applied to 26 *A. bisporus* accessions. The average PIC of 0.501 was higher than that of a previous study (PIC value, 0.366) using 6 *A. bisporus* accessions (Supplemental Table S4). This discrepancy can be explained by the use of relatively higher number of accessions and markers in the analysis. The analysis of 26 *A. bisporus* accessions by 121 SSR markers (present study) and 20 SSR markers (previous study) showed the average PICs of 0.569 and 0.501, respectively (Supplemental Tables S3 and S4). The results suggest that the 121 SSR markers developed in this study are more suitable in the evaluation of genetic diversity.

The results suggest that the 121 SSR markers developed in this study are more suitable in the evaluation of genetic diversity. Fu et al. [31] selected 17 SSR markers based on 15 cultivated *A. bisporus* accessions and 13 wild species accessions. With a mean PIC value of 0.618, these markers exhibited higher diversity than those developed in the present study. The comparatively high PIC value obtained by Fu et al. [31], despite the small number of markers, may have resulted from the high proportion of wild species accessions. Foulongne-Oriol et al. [26] evaluated the diversity of 33 SSR markers in 8 *A. bisporus* species, 13 wild species accessions, 2 *A. bisporus var. burnettii*, and 1 *A. bisporus var. eurotetrasporu*, and the mean H_E value (0.553) was lower than that in the present study (0.619). Although most *A. bisporus* accessions used in this study were genetically close cultivated species, the 121 SSR markers showed higher diversity than the previous markers in the existing wild species and intra-species. Therefore, even if new *A. bisporus* cultivars are developed in the future, these newly developed SSR markers can be efficiently used to identify new cultivars from genetically similar resources.

Previous studies obtained a PIC value of 0.612 for shiitake mushrooms [22], and PIC and H_E values of 0.511 [21] and 0.67 [23], respectively, for pine mushrooms, indicating that the markers developed for other edible mushrooms showed a similar trend to that observed in the present study. Assessing the diversity of germplasm using DNA markers is very important and essential for selecting breeding materials. The SSR markers already developed in shiitake and pine mushrooms have been widely applied to the selection of breeding materials and analysis of the relationship between resources. The 121 SSR markers developed in this study, with similar polymorphic parameters, can be applied to evaluate the diversity of *A. bisporus* and to select breeding materials.

Marker diversity analysis according to SSR motif type showed that the mean PIC value was highest in tri-nucleotide repeats (0.609), followed by tetra-nucleotide repeats (0.55) and di-nucleotide repeats (0.547). However, there was no significant difference in PIC value according to the motif type (Table 1). Tóth et al. reported that mono-nucleotide repeats were the most frequent SSRs in fungi based on enriched genomic libraries, followed by tri-, hexa-, penta- and di-nucleotide repeats [32]. However, Wang et al. [25] investigated the SSR distribution in the *A. bisporus* genome and found that mono-nucleotide repeats were the most abundant, followed by tri-, di-, tetra-, and hexa-nucleotide. SSR distribution in *A. bisporus* re-sequencing data also revealed that tri-nucleotide repeats were the most abundant, when mono-nucleotide repeats were excluded, followed by di-, tetra-, and hexa-nucleotide repeats [28]. *A. bisporus* is classified as a fungus but differs in genetic composition (distribution of SSRs) from other fungi that are generally not edible. Therefore, we intensively extracted SSR marker motifs from the di- to tetra-nucleotide repeats to increase the efficiency of SSR marker selection. As a result, we selected multiple tri-nucleotide repeat markers with high polymorphism (PIC value, 0.609) and it is possible to apply these markers to evaluate the diversity and relationships of genetically similar *A. bisporus* germplasm.

Pearson's correlation coefficient between the polymorphic parameters (M_{AF}, N_G, N_A, H_O, and PIC) of the 121 SSR markers is shown in Table 2. M_{AF} was negatively correlated with N_G (r = -0.683), N_A ($r = -0.600$), H_O ($r = -0.584$), and PIC ($r = -0.941$). N_G, N_A, H_O, and PIC were positively correlated with the other parameters except M_{AF}. The highest negative correlation was observed between PIC and M_{AF} ($r = -0.947$), while the highest

Table 1. The variation of polymorphism parameters according to SSR motif type.

Repeat motif	Number of markers	M_{AF}	N_G	N_A	H_O	PIC
Di-nucleotide	23	0.524^{ns}	5.783^{ns}	5.174^{ns}	0.193^{ns}	0.547^{ns}
Tri-nucleotide	80	0.464^{ns}	7.025^{ns}	6.013^{ns}	0.308^{ns}	0.609^{ns}
Tetra-nucleotide	18	0.511^{ns}	5.722^{ns}	5.222^{ns}	0.181^{ns}	0.550^{ns}

^{ns}not significant within each column.

Table 2. Pearson's correlation between polymorphism parameters of 121 SSR markers.

	M_{AF}	N_G	N_A	H_O	PIC
M_{AF}	–	−0.683**	−0.600**	−0.584**	−0.941**
N_G		–	0.856**	0.671**	0.803**
N_A			–	0.593**	0.752**
H_O				–	0.630**
PIC					–

**Significant at $\alpha = 0.01$.

positive correlation was between N_A and N_G ($r = 0.856$) (Table 2). Pearson's correlation coefficient between M_{AF} and PIC was the highest ($r = -0.941$). This suggests that the frequency of alleles amplified by the marker (M_{AF}) affected the elements of polymorphism (PIC). This further suggests that the two parameters are closely related. Pearson's correlation coefficient between N_A and N_G (0.856) was the result of more diversified genotypes as the fragment bands (number of alleles) generated by the DNA marker increased [33]. In general, the PIC value is used as an indicator for selecting polymorphism markers, which can differ by M_{AF}, N_A, and N_G. The present study also revealed that M_{AF}, N_A, and N_G strongly correlate with PIC. Therefore, it will be easier to select markers with more polymorphisms that target diverse accessions by considering this relationship.

The results of the cluster analysis indicated that 26 *A. bisporus* accessions were classified into 3 groups. Group I contained 10 accessions, comprising 2 accessions from Korea (KOR), 3 accessions from the Netherlands (NLD), and 1 accession each from China (CHN), the United States America (USA), Germany (DEU), Japan (JPN) and Peru (PER). The pileus phenotypes of these 10 Group I accessions were white (7), brown (2), and light brown (1). Group II consisted of 8 accessions, including 3 from the USA, 2 from KOR and NLD, and 1 from DEU; the pileus phenotypes were white (4), brown (3), and light brown (1). Group III contained 4 accessions from DEU and 2 accessions from the USA and KOR; the pileus phenotypes were white (4), brown (3), and cream (1). All 26 *A. bisporus* accessions were distinguished using the 121 SSR markers developed in this study, but no clusters were formed according to their geographical origin or pileus color (Figure 1). Cluster analysis showed that all 26 *A. bisporus* accessions were differentiated by the newly developed SSR markers. However, SSR was selected based on non-coding region, and not based on genes related to qualitative traits. Thus, the phenotype of the detected sequence was not identified. Therefore, no clusters were observed that distinguished qualitative traits, such as pileus color, or resource characteristics, such as origin. Future

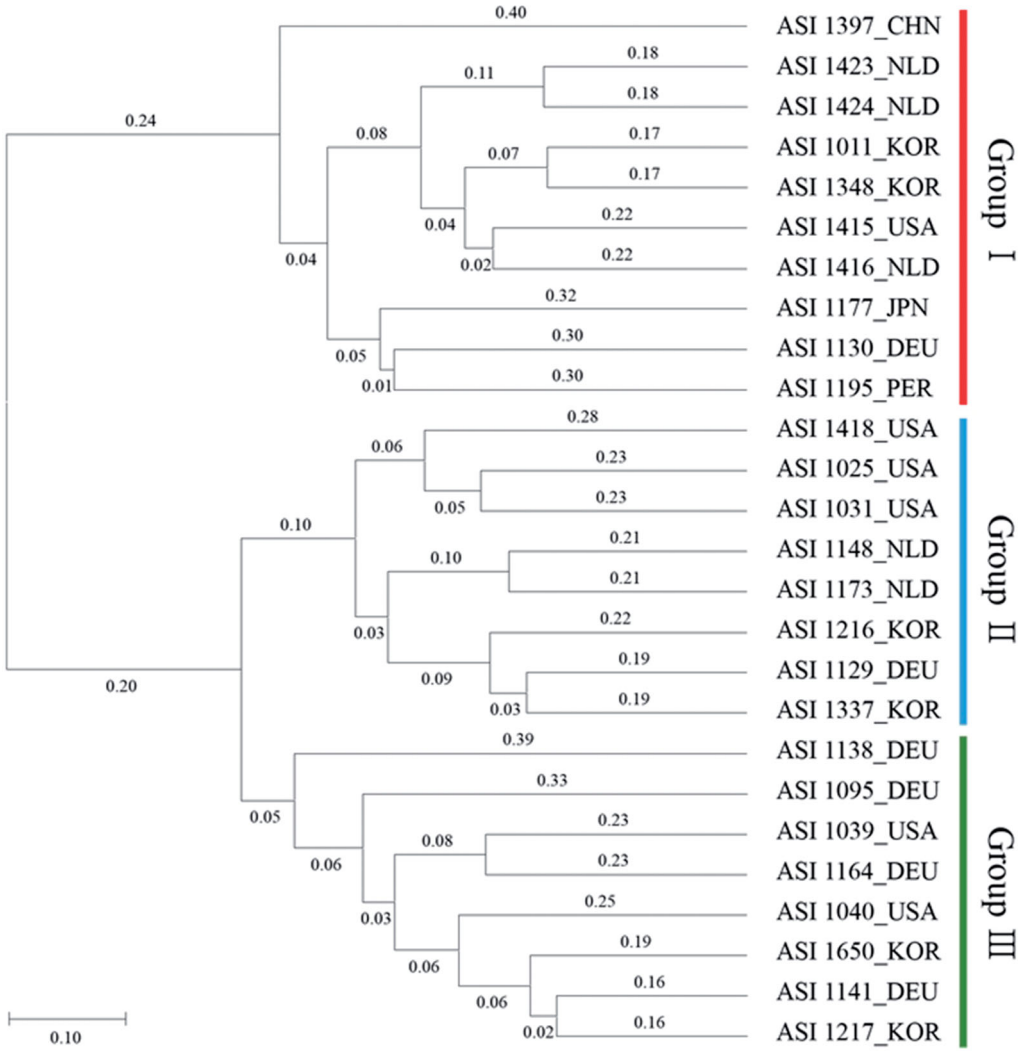

Figure 1. UPGMA analysis of 26 A. bisporus accessions based on combined SSR data analyzed using C.S. Chord 1967 distance.

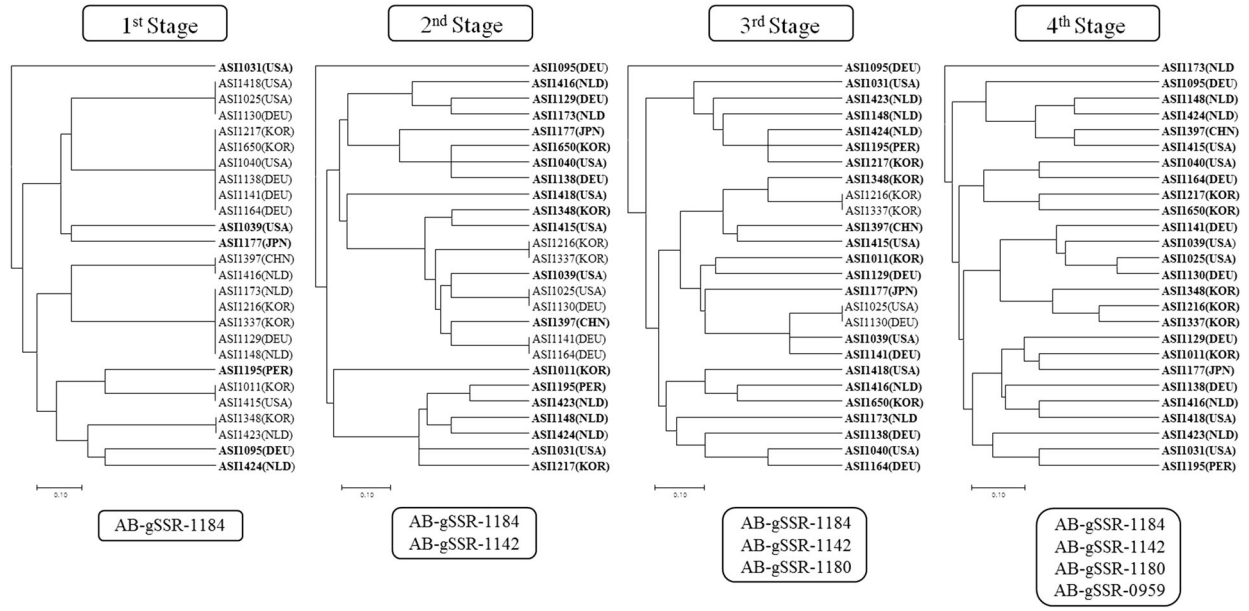

Figure 2. Diagrammatic display of molecular discrimination by phylogenetic trees using 26 *A. bisporus* accessions at each stage by four SSR markers.

experiments on germplasm accessions showing varying and distinct traits will allow for the selection and grouping of SSR markers according to specific phenotypes.

The following are the results of the molecular discrimination of 26 *A. bisporus* accessions with 4 SSR markers. Six accessions were identified by the AB-gSSR-1184 marker (Stage 1) from 26 *A. bisporus* accessions. Twenty unidentified accessions were grouped in 6 categories based on the genotype. Stage 2 added the AB-gSSR-1142 marker and identified 14 additional accessions from the 20 unidentified accessions, which were not identified in Stage 1. Stage 3 added the AB-gSSR-1180 marker and identified 2 additional accessions from the 6 accessions, which were not identified in Stage 2. Finally, Stage 4 added the AB-gSSR-0959 marker and identified all 26 *A. bisporus* accessions (Figure 2). But, the SSR marker combination may be different in according to accession changes.

In this study, 121 SSR markers were developed based on the whole genome of *A. bisporus*. The developed SSR markers differentiated genetically close resources and exhibited high polymorphism. Our results are expected to be useful for analyses of genetic diversity and population structure, cultivar discrimination, and QTL analysis of *A. bisporus*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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