Ribosomal Intergenic Spacer 1 Based Characterization of Button Mushroom (Agaricus bisporus) Strains

Hyuk Woo Kwon¹, Min Ah Choi², Dae Wook Kim¹, Youn-Lee Oh¹, Min Woo Hyun², Won-Sik Kong² and Seong Hwan Kim¹,*
¹Department of Microbiology and Institute of Biodiversity, Dankook University, Cheonan 31116, Korea
²Mushroom Research Division, National Institute of Horticultural and Herbal Science, RDA, Eumseong 27709, Korea

Abstract Breeding the button mushroom requires genetic information about its strains. This study was undertaken to genetically characterize four domestically bred button mushroom strains (Saea, Saejung, Saedo, Saeyeon cultivars) and to assess the possibility of using the intergenic spacer 1 (IGS1) region of rDNA as a genetically variable region in the genetic characterization. For the experiment, 34 strains of Agaricus bisporus, two strains of A. bitorquis, and one strain of A. silvaticus, from 17 countries were used. Nucleotide sequence analysis of IGS1 rDNA in these 37 Agaricus strains confirmed that genetic variations exist, not only among the four domestic strains, but also between the four domestic strains and foreign strains. Crossing two different haploid strains of A. bisporus seems to generate genetic variation in the IGS1 region in their off-spring haploid strains. Phylogenetic analysis based on the IGS1 sequence revealed all A. bisporus strains could be differentiated from A. silvaticus and A. bitorquis strains. Five genetic groups were resolved among A. bisporus strains. Saejung and Saeyeon cultivars formed a separate genetic group. Our results suggest that IGS1 could be complementarily applied in the polymorphism analysis of button mushroom.

Keywords Agaricus bisporus, Dikaryotic strain, Ribosomal intergenic spacer 1

Button mushroom, Agaricus bisporus (J. Lange) Imbach, an edible basidiomycete mushroom, is the most widely cultivated mushroom in the world. With its popularity in cultivation, diverse trials have been performed to breed noble cultivars. The first hybrid strains were released in 1981 [1]. However, the diversity of cultivars with different traits is still limited due to obstruction in the genetic manipulation of mating between A. bisporus strains. The bisporic production of basidiospores, which leads to formation of the secondary homothallic mushroom species, is the main reason for the obstruction [2, 3]. In addition, the narrow genetic base of the commercially available mushroom cultivars is also hindering the choice of parental strains for breeding. Foulone-Oriol et al. [4] studied simple sequence repeat markers and found that there is homogeneity within the actual commercial strains of button mushroom. Interestingly, however, they also found that a hidden diversity exists beyond the apparent uniformity of the mushroom. It is obvious that the exploitation of genetic resources for genetic variability could increase the opportunity of breeding new varieties with different properties. Therefore, information on genetic characterization of parental genotypes used for crossing would provide a sound basis to operate breeding programs for button mushroom.

The intergenic spacer (IGS) between the 28S and 18S rRNA genes is useful for examination of close relationships in edible mushrooms such as Lentinula edodes [5], Pleurotus eryngii [6], and Auricularia auricula-judae [7]. Recently, domestic breeding has produced several cultivars of button mushroom [8]. However, basic useful data showing their genetic relationships with other strains from diverse origins is very limited. Therefore, this study was undertaken to genetically characterize the domestically bred strains, together with foreign strains, by analyzing the divergences in their nucleotide sequences of IGS1.
Table 1. List of Agaricus strains used in this study

| No. | ASI No. | Nomenclature | GenBank accession No. | Color | IGS1 size (bp) | Source |
|-----|---------|--------------|-----------------------|-------|----------------|--------|
| 01  | ASI1151 | *Agaricus bitorquis* | KY078324 | White | 1,263 | Korea |
| 02  | ASI1337 | *A. bisporus* | KY078318 | White | 1,210 | |
| 03  | ASI1338 | *A. bisporus* | KY078317 | White | 1,212 | |
| 04  | ASI1347 | *A. bisporus* | KY078347 | White | 1,121 | |
| 05  | ASI1348 | *A. bisporus* | KY078325 | White | 1,211 | |
| 06  | ASI1246 | *A. bisporus* | KY078314 | - | 1,212 | |
| 07  | ASI1146 | *A. bisporus* | KY078335 | Brown | 1,212 | |
| 08  | ASI1153 | *A. bisporus* | KY078321 | Cream | 1,213 | |
| 09  | ASI1038 | *A. bisporus* | KY078313 | White | 733 | USA |
| 10  | ASI1031 | *A. bisporus* | KY078328 | White | 1,202 | |
| 11  | ASI1032 | *A. bisporus* | KY078337 | Cream | 1,185 | |
| 12  | ASI1072 | *A. bisporus* | KY078334 | White | 1,208 | Denmark |
| 13  | ASI1024 | *A. bisporus* | KY078333 | White | 1,212 | Taiwan |
| 14  | ASI1047 | *A. bisporus* | KY078322 | White | 1,150 | Japan |
| 15  | ASI1177 | *A. bisporus* | KY078323 | Cream | 1,212 | |
| 16  | ASI1050 | *A. bisporus* | KY078338 | Brown | 1,178 | France |
| 17  | ASI1054 | *A. bisporus* | KY078339 | White | 1,143 | |
| 18  | ASI1060 | *A. bisporus* | KY078320 | White | 1,216 | India |
| 19  | ASI1085 | *A. bisporus* | KY078340 | White | 1,175 | Canada |
| 20  | ASI1086 | *A. bisporus* | KY078315 | Brown | 1,212 | |
| 21  | ASI1164 | *A. bisporus* | KY078336 | Brown | 1,212 | Germany |
| 22  | ASI1095 | *A. bisporus* | KY078312 | White | 1,213 | |
| 23  | ASI1138 | *A. bitorquis* | KY078319 | White | 1,257 | |
| 24  | ASI1096 | *A. bisporus* | KY078329 | White | 1,213 | Switzerland |
| 25  | ASI1118 | *A. bisporus* | KY078326 | - | 1,213 | UK |
| 26  | ASI1195 | *A. bisporus* | KY078327 | - | 1,157 | Peru |
| 27  | ASI1320 | *A. bisporus* | KY078331 | Brown | 1,210 | Netherlands |
| 28  | ASI1322 | *A. bisporus* | KY078330 | - | 1,216 | |
| 29  | ASI1324 | *A. bisporus* | KY078342 | White | 1,165 | Australia |
| 30  | ASI1323 | *A. bisporus* | KY078341 | Brown | 1,134 | New Zealand |
| 31  | ASI1326 | *A. bisporus* | KY078343 | White | 1,190 | |
| 32  | ASI1328 | *A. bisporus* | KY078344 | - | 1,178 | |
| 33  | ASI1329 | *A. bisporus* | KY078316 | - | 1,150 | Brazil |
| 34  | ASI1330 | *A. bisporus* | KY078345 | - | 1,186 | |
| 35  | ASI1336 | *A. bisporus* | KY078346 | Brown | 1,177 | |
| 36  | ASI1339 | *A. bisporus* | KY078332 | - | 1,215 | Vietnam |
| 37  | ASI34010 | *A. silvaticus* | KY078348 | - | 569 | USA |

ASI, Agricultural Science Institute; IGS1, intergenic spacer 1. Acronym for mushroom strains used in the Mushroom Science Division in the Rural Development Administration, Korea.

Fig. 1. The pedigree of the four domestically bred strains of *Agaricus bisporus*, ASI1338, ASI1347, ASI1348, and ASI1337. KMCC, Korea Mushroom Culture Collection.
The sources and colors of the 34 strains of *Agaricus bisporus*, two strains of *Agaricus bitorquis*, and one dikaryotic strain of *Agaricus silvaticus* from 17 countries, used in this study, are given in Table 1.ASI1337 (Saea), ASI1338 (Saejeong), ASI1347 (Saeyeon), and ASI 1348 (Saedo) are domestically bred cultivars (Fig. 1). All these diploid strains were obtained from the Mushroom Research Division, National Institute of Horticultural and Herbal Sciences, RDA, Eumseong, Korea. To analyze the IGS1 region, all strains were cultured on cellophane-layered corn meal agar at 25°C for 7 days, and their mycelia were subjected to genomic DNA extraction by the method described by Kim et al. [9]. Primers LR12R (5'-GAACGCCTCTAAGT-CAGAATCC-3') and 5SRNA (5'-ATCAGACGGGATGC-GGT-3') were used for PCR amplification of the IGS1 region [10]. The PCR products were electrophoresed on 0.75% agarose gels to check for the presence of amplified DNA, subcloned into TA cloning vector using the InsTAclone PCR Cloning Kit (Seoulin Co, Seoul, Korea) according to the manufacturer's protocol, and sequenced at Macrogen Corp. (Seoul, Korea).

All the determined nucleotide sequences were verified as fungal IGS1 sequences by BLAST searches in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The 37 IGS1 sequences generated in this study were deposited in the GenBank database with accession numbers KY078312 to KY078348 (Table 1). The sizes of the IGS1 nucleotide sequences are given in Table 1. The IGS1 region has no repeats. In *A. bisporus*, the shortest size was 733 bp (ASI1038) and the longest size was 1,212 bp (ASI1060). The sizes of four domestically bred cultivars were not identical. They were 1,121 bp in Saeyeon (ASI1347), 1,210 bp in Saea (ASI1337), 1,211 bp in Saedo (ASI 1348), and 1,212 bp in Saejung (ASI1338). These four domestic cultivar strains were bred using a haploid strain derived from a basidiospore of a summer button mushroom, were larger than the IGS1 sizes of *A. bisporus* strains. The IGS1 size of strains from Australia, Brazil, Chile, and New Zealand, and France ranged from 1,134 to 1,190 bp. Meanwhile, the IGS1 size of *A. silvaticus* (569 bp) was shorter than all the *A. bisporus* and *A. bitorquis* strains. The IGS1 sizes of two strains of *A. bitorquis* (ASI1151, ASI1138), which is called a summer button mushroom, were larger than the IGS1 sizes of *A. bisporus* and *A. silvaticus* strains. There were no noticeable patterns showing a relation between the IGS1 sizes and the different colors in *A. bisporus* strains. Portobello strains (ASI 1216, 1178, 1186) also showed no special pattern in relation to IGS1 sizes. It clearly indicates that all the *A. bisporus* strains are a single species with various colors and maturities.

In addition to variation in sizes, variation was also observed in the IGS1 nucleotide sequence identities among the *Agaricus* strains as shown in Table 2. The domestic strain Saea (ASI1337) showed 97.93 to 99.92% identity with Saeyeon (ASI1347), Saedo (ASI 1348), and Saejung (ASI1338). There were no 100% identities among Saeyeon (ASI1347), Saedo (ASI 1348), and Saejung (ASI1338) strains. This is an interesting result because Saeyeon (ASI1347) and Saejung

Table 2. Nucleotide sequence identity of the IGS1 rDNA region between four domestically bred *Agaricus bisporus* strains and other strains of *A. bisporus*, *A. bitorquis*, and *A. silvaticus* from foreign countries

| Country          | 1     | 2     | 3     | 4     |
|------------------|-------|-------|-------|-------|
| Domestic strains | 100   | 99.82 | 99.91 | 100   |
| 1                | 99.82 | 99.91 | 100   |       |
| 2                | 99.82 | 99.91 | 100   |       |
| 3                | 99.82 | 99.91 | 100   |       |
| 4                | 99.82 | 99.91 | 100   |       |
| 5                | 99.82 | 99.91 | 100   |       |
| 6                | 99.82 | 99.91 | 100   |       |
| 7                | 99.82 | 99.91 | 100   |       |
| 8                | 99.82 | 99.91 | 100   |       |
| 9                | 99.82 | 99.91 | 100   |       |
| 10               | 99.82 | 99.91 | 100   |       |
| 11               | 99.82 | 99.91 | 100   |       |
| 12               | 99.82 | 99.91 | 100   |       |
| 13               | 99.82 | 99.91 | 100   |       |
| 14               | 99.82 | 99.91 | 100   |       |
| 15               | 99.82 | 99.91 | 100   |       |
| 16               | 99.82 | 99.91 | 100   |       |
| 17               | 99.82 | 99.91 | 100   |       |
| 18               | 99.82 | 99.91 | 100   |       |
| 19               | 99.82 | 99.91 | 100   |       |
| 20               | 99.82 | 99.91 | 100   |       |
| 21               | 99.82 | 99.91 | 100   |       |
| 22               | 99.82 | 99.91 | 100   |       |
| 23               | 99.82 | 99.91 | 100   |       |
| 24               | 99.82 | 99.91 | 100   |       |
| 25               | 99.82 | 99.91 | 100   |       |
| 26               | 99.82 | 99.91 | 100   |       |
| 27               | 99.82 | 99.91 | 100   |       |
| 28               | 99.82 | 99.91 | 100   |       |
| 29               | 99.82 | 99.91 | 100   |       |
| 30               | 99.82 | 99.91 | 100   |       |
| 31               | 99.82 | 99.91 | 100   |       |
| 32               | 99.82 | 99.91 | 100   |       |
| 33               | 99.82 | 99.91 | 100   |       |
| 34               | 99.82 | 99.91 | 100   |       |
| 35               | 99.82 | 99.91 | 100   |       |
| 36               | 99.82 | 99.91 | 100   |       |
| 37               | 99.82 | 99.91 | 100   |       |

Domestically bred strains: 1, ASI1338; 2, ASI1347; 3, ASI1348; 4, ASI1337. *A. bisporus* strains: 5, ASI1038; 6, ASI1047; 7, ASI1031; 8, ASI1118; 9, ASI1060; 10, ASI1096; 11, ASI1024; 12, ASI1339; 13, ASI1085; 14, ASI1054; 15, ASI1336; 16, ASI1329; 17, ASI1330; 18, ASI1323; 19, ASI1328; 20, ASI1246; 21, ASI1326; 22, ASI1195; 23, ASI1095; 24, ASI1032; 25, ASI1164; 26, ASI1146; 27, ASI1177; 28, ASI1086; 29, ASI1153; 30, ASI1322; 31, ASI1320; 32, ASI1072; 33, ASI1050; 34, ASI1324. *A. bitorquis* strains: 35, ASI1138; 36, ASI1315. *A. silvaticus* strain: 37, ASI4010.
IGS1 Based Characterization of *Agaricus bisporus* Strains

ASI1338 (Korea, white, Saejeong)  
ASI1347 (Korea, white, Saejeon)  
ASI1032 (USA, cream)  
ASI1072 (Denmark, white)  
ASI1195 (Peru)  
ASI1153 (Korea, cream)  
ASI1086 (Canada, brown)  
ASI1329 (Brazil)  
ASI1177 (Japan, cream)  
ASI1322 (Netherlands, portobello)  
ASI1146 (Korea, brown)  
ASI1095 (Germany, white)  
ASI1050 (France, brown)  
ASI1246 (Korea)  
ASI1337 (Korea, white, Saeja)  
ASI1348 (Korea, white, Saedo)  
ASI1320 (Netherlands, brown)  
ASI1164 (Germany, brown)  
ASI1323 (Northern New Zealand, brown)  
ASI1324 (Australia, white)  
ASI1326 (Northern New Zealand, white)  
ASI1328 (Southern New Zealand, portobello)  
ASI1330 (Brazil, portobello)  
ASI1336 (Brazil, brown)  
ASI1139 (Vietnam)  
ASI1024 (Taiwan, white)  
ASI1054 (France, white)  
ASI1031 (USA, white)  
ASI1085 (Canada, white)  
ASI1060 (India, white)  
ASI1096 (Switzerland, white)  
ASI1047 (Japan, white)  
ASI1118 (UK)  
ASI1138 (Germany, white)  
ASI1151 (Korea, white)  
ASI1038 (USA, brown)  
ASI134010 (USA)

**Agaricus bisporus**  
**Agaricus bitorquis**  
**Agaricus silvaticus**

Fig. 2. Phylogenetic tree based on intergenic spacer 1 (IGS1) rDNA region sequences of diploid button mushroom strains of *Agaricus bisporus*, *A. bitorquis*, and *A. silvaticus*. The neighbor-joining tree was constructed using MEGA program with 1,000 bootstrap resampling. The IGS1 sequence of *A. silvaticus* was used as outgroup.

(ASI1338) strains were bred using haploid strains derived from basidiospores of the fruit body of Saeja (ASI1337) as one of parental strains (Fig. 2). It indicates that the IGS1 sequence of progenies of a parental strain could differ from that of their parental strains. Namely, crossing two different haploid strains of *A. bisporus* would generate genetic variation in the IGS1 region in their off-spring haploid strains. Similar results were shown in the previous work on the IGS1 sequence analysis of progenies of two different haploid strains [10]. None of strains in Table 1 showed 100% identity with Saeyeon (ASI1347) and Saejung (ASI1338) strains. These two domestic cultivars shared 69.99% to 99.91% identity with other *A. bisporus* strains, 70.50% to 77.55% with *A. bitorquis* strains, and 59.55% to 58.28% with *A. silvaticus* strain, indicating that these cultivars are distinguishable from 35 strains. The Saedo (ASI 1348) strain shared 100% identity with the ASI1323 strain from New Zealand, 72.09% to 99.92% with other *A. bisporus* strains, 72.35% to 78.49% with *A. bitorquis* strains, and 60.67% with *A. silvaticus* strain. The Saeyeon (ASI1337) strain shared 100% identity with 13 strains of *A. bisporus*, indicating that it has more homology with other *A. bisporus* strains than the Saedo (ASI 1348) strain does. These results also showed that the button mushroom has divergence in the IGS1 sequence among strains, and this divergence is detectable although the mushroom is known to have narrow genetic base. Consequently, it is assumed that genotyping a button mushroom cultivar based on the nucleotide sequence of IGS1 could be possible. The polymorphic results of IGS analysis by PCR-restriction fragment length polymorphism method in *A. bisporus* strains support this possibility [11].

Phylogenetic analysis was performed using MEGA 6 program [12]. Alignments of IGS1 nucleotide sequences in 37 test strains were made using the Clustal W algorithm.
Based on the IGS1 sequences, a neighbor-joining tree was constructed with the maximum likelihood method [12]. Kimura-2-parameter and close-neighbor-interchange algorithms were used in the method. Transition and transversion were set as the same weight. Tree topology was evaluated with 1,000 bootstrap replicates. The resulting phylogenetic tree shown in Fig. 2 indicates that all A. bisporus strains could be differentiated from A. silvaticus and A. biturquis strains. A. biturquis strains were more closely related to A. bisporus strains than to A. silvaticus strains. Five genetic groups were resolved among A. bisporus strains. The domestic strains Saeyeon (ASI1347) and Saejung (ASI1338) formed one of the five genetic groups, being separated from other A. bisporus strains. Among A. bisporus strains, only the ASI 1038 strain was found at a distantly separated position. Despite its being the parentage strain of the four domestically bred A. bisporus strains, this ASI1038 strain was distantly related to the four domestically bred A. bisporus strains. These results demonstrate that the IGS1 rDNA region in A. bisporus is not inheritable with entirely homologous sequences [10]. On the contrary, it seems that it is a quickly shifting region across the rDNA unit during mating events in the process of breeding.

In conclusion, the IGS1 analysis in this study reveals that genetic variation exists in nucleotide sequence identities and lengths among the four newly bred domestic cultivar strains, and between the four domestic strains and all the foreign strains. This is the first report of a comparative strains, and between the four domestic strains and all the foreign strains, and among the newly bred diploid strains shown in Table 1 [9], will be used as a guide to the management of strain resources of the button mushroom.

ACKNOWLEDGEMENTS

This research was supported by the Golden Seed Project (Center for Horticultural Seed Development, No. 213003-04-2-CGJ00), Ministry of Agriculture, Food and Rural Affairs (MAFRA), Ministry of Oceans and Fisheries (MOF), Rural Development Administration (RDA) and Korea Forest Service (KFS).

REFERENCES

[13].

1. Fritsche G. Breeding mushrooms. Mushroom J 1986;157:4-

2. Raper CA, Raper JR, Miller RE. Genetic analysis of the life cycle of Agaricus bisporus. Mycologia 1972;64:1088-117.

3. Kerrigan RW, Royer JC, Baller LM, Kohli Y, Horgen PA, Anderson JB. Meiotic behavior and linkage relationships in the secondarily homothallic fungus Agaricus bisporus. Genetics 1993;133:225-36.

4. Foulone-Oriol M, Rodier A, Caumont P, Spataro C, Savoie JM. Agaricus bisporus cultivars: hidden diversity beyond apparent uniformity? In: Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7); 2011 Oct 4-7; Arcachon, France. p. 9-16.

5. Saito T, Tanaka N, Shinozawa T. Characterization of subrepeat regions within rDNA intergenic spacers of the edible basidiomycete Lentinula edodes. Biosci Biotechnol Biochem 2002;66:2125-33.

6. Huang CY, Zhang JX, Zheng SY, Guan GP, Zhang RY. Analysis of intergenic spacer 2 diversity of ribosome DNA for strains of Pleurotus eryngii. J Agric Biotechnol 2005;13:592-5.

7. Bonuso E, Zambonelli A, Bergemann SE, Lotti M, Garbelotto M. Multilocus phylogenetic and coalescent analyses identify two cryptic species in the Italian bianchetto truffle, Tuber borchii Vittad. Conserv Genet 2010;11:1453-66.

8. Min KJ, Kim JK, Kwak AM, Kong WS, Oh YH, Kang HW. Genetic diversity of Agaricus bisporus strains by PCR polymorphism. Kor J Mycol 2014;42:1-8.

9. Kim SH, Uzunovic A, Breuil C. Rapid detection of Ophiostoma piceae and O. quercus in stained wood by PCR. Appl Environ Microbiol 1999;65:287-90.

10. Kwon HW, Choi MA, Yun YH, Oh YL, Kong WS, Kim SH. Genetic and biochemical characterization of monokaryotic progeny strains of button mushroom (Agaricus bisporus). Mycobiology 2015;43:81-6.

11. Bunyard BA, Nicholson MS, Roys DJ. Phylogeny of the genus Agaricus inferred from restriction analysis of enzymatically amplified ribosomal DNA. Fungal Genet Biol 1996;20:243-53.

12. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725-9.

13. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80.

14. Schmidt O, Moreth U. Ribosomal DNA intergenic spacer of indoor wood-decay fungi. Holzforschung 2008;62:759-64.

15. Bertoldo C, Gilardi G, Spadaro D, Garibaldi A, Gullino ML. Assessment of genetic variability of some strains of Fusarium spp. isolated from Lisianthus by analysis of TEF sequences, IGS and RAPD. Prot Cult 2011(2):74.