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Research Note

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

Hyuk Woo Kwon[1], Min Ah Choi[1], Dae Wook Kim[1], Youn-Lee Oh[2], Min Woo Hyun[2], Won-Sik Kong[2] and Seong Hwan Kim[1,*]

[1]*Department of Microbiology and Institute of Biodiversity, Dankook University, Cheonan 31116, Korea*
[2]*Mushroom 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.

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***Corresponding author**
E-mail: piceae@dankook.ac.kr

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Table 1. List of *Agaricus* strains used in this study

No.	ASI No.	Nomenclature	GenBank accession No.	Color	IGS1 size (bp)	Source
1	ASI1151	*Agaricus bitorquis*	KY078324	White	1,263	Korea
2	ASI1337	*A. bisporus*	KY078318	White	1,210	
3	ASI1338	*A. bisporus*	KY078317	White	1,212	
4	ASI1347	*A. bisporus*	KY078347	White	1,121	
5	ASI1348	*A. bisporus*	KY078325	White	1,211	
6	ASI1246	*A. bisporus*	KY078314	-	1,212	
7	ASI1146	*A. bisporus*	KY078335	Brown	1,212	
8	ASI1153	*A. bisporus*	KY078321	Cream	1,213	
9	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.

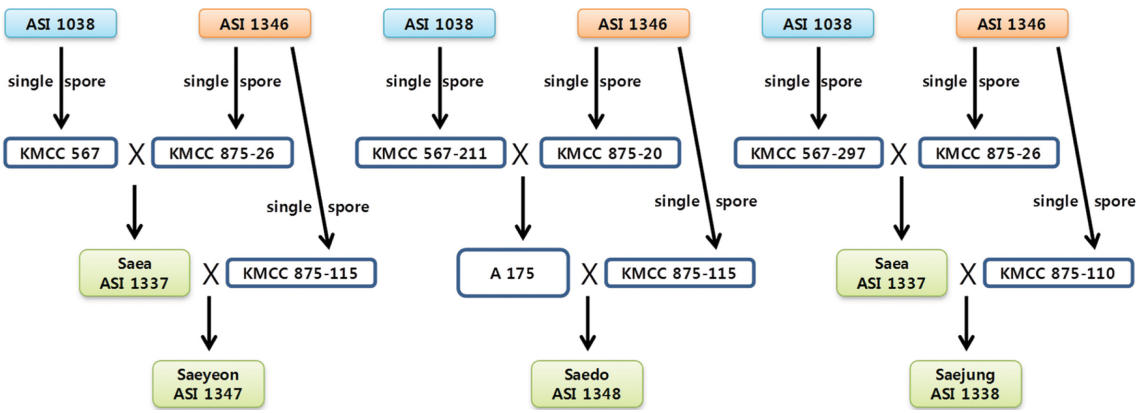

Fig. 1. The pedigree of the four domestically bred strains of *Agaricus bisporus*, ASI1338, ASI1347, ASI1348, and ASI1337. KMCC, Korea Mushroom Culture Collection.

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 the strains were cultured on cellophane-layered corn meal agars 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 1,216 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 from the fruit body of the 733 bp ASI1038 strain as one of parental strains. Thus, variation in the IGS1 size likely results from the mating process of the parental strains. Among the IGS1 sizes of *A. bisporus* strains, 1,212 bp was the dominant size. This dominant IGS1 size was found in the strains from Canada, Germany, Korea, and Taiwan. The IGS1 size of strains from Australia, Brazil, Chile, 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

	1	2	3	4
1	100			
2	99.82	100		
3	97.93	99.91	100	
4	97.93	99.82	99.92	100
5	69.99	70.15	72.09	72.09
6	93.41	94.60	94.90	94.90
7	94.50	96.51	95.33	95.42
8	93.29	94.83	95.29	95.20
9	94.88	96.70	97.03	97.11
10	96.78	98.57	98.76	98.84
11	96.94	99.55	99.01	99.09
12	97.52	99.55	99.67	99.75
13	98.98	99.55	99.66	99.74
14	99.39	99.64	99.65	99.56
15	98.98	99.82	99.83	99.91
16	99.56	99.82	99.91	100
17	98.82	99.82	99.92	100
18	99.74	99.91	100	100
19	98.39	99.82	99.24	99.32
20	97.19	99.73	99.26	99.34
21	98.49	99.82	99.75	99.83
22	99.39	99.73	99.83	99.91
23	97.77	99.73	99.83	99.92
24	98.56	99.82	99.75	99.83
25	97.77	99.82	99.92	100
26	97.77	99.82	99.92	100
27	97.85	99.82	99.92	100
28	97.93	99.82	99.92	100
29	97.85	99.82	99.92	100
30	97.77	99.82	99.92	100
31	97.93	99.82	99.92	100
32	98.01	99.82	99.92	100
33	99.15	99.82	99.92	100
34	99.23	99.82	99.91	100
35	70.50	70.94	72.35	72.27
36	76.55	77.55	78.49	78.39
37	59.55	58.28	60.67	60.67

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, ASI1151. *A. silvaticus* strain: 37, ASI34010.

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 Saea (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 Saea (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

[13]. 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. bitorquis* strains. *A. bitorquis* 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 genetic analysis of *A. bisporus* diploid strains of domestic and foreign origin. Because of rapid evolution in the rDNA complex [14, 15], we expect the IGS1 could be complementarily applied to the polymorphism analysis of button mushroom in conjunction with breeding programs. The IGS1 genetic data collected in this work, together with the previous work on biochemical characterization of the *A. bisporus* diploid strains shown in Table 1 [9], will be used as a guide to the management of strain resources of the button mushroom.

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