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RESEARCH ARTICLE

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Evaluating Genetic Diversity of *Agaricus bisporus* Accessions through Phylogenetic Analysis Using Single-Nucleotide Polymorphism (SNP) Markers

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ABSTRACT

Agaricus bisporus, commonly known as the button mushroom, is widely cultivated throughout the world. To breed new strains with more desirable traits and improved adaptability, diverse germplasm, including wild accessions, is a valuable genetic resource. To better understand the genetic diversity available in *A. bisporus* and identify previously unknown diversity within accessions, a phylogenetic analysis of 360 *Agaricus* spp. accessions using single-nucleotide polymorphism genotyping was performed. Genetic relationships were compared using principal coordinate analysis (PCoA) among accessions with known origins and accessions with limited collection data. The accessions clustered into four groups based on the PCoA with regard to genetic relationships. A subset of 67 strains, which comprised a core collection where repetitive and uninformative accessions were not included, clustered into 7 groups following analysis. Two of the 170 accessions with limited collection data were identified as wild germplasm. The core collection allowed for the accurate analysis of *A. bisporus* genetic relationships, and accessions with an unknown pedigree were effectively grouped, allowing for origin identification, by PCoA analysis in this study.

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1. Introduction

Agaricus bisporus is a saprophytic mushroom that traditionally grows in clusters in substrates such as horse manure, lawns, and agricultural waste and along with several plant genera in nature, including *Cupressus*, *Picea*, and *Prosopis* [1,2]. About 300 years ago, *A. bisporus* was first artificially cultivated in France and then spread throughout the world in the subsequent centuries [3]. The button mushroom as we know it consists of three cultivars: var. *bisporus*, var. *burnetti*, and var. *eurotetrasporus* that can be differentiated based on their typical spore number production [4]. At present, the cultivars of *A. bisporus* are some of the most widely consumed mushrooms in the world.

Since the first hybrid strain of *A. bisporus*, Horst U1, was marketed in 1980 [5], many cultivars have been developed by several companies (Sylvan, Amycel, Italspawn, etc.). The extensive use of hybrid cultivars of *A. bisporus* in cultivation has led to the concern of a potential escape of genes or genotypes from artificial mushroom production into natural settings where it could overtake and dilute wild germplasm populations. Furthermore, wild populations are not well characterized, especially since records and labeling of germplasm collected in the wild are often incomplete, making it difficult to obtain accurate information and document the diversity of a given region based on these accessions. Moreover, *A. bisporus* has limited morphological distinctiveness regarding mushroom fruiting bodies, and wild accessions cannot reliably be distinguished from cultivated cultivars. The lack of distinctive morphological differences also causes some difficulty in selecting diverse parental genotypes for breeding, especially if a breeder wants to include wild germplasm within a breeding program. Therefore evaluation of genetic diversity is needed for identifying of accession and selection of parental genotypes.

A core collection is a subset of accessions that represents the greatest possible genetic diversity contained in an entire collection with the least number of redundancy and it was established for efficient management of germplasm in many crops [6,7]. A core collection is conventionally grouped based on morphological and agronomic

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characteristics but both of traits were affected by environmental variation [8]. Conversely, molecular markers can directly reflect genotypes of accession. In recent years, SNPs have started to replace SSR (simple sequence repeat)s in studies of genetic diversity by the advent of next-generation sequencing. Though SNPs arise twice as repeatedly in intergenic and non-coding regions of the genome than in coding regions, genome-wide association studies showed that SNPs located in non-coding regions are often physically linked to functional or regulatory genomic sites, thus reflecting, for example, selection signatures [9,10]. However, high number of highly polymorphic SNPs needs to resolve exact genetic diversity. Based on molecular marker data, Kim et al. [11] developed software named PowerCore that it allows all characteristics for qualitative traits and quantitative ones to be captured in a minimum number of accessions. Therefore its program is used with many economically important crops. Principal Coordinates Analysis (PCoA, = Multidimensional scaling, MDS) is based on non-euclidean distances from quantitative, semi-quantitative, qualitative, and mixed variables and a method to explore and to visualize similarities or dissimilarities of data. It used for classification in many crops including mushrooms [12] because it can be calculated genetic distance of population. This method is a descriptive model, and various analysis methods must be supplemented for accurate interpretation of the results, and about 40–50 quantitative traits are required [13].

This study aimed to evaluate the available genetic diversity of *A. bisporus*, to uncover the origin of accessions with incomplete collection data using SNP genotyping, and to perform a phylogenetic analysis by comparing genetic relationships and principal coordinate analysis (PCoA) among accessions. In addition, a core collection of *A. bisporus* with significant variation among genotypes was constructed to facilitate the efficient use of time and resources in genetic analyses and may serve as a practical tool for genetic future studies.

2. Materials and methods

2.1. Mushroom strains

A total of 360 strains were included in this study. A diverse set of 190 accessions of *Agaricus* spp., which consisted of wild germplasm, traditional cultivars, and present-day and Chinese hybrids, were obtained from the Wageningen UR Plant Breeding collection (Wageningen University, The Netherlands). An additional 170 accessions of *Agaricus* spp., referred to in this study as the Rural Development Administration (RDA) collection were obtained from the Mushroom Research Division of the National Institute of Horticultural and Herbal Science (NIHHS) (Rural Development Administration, Korea).

2.2. DNA extraction

DNA was extracted according to Sonnenberg et al. [14]. The lyophilized mycelium that grow for 2 weeks on malt extract-mycological peptone (MMP) agar plates covered with cellophane milled in an eppendorf tube and added one ml of DNA extraction buffer (200 mM Tris/HCl, pH 8.0; 25 mM EDTA; 250 mM NaCl) in the tube and vortex well. Consecutively, 700 µL of phenol and 300 µL of chloroform:isoamylalcohol (24:1 v/v) were treated in the mixture. After vortexing in each step, mixture was centrifuged for 1 h at 14,000 rpm at 4 °C. The upper phase (around 900 µL) mixtures were carried over to a new 2 mL eppendorf tube, added 12 µL of RNase (10 mg/ml), mixed gently by turn over the tubes and incubated for 30 min by 37 °C. One vol. of chloroform:isoamylalcohol (24:1 v/v) was added, gently by turn over the tubes and centrifuged for 30 min at 14,000 rpm by 4 °C. The 730 µL of the mixtures was carried over to a new 1.5 mL eppendorf tube. The mixture that added 0.55 vol. isopropanol (–20 °C) were mixed gently by turn over the tube, centrifuged for 10 min at 14,000 rpm at 4 °C and discarded the supernatant. One mL of 70% ethanol were mixed gently to wash DNA, centrifuged for 10 min at 14,000 rpm at 4 °C and discarded the supernatant. The pellets (DNA) were dried in a fumehood for 30 min and dissolved in 30 µL TE buffer.

2.3. SNP genotyping

A total heterokaryons of 360 accessions were genotyped with a KASPar SN0P genotyping system (KBiosciences, Hertfordshire, UK) that based on competitive allele-specific PCR and one of the uniplex SNP genotyping platforms. Since genotype of used heterokaryons is heterzygours, a mixed fluorescent were generated. We also used previously published KASP (Kompetitive Allele Specific PCR) markers [14,15]. Selected genetically distantly related five strains used for the analysis to select informative SNPs (Table 1). One to three SNPs of each

Table 1. Information of selected genetically distantly related five constituent homokaryons.

	Constituent homokaryon	Strain type	Life cycle
Horst U1	H39	Present-day white hybrid	*bisporus*
Bisp 141	Bisp141–3	Brown wild isolate	*burnetti*
Bisp 53	MES 09143	Brown wild isolate	*bisporus*
Bisp 170	Z8	White wild isolate	*bisporus*

Table 2. Number of KASPar SNPs selected for each chromosome for five of the *Agaricus spp.* strains evaluated.

Chromosome number	Strains					Total SNPs per chromosome
	141–3	H39	JB137	MES09143	Z8	
Ch1	2	2	2	1	0	7
Ch2	2	2	2	2	2	10
Ch3	2	2	2	2	0	8
Ch4	2	2	2	2	1	9
Ch5	2	2	2	2	1	9
Ch6	2	2	2	2	0	8
Ch7	1	2	2	1	0	6
Ch8	1	2	2	2	2	9
Ch9	2	2	2	2	1	9
Ch10	3	2	3	3	1	12
Ch11	2	2	3	2	0	9
Ch12	1	2	3	2	1	9
Ch13	2	2	0	1	1	6
Total SNPs per strain	24	26	27	24	10	111

chromosome were selected unique for one of the homokaryones. After evaluation of the KASP markers, 111 SNPs were selected across the genome for further analysis (Table 2).

2.4. Phylogenetic analysis

Phylogenetic analyses of the accessions of both whole and core collections were conducted in MEGA X. Dendrograms were generated using a neighbor-joining method within MEGA X (bootstrap 1,000) [16]. The accessions comprising the core collection were selected using PowerCore program, which effectively reduces the number of core entries to those with the most diverse alleles and eliminates redundancy that comes from uninformative alleles [17].

2.5. Principal coordinate analysis

The PCoA was performed in R using the "Exploratory Analysis of Genetic and Genomic Data" (adegenet) package using default settings. The SNP sequences of the accessions were analyzed with the Dudi.pco function that computes measures of genetic distances between populations in the adegenet package [18]. Since this function calculated non-euclidian distance, it is a different principal component analysis (PCA).

3. Results

3.1. SNP analysis

Previously published KASP markers were used in a diverse set of *A. bisporus* strains [15]. In this study, 111 SNPs were identified as informative and useful for the analysis of a large set of germplasm including 170 accessions of *A. bisporus* strains. from the RDA collection, which mostly consisted of strains used in Korean breeding programs.

3.2. Genetic diversity of all accessions

The 360 accessions were divided into four groups following phylogenetic analysis hereby referred to as Groups A, B, C, and D (Figure 1). Group A mostly consisted of commercial cultivars, including the hybrids TripleX, Delta, Brawn, and Heirloom (Amycel Inc., San Juan Bautista, CA), Chinese hybrid cultivars W192 and W2000 (Institute of Edible Fungi, Fujian Academy of Agricultural Sciences, Fuzhou, China), and 169 Korean RDA accessions. Interestingly, the closely related species *A. blazei* and *A. arevensis* were also included in Group A. Group B consisted of commercial accessions and wild germplasm. The commercial cultivars included FB30 and FB5 (Italspawn, Treviso, Italy), U1 and A15 (Sylvan Spawn, Cambridgeshire, UK), and Le Lion C9 (Société Blanc de Semis, France). The wild accessions originated from the United States, Canada, China, Romania, and the United Kingdom.

Both Groups C and D were comprised of wild germplasm. Group C included strains from Belgium, the Czech Republic, the United Kingdom, France, Italy, the Netherlands, Russia, Spain, and the United States. Group D was comprised of accessions originating from the United States, Canada, Greece, Mexico, and France, and all of the strains were tetrasporus except for bisp172. This group also included *A. bitorquis* (KMCC00667) (Figure 1).

3.3. Genetic diversity of the core collection

A total of 67 strains were selected for the core collection with the PowerCore program from the initial 360 strains and used to analyze phylogenetic relationships in more detail. All of the selected accessions from the initial Group D were also included in the core collection Group D. However, selected accessions initially from Groups A, B, and C were subdivided (Figure 2). Group A was subdivided into 2 groups (A-1 and A-2), where the 169 Korean

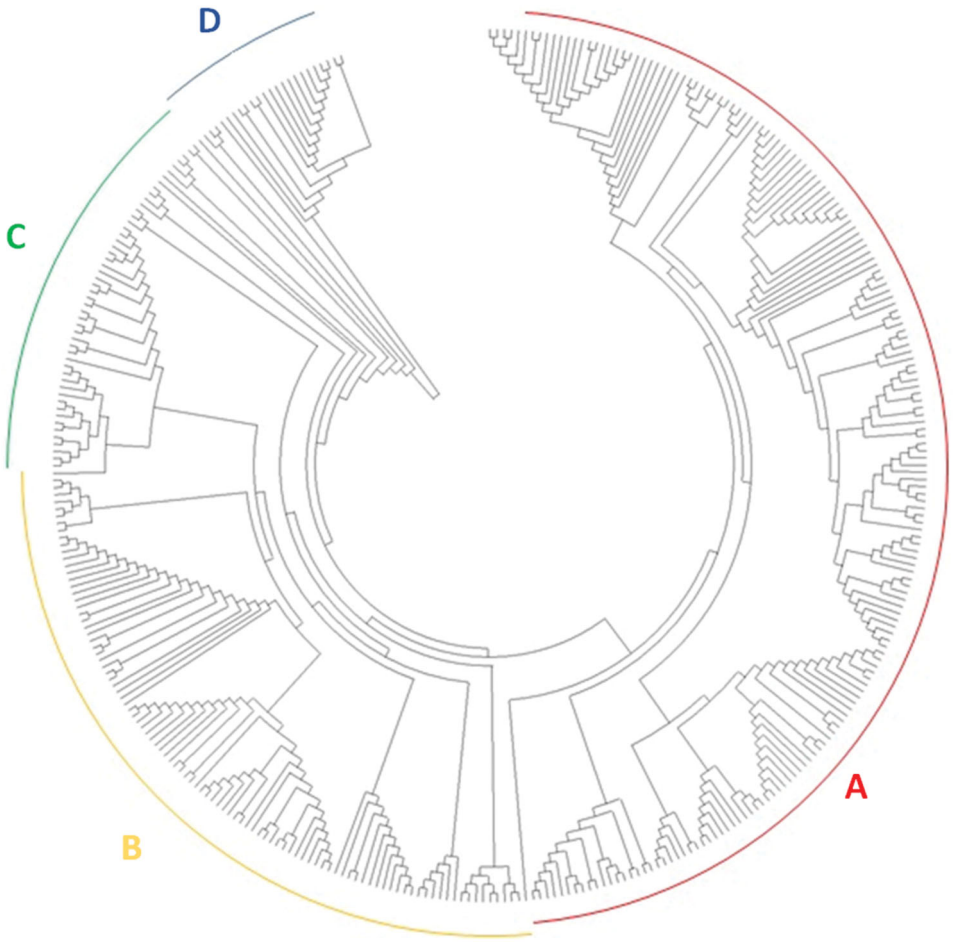

Figure 1. Dendrogram of all 360 *Agaricus* spp. accessions based on genotyping using 111 SNP markers. The dendrogram was generated using the neighbor-joining method in MEGA X with a bootstrap of 1000 [8].

RDA accessions comprised the A-1 group and a Chinese accession was the sole member of the A-2 group. The B-1 group mostly consisted of white accessions except for bisp010, whereas brown cultivars were included in Groups B-2, C-1, and C-2.

Interestingly, bisp073, bisp087, bisp265, KMCC00667, KMCC00937, and R20 HK (*A. arvensis*) clustered into a completely separate group, hereby referred to as Group E, which was not present in the results following the analysis of the entire collection. Initially, the three accessions, bisp073, KMCC00937, and R20 HK, were in Group A, bisp265 was in Group C, and bisp073 and KMCC00667 were in Group D (Figures 1 and 2). KMCC00667 and KMCC00937 revealed different genetic patterns than the other Korean RDA accessions.

3.4. Principal coordinate analysis

The 360 accessions were divided into four clusters (A, B, C, and D) in the PCoA analysis. The A cluster consisted of 168 Korean RDA collection accessions, a Chinese hybrid, a commercial hybrid, and *A. blazei*. The B cluster mostly consisted of wild germplasm, a traditional cultivar, and a present-day commercial hybrid. Clusters C and D consisted of wild germplasm, including KMCC00937 and KMCC00667 from the Korean RDA collection. Moreover, nine accessions did not partition into any cluster, i.e., bisp009, bisp030, bisp061, bisp071, bisp072, bisp078, bisp084, bisp103, and bisp105. These accessions were collected in North America except for bisp061 (Figure 3).

For the PCoA of the accessions from the whole collection, the accessions clustered into seven groups: those from the RDA collection, Chinese hybrids, wild germplasm, traditional cultivars, present-day hybrids, *A. blazei*, and *A. arvensis* (Table 3).

4. Discussion

Breeding new mushroom hybrid cultivars relies on integrating wild germplasm through the selection of appropriate parental lines [19], especially in *A. bisporus*, which is homothallic [14]. Therefore, identifying sources of wild germplasm is important for maintaining breeding programs. In this study, the genetic diversity of a diverse set of accessions of *Agaricus* spp. was evaluated and used to better characterize accessions with incomplete collection data

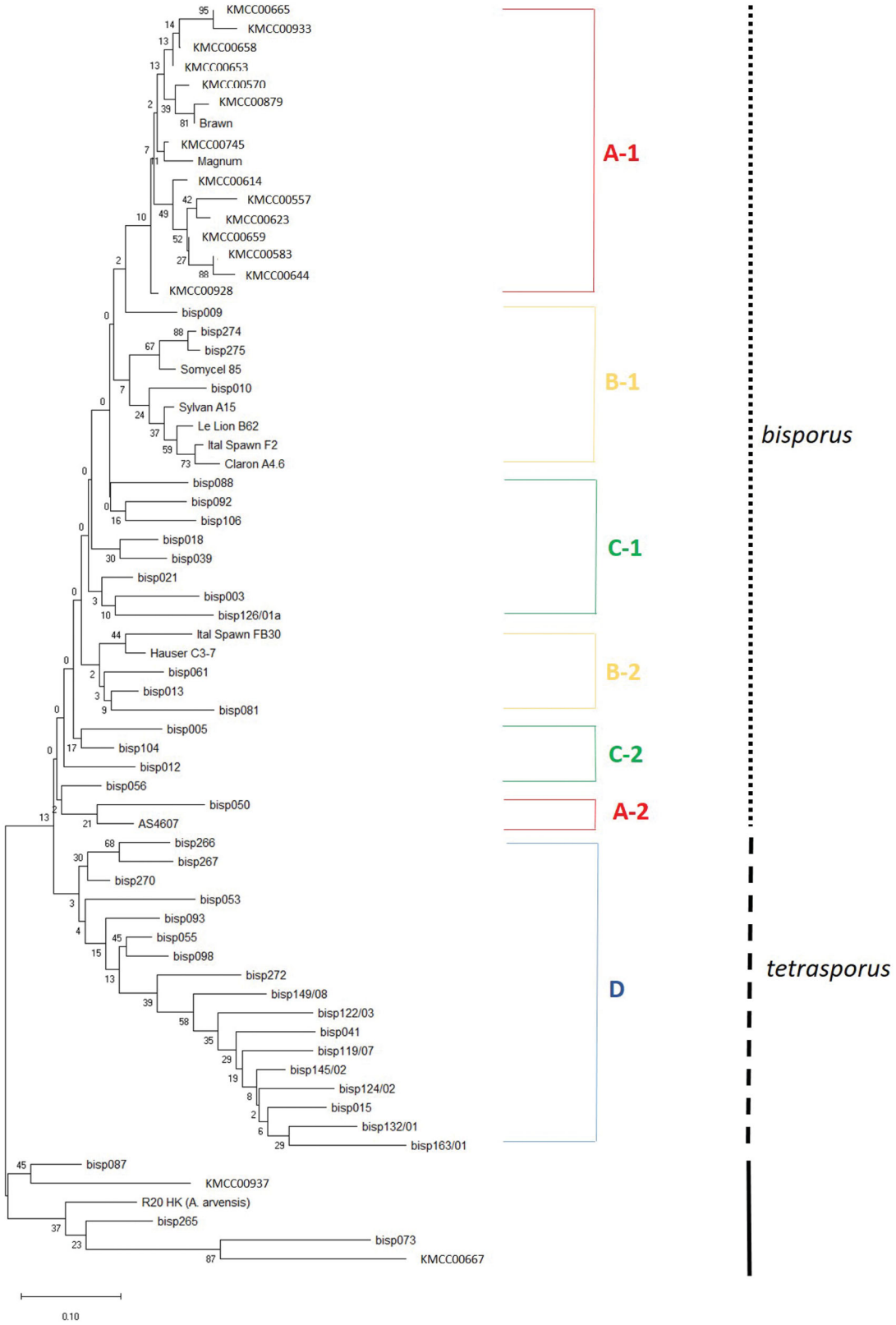

Figure 2. Dendrogram of 67 selected accessions from the original 360 *Agaricus* spp. accessions selected using the PowerCore program [9] representing a core collection of entries with the most diverse alleles while eliminating redundancy from non-informative alleles.

through the comparison of genetic relationships and PCoA based on SNP genotyping.

The whole collection of 360 accessions was divided into four groups based on phylogenetic analysis. Group A mostly consisted of commercial accessions, Group B contained commercial accessions and wild germplasm, Group C was made up of wild germplasm, and Group D was made up of tetrasporus wild germplasm. The 169 accessions from the Korean RDA collection with incomplete collection data were all in Group A with the exception of KMCC00667 (*A. bitorquis*), which was in

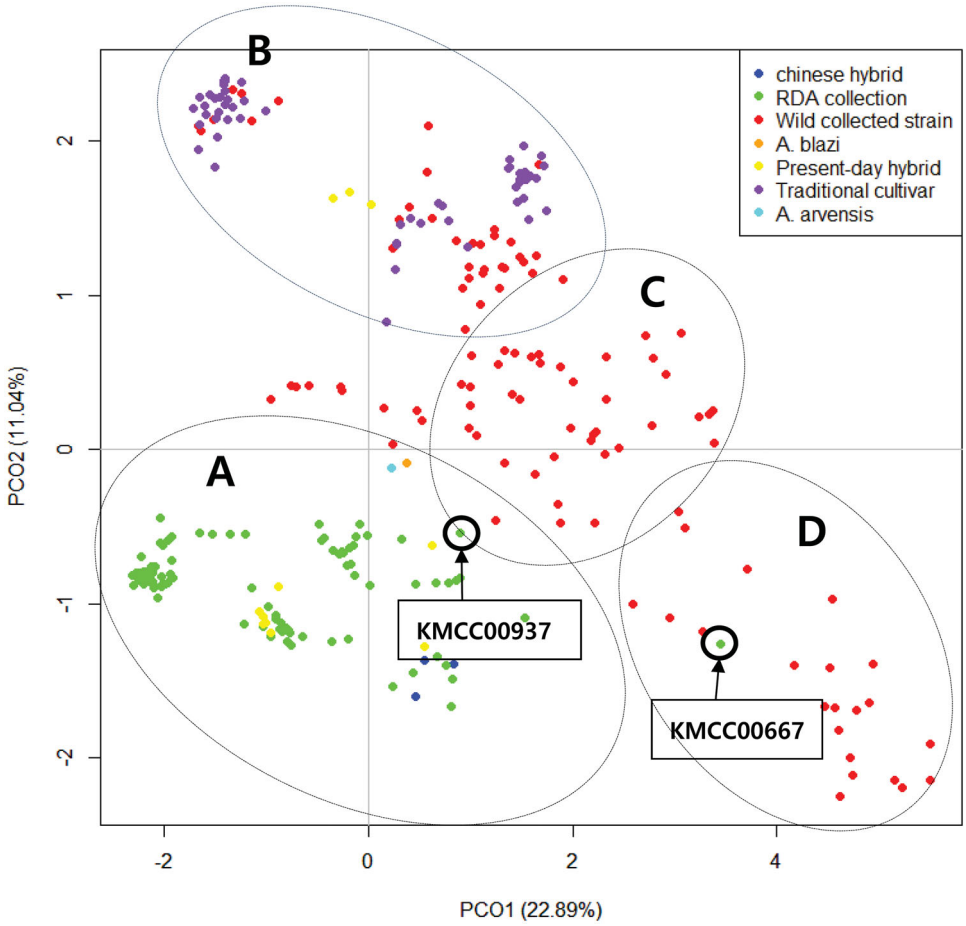

Figure 3. Principal component analysis plot of the 360 *Agaricus* spp. accessions. Grouping was performed based on SNP sequences analyzed with the Dudi.pco function in the adegenet package from the R software [10].

Group D. Based on these findings, accession KMCC00667 should be considered as wild germplasm within the Korean RDA collection.

The core collection was divided into seven groups (A-1, A-2, B-1, B-2, C-1, C-2, and D) based on phylogenetic analysis. Groups B-1 and B-2 were comprised of commercial cultivars, whereas Groups C-1 and C-2 were comprised of wild germplasm. Based on the phylogenetic tree (Figure 1), the wild germplasm within Group C appears to be the progenitors of the cultivars with Group B. Furthermore, the core collection primarily grouped based on the number of spores produced. Interestingly, Group E was comprised of *A. bitorquis* (KMCC00667, bisp073), *A. arvensis* (R20 HK), *A. bisporus var. eurotertrasporus* (bisp265), *A. bisporus* var. *bisporus* (bisp087), and KMCC00937.

The strains of *A. bitorquis* and *A. arvensis* are heterothallic, while *A. bisporus var. eurotertrasporus* is homothallic with four-spored basidia [4,19–21]. The button mushroom, *A. bisporus* var. *bisporus*, shows secondary (pseudo) homothallism with two-spored basidia. However, *A. bisporus* var. *burnetti* has limited pseudo-homothallism along with a low percentage of two-spored basidia. Also, *A. bisporus* var. *bisporus* has limited heterothallism with a low percentage of four-spored basidia. The difference in grouping and the presence of Group E in the core collection phylogenetic analysis results compared to the results of the entire collection are likely due to the amphitallic nature of the sexual reproduction of both varieties [22].

The results of PCoA and phylogenetic analyses of all accessions showed a similar trend overall, although the grouping was slightly different between the accessions except for the D group. However, nine accessions did not cluster in the PCoA. These strains were included in Group B of the phylogenetic analysis, which was composed of wild germplasm and commercial cultivars, when the entire collection was analyzed. The nine accessions show a genotype that is an intermediate between wild germplasm and commercial cultivars. According to Callac, since several accessions of wild germplasm collected in nature were cultivated between the seventeenth and nineteenth centuries, most differences between wild germplasm and cultivars have disappeared [23]. Furthermore, KMCC00937 within the RDA collection was an outlier and identified as a previously unknown source of wild germplasm.

The results of this study showed that, although there are few morphological differences between

Table 3. List of 67 strains of *Agaricus* spp. included within the core collection of this study, including their classification, species, the name of the strain, regional origin if known, cap color and the typical number of spores produced per basidium if known.

Classification	Species	Strain name	Origin	Cap color	Number of spores per basidium
A. arvensis	*A. arvensis*	R20_HK			
Chinese hybrid	*A. bisporus var. bisporus*	AS4607	China	White	
RDA collection	*A. bisporus var. bisporus*	KMCC00557		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00570		Brown	
RDA collection	*A. bisporus var. bisporus*	KMCC00583		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00614		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00623		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00644		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00653		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00658		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00659		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00665		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00667		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00745		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00879		Whte	
RDA collection	*A. bisporus var. bisporus*	KMCC00928		White	
RDA collection	*A. bisporus var. bisporus*	KMCC00933		Brown	
RDA collection	*A. bisporus var. bisporus*	KMCC00937		White	
Wild	*A. bisporus var. bisporus*	bisp003	Netherlands	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp005	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp009	USA	Brown	2(3)
Wild	*A. bisporus var. bisporus*	bisp010	USA	Dark brown	2
Wild	*A. bisporus var. bisporus*	bisp012	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp013	Canada	Brown	2
Wild	*A. bisporus var. bisporus*	bisp015	USA	Dark brown	4
Wild	*A. bisporus var. bisporus*	bisp018	USA	Light brown	2(3)
Wild	*A. bisporus var. bisporus*	bisp021	USA	Light brown	4
Wild	*A. bisporus var. bisporus*	bisp039	USA	Dark brown	2(3)
Wild	*A. bisporus var. bisporus*	bisp041	USA	Light brown	3(4)
Wild	*A. bisporus var. bisporus*	bisp050	China	Off-white	2
Wild	*A. bisporus var. bisporus*	bisp053	Canada	Off-white	2(3)
Wild	*A. bisporus var. bisporus*	bisp055		Light brown	2
Wild	*A. bisporus var. bisporus*	bisp056		Light brown	2(3)
Wild	*A. bisporus var. bisporus*	bisp061	Romania	Brown	2(3–4)
Wild	*A. bisporus var. bitorquis*	bisp073	USA	White	4
Wild	*A. bisporus var. bisporus*	bisp081	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp087	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp088	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp092	USA	Light brown	2(3)
Wild	*A. bisporus var. bisporus*	bisp093	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp098	USA	Brown	2–3
Wild	*A. bisporus var. bisporus*	bisp104	USA	Light brown	2
Wild	*A. bisporus var. bisporus*	bisp106	USA	Light brown	2(3)
Wild	*A. bisporus var. bisporus*	bisp119/07	USA	Brown	4
Wild	*A. bisporus var. bisporus*	bisp122/03	USA	Dark brown	4–3
Wild	*A. bisporus var. bisporus*	bisp124/02	USA	Brown	4
Wild	*A. bisporus var. bisporus*	bisp126/01a	USA	Dark brown	4
Wild	*A. bisporus var. bisporus*	bisp132/01	USA	Brown	4
Wild	*A. bisporus var. bisporus*	bisp145/02	USA	Light brown	3–4
Wild	*A. bisporus var. bisporus*	bisp149/08	USA	Brown	3–4(2)
Wild	*A. bisporus var. bisporus*	bisp163/01	USA	Dark brown	4–3
Wild	*A. bisporus var. eurotetrasporus*	bisp265	France		4
Wild	*A. bisporus var. eurotetrasporus*	bisp266	Greece		4
Wild	*A. bisporus var. eurotetrasporus*	bisp267	Greece		4
Wild	*A. bisporus var. eurotetrasporus*	bisp270	Mexico		4
Wild	*A. bisporus var. eurotetrasporus*	bisp272	Greece		4
Wild	*A. bisporus var. eurotetrasporus*	bisp274	China		4
Wild	*A. bisporus var. eurotetrasporus*	bisp275	China		4
Traditional cultivar	*A. bisporus var. bisporus*	Claron A4.6		Off-white	
Traditional cultivar	*A. bisporus var. bisporus*	Hause C3–7		Brown	
Traditional cultivar	*A. bisporus var. bisporus*	ItalSpawn F2		Off-white	
Traditional cultivar	*A. bisporus var. bisporus*	ItalSpawn FB30		Brown	
Traditional cultivar	*A. bisporus var. bisporus*	Le Lion B62		Off-white	
Traditional cultivar	*A. bisporus var. bisporus*	Somycel 85		White	
Present-day hybrid	*A. bisporus var. bisporus*	Brawn		Brown	
Present-day hybrid	*A. bisporus var. bisporus*	Magnum		White	
Present-day hybrid	*A. bisporus var. bisporus*	Sylvan A15		White	

wild accessions and commercial cultivars of *A. bisporus*, genetically they can be distinguished with a limited number of SNP markers using multiple methods. Furthermore, based on phylogenetic analysis of SNP data, a core collection of *A. bisporus* with significant variation among genotypes was

constructed. This core collection will be a useful tool in future studies for focusing research and using time and resources efficiently.

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Disclosure statement

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