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Stain improvement in the white button mushroom 'Seolgang' and its varietal characteristics in *Agaricus bisporus*

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ABSTRACT: The button mushroom (*Agaricus bisporus*) is one of the most widely cultivated important edible mushroom species. In the breeding of new button mushroom, 'Seolgang' was developed by crossing two monokaryons 'CM020913-27' and 'SSU423-31'. Because of the secondarily homothallism, only a small percentage of the basidia produce 3 or 4 spores, which are mostly haploid (n) and do not fruit. Single spore cultures derived from these types of spores produce a vegetative mycelium that also contain a variable number of genetically identical nuclei per cell called monokaryon. The lack of clamp connections between monokaryon and dikaryon required a series of mycelial culture and fruiting test. After crossing, hybrids were cultivated on a small scale and on a commercial scale at a farm. For this, the spawn was made by a commercial spawn producer and the spawned compost by a commercial compost producer. Mycelial growth of 'Seolgang' on CDA was better at 20℃ and 25℃ when it was compared with that of '505 Ho'. The mature cap shape of new strain 'Seolgang' is oblate spheroid and the immature cap shape is round to oblate spheroid. The cap diameter was 41.2 mm on average. In comparison with white strain '505 Ho', the strain had a yield that was 9% higher. It produced fruiting bodies which had a higher weight on average per fruiting body and were 19% firmer with a good shelf life. Days of fruiting body were 3-4 days later than those of '505 Ho'. The physical characteristics such as elasticity, chewiness, adhesiveness were better than that of '505 Ho'. Genetic analysis of the new strain 'Seolgang' showed different profiles compared to '505 Ho', CM02913-27, SSU413-31, when RAPD primers A02 and O04 were used.

KEYWORDS : *Agaricus bisporus*, Button mushroom, Dikaryon, Mating, Monokaryon, Secondarily homothallism

INTRODUCTION

Mushrooms have been sought and cultivated for the production of food consumption due to its nutritive and medicinal values (Fan *et al.*, 2006). They also can be used for ecosystems by protecting, regenerating, and detoxificating after human impact as feed or fertilizers (Barton, 1996; Kothe, 2001). The button mushroom is one of the most widely cultivated important edible mushroom species placing first followed by *Pleurotus ostreatus* and *Lentinus edodes*. It is an edible basidiomycete mushroom native to grasslands in Europe and North America cultivated in more than 70 countries (Cappelli, 1984) increasing its popularity in Eastern Countries such as China and Korea.

The majority of *A. bisporus*, including cultivated and many wild strains are predominantly secondarily homothallic (Khush *et al.*, 1995), in which a fertile dikaryotic mycelium is established from a basidiospore carrying two meiotic nuclei of different mating types. In a number of cases, the two nuclei in the basidiospores of *A. bisporus* are non-sisters and carry compatible mating types with a bipolar mating system comprising multiple alleles. The bisporic spores, therefore, give rise to fertile dikaryotic progeny (n+n) capable of fruiting bodies (Kerrigan *et al.*, 1993; Langton and Elliott, 1980). In such result, over 90% of the basidiospores produced remain heterozygous at loci accompanied by low recombination frequencies.

A. bisporus was composed of 13 chromosomes that account for a total genomic size of about 34.2 Mbp per haploid genome ranging 1.4 Mbp to 3.65 Mbp and the mating locus MAT was on chromosome I and the cap color locus *PPC1* on chromosome VIII in the construc-

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tion of a genetic linkage map (Foulongne-Oriol *et al.*, 2010; Kerrigan *et al.*, 1993; Sonnenberg *et al.*, 1991, 1996; Xu *et al.*, 1993). The *PPC1* locus was found to be recessive for the white color (Callac *et al*, 1998). Quantitative trait locus mapping of yield-related traits in *A. bisporus* was also identified recently (Foulongne-Oriol *et al.*, 2012)

In the breeding program, *A. bisporus* has certain inherent difficulties for a breeding program because uninucleate monokaryotic materials for outcrossing are very laborious and time consuming to obtain (Horgen and Anderson, 1992). Clamp connections also are not produced and this makes the identification of compatible reactions a problem. However, there is some opportunity for crossing between monokaryons. A minority of basidia produce three or four spores, some of which are monokaryotic and self-sterile on germination (Kerrigan *et al.*, 1993). Such monokaryons can be crossed with an appropriate breeding partner resulting in improved strains.

A dikaryon does no longer accept other nuclei, but it can donate nuclei to a monokaryon. The dikaryotization of a monokaryon by a dikaryon was first demonstrated in *Corprinus lagopus* and was called as the 'Buller phenomenon' (Quintanilha, 1937) or 'di-mon mating' (Papazian, 1950). This mode of dikaryotization produces a new pair of conjugate nuclei following nuclear migration from the dikaryon (Clark and Anderson, 2004; Xu *et al.*, 1996). There is also evidence that genetic exchange can occur between dikaryons. In *Heterobasidion annosum*, parings of dikaryons yield subcultures that are non parental with respect to somatic incompatibility reactions. Furthermore, in the cultivated button mushroom parings of dikaryons with other dikaryons produced recombinant genotypes (Xu *et al.*, 1996).

In the last two decades, various molecular makers have been used in the breeding of edible fungi. The infrequent monokaryons of different *A. bisporus* strains were identified from dikaryons using quick and reliable DNA markers, such as inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD) markers (Kerrigan *et al.*, 1993; Khush *et al.*, 1992; Moore *et al.*, 2001). Compatible matings were identified by the formation of genetically stable dikaryons which were verified by restriction fragment length polymorphisms (RFLP) (Xu *et al.*, 1993). Therefore, the use of molecular markers will allow for a fast, easy, and cheap method for the screening and selection with desirable traits without waiting for the fruiting stages in mushroom breeding.

MATERIALS AND METHODS

Single basidiospore isolates and monokaryons

Basidiospores were obtained from freshly collected fruiting bodies of *A. bisporus* strains CM020913 and SSU423. They were suspended in sterile distilled water. Suspensions were spread on 9cm petri dishes with CDA and incubated at 25℃. The colonies of single spore isolates (SSIs) were transferred onto new petri dishes of CDA in sterile condition and incubated at 25℃ in dark. After 30 days of SSIs incubation, colony diameter and morphology type of each isolates was determined. Based on growth rate, colony morphology, putative monokaryons were selected and fruiting trials were carried out. After this crossing dikaryotic mycelia were placed in petri dishes containing CDA medium.

Media and culture.

Mycelia of *A. bisporus* strains were cultured vegetatively at 25℃ on compost dextrose agar (CDA) medium. For CDA 20g compost was added to 1L distilled water, boiled for 15 min and 10g dextrose and 15g agar were added to liquid extract. The volume for 1L was made with distilled water. The medium was sterilized at 121℃ for 30 minutes, and then preserved at 4℃. Agar blocks taken from actively growing colonies on CDA plates were inoculated into different culture media for mycelial growth (Table 1).

Fruiting trial

Mycelial cultures were transferred to 450g of sterile cooked wheat grain, buffered with 5% CaCO3. Colonized grains (40g) were used to inoculate plastic container (57×41×18cm) containing a complex biologically modified, straw-based substrate. Pasteurized soil was used as the casing material. Case-run, initiation and maturation all occurred in controlled conditions.

Table 1. Culture media and their constituents used for mycelial growth.

Composition	Media[a] (g/l)		
	CDA	PDA	MCM
potato		200	
dextrose	10	20	20
peptone	1		
malt extract	7		
yeast extract			2
dry compost	40		
KH^2PO^4	1		0.4
K^2HPO^4			1
$MgSO^4$	0.5		0.5
agar	20	20	20

[a]CDA, compost dextrose agar; PDA, potato dextrose agar; MCM, mushroom complete media.

Two replications were used for each isolate. Dikaryotic strains were cultivated on a small scale and on a commercial scale at a farm. For this, the spawn was made by a commercial spawn producer and the spawned compost by a commercial compost producer.

Determination of color value

The surface color and L-value of button mushrooms were measured by a Chroma Meter CR-200 (Minolta Camera Co. Japan). Three random locations were measured on the cap of fruiting body and they were compared with the white color values of L (brightness)=97, a (greenness) =0 and b (yellowness) =1.95 using ΔE as described by the following equation $\Delta E = \sqrt{(L-L')^2+(a-a')^2+(b-b')^2}$, where ΔE is degree of overall color change in comparison with ideal color values.

Nutrient content determination

Prior to nutrient analysis, moisture content for the mushroom samples were determined by the direct oven drying method. The weight loss after oven drying of each sample of 2 g at 105℃ to constant weight was expressed as % moisture content. Crude protein content was determined using the Kjeldahl method. A 0.5 g ground sample from each of the mushroom species was digested in Kjeldahl flask using 98% sulphuric acid after which it was steam-distilled. The resulting distillate was titrated to pink or wine-red colour using 0.01 M hydrochloric acid and the protein percentage was calculated. Crude fat was determined by using the Soxhlet extraction method using petroleum ether as the solvent. Ash content of 1 g powdered sample was determined as the residue of incineration at 550℃ in a muffle furnace. Total carbohydrate was determined by 2 g of each sample in 50 ml distilled water of which 0.2 ml was diluted ten-fold. To 1 ml of the resulting solution and serial dilutions of glucose stock (10 mg/100 ml) solution, 4 ml of anthrone reagent was added and absorbed the solutions were measured by a spectrophotometer at 620 nm against a reagent blank.

Texture profile analysis

Instrumental texture profile analysis (TPA) is one of the methods to determine the texture by simulating or imitating the repeated biting or chewing of a sample. In the present work TPA was performed to the cap of mushrooms to evaluate the influence of this processing operation in the textural properties. TPA was carried out using a texture analyser TA-Plus (Lloyd Instruments Ltd., UK) by two compression cycles between parallel plates performed using a 5 mm diameter plunger with a 5 seconds interval between cycles. Middle area of the cap of the fruit body in the fresh state was the object of probe with 4 analyses. The parameters that have been used were 5 kg force load cell and 2.0 mm/sec test speed. The textural properties

hardness, gunmminess, adhesiveness, springiness, cohesiveness, and chewiness were then calculated.

DNA preparation

Mycelia cultured in PDA medium at 25℃ for 14 days were filtered through Miracloth (pore size 10μm) and washed at least three times with distilled water. The mycelia were then squeezed with a dry paper towel and this mycelial mat was centrifuged for 5 min at 10,000 rpm followed by a washing with 50μL TE beffer. The resulting pellet was grinded in extraction buffer (200 mM Tris-HCl, pH 8.5, 25 mM EDTA, 250 mM NaCl, 0.5% sodium dodecyl-sulphate) followed by addition of 150 μL of 3M sodium acetate. The lysates were incubated at 20℃ for 10 min followed by centrifugation. DNA was precipitated from the supernatant by adding equal volumes of isopropanol and resultant pellet was washed with 70% ethanol. The genomic DNA was air dried and dissolved in 20 μL of TE buffer. DNA quantification was performed spectrophotometer (Smart Spec Plus, BIO-RAD Co.) and a dilution of 20 ng/μL was used as template genomic DNA for the PCR.

DNA polymorphic analysis

The PCR amplifications was performed in Maxime PCR PreMix Kit that contained 20 ng of genomic and 10 pm of primer using iCycle (BIO-RAD Co.). After initial denaturation at 94℃ for 5 min, the mixture was subjected to 40 cycles of 1 min of denaturation at 94℃, 1 min of annealing at 43℃, and 2 min extension at 72℃. These were performed before a final extension of 5 min at 72℃ and subsequent cooling at 4℃. The electrophoresis was performed in 1.5% agarose gels, that was stained in 10% ethidium bromide solution and bands were observed in an UV transilluminator. A total of 160 random primers of 10 nucleotides (Operon primer kit A~H) were used for the RAPD analysis.

RESULT AND DISCUSSION

Although *A. bisporus* has been a important cultivated crop world wide, the strain improvement of this species has remained poor because of two main reasons: the predominantly homothallic life cycle producing self-fertile binucleate spores and the lack of morphological features allowing the recognition of the monokaryotic status making the isolation of monokaryons very difficult. This type of sexual behavior hampers outcrossing and limits breeding success (Elliott and Langton, 1981). The early strain improvement was based on selection giving higher yields than the

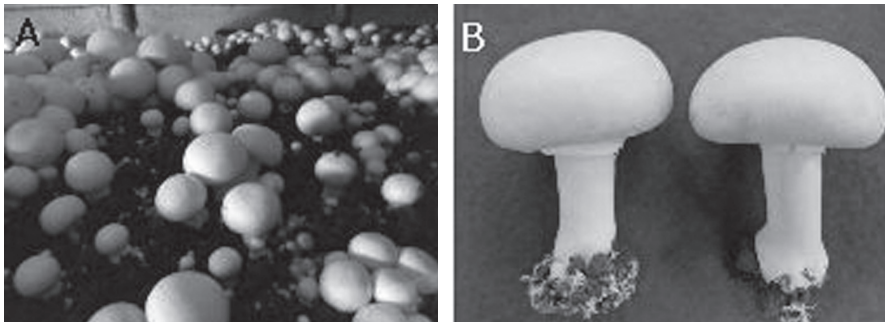

Fig 1. Cultivation of *A. bisporus* strain 'Seolgang' (A) and fruting bodies of 'Seolgang' (left) and '505 Ho' (right) (B).

Table 2. Inherent characteristics of *A. bisporus* strain 'Seolgang' compared to '505 Ho'.

Strain	Temperature of mycelial growth(℃)	Temperature of fruiting body(℃)	Color of pileus	Shape of pileus
Seolgang	25	16-17	White	Round oblate
505 Ho	25	14-16	White	Flat oblate

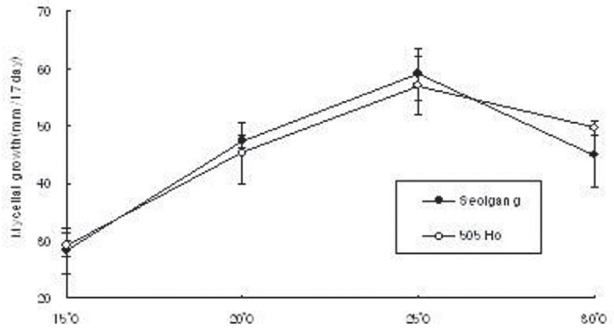

Fig 2. Mycelial growth at different temperatures of *A. bisporus* strain 'Seolgang' and '505 Ho'.

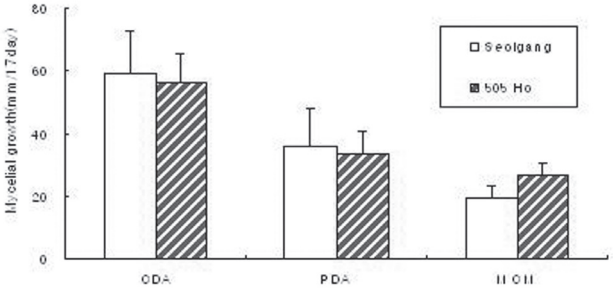

Fig. 4. Mycelial growth on different media of *A. bisporus* strain 'Seolgang' and '505 Ho'.

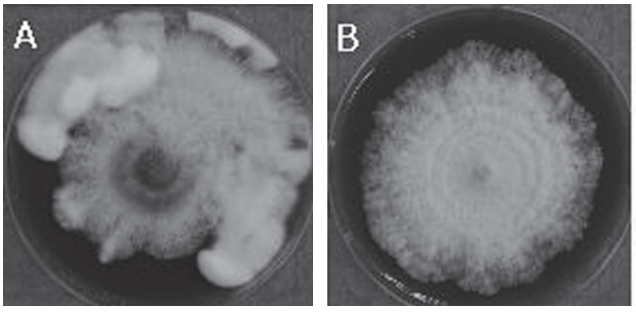

Fig 3. Mycelial growth at 25℃ on CDA after 23 days of *A. bisporus* strain 'Seolgang' (A) and '505 Ho' (B).

parental strains because the fertile single spores from an individual sporophore differed in growth rate, appearance of the mycelia, sporophore morphology and in productivity. Strain selection based on single spores, multispores or tissue culture may give improvement in the short term but it is unlikely to be as effective as controlled crossing. The new strains are needed to be measured appropriate parameters. The morphological, agronomical and chemical data obtained in the analysis of the new mushroom variety should be compared to those obtained from an appropriate variety grown under identical conditions. The mature pileus of new hybrid strain 'Seolgang' is oblate spheroid in the shape and white in the color (Fig. 1). In '505 Ho' it is white in the color of pileus and the shape is rather flat compared to new strain. The cultivation temperature for mycelia growth was similar at 25℃ but appropriate temperature for fruiting body formation was a little bit higher (Table 2).

Mycelial growth at different temperatures on CDA medium increased from 15 to 25℃ and decreased between 25 and 30℃. In the range of temperature between 20 and 25℃ 'Seolgang' was better in mycelial growth but was less out of this range compared to '505 Ho' (Fig. 2). As seen in Fig. 3, the morphology of mycelial colony was fluffy and the mycelial density was higher in 'Seolgang' (A) than that of '505 Ho' (B) when they were grown at 25℃ on CDA for 23 days. Dikaryotic mycelium of 'Seolgang' tended to produce more dense and abundant in aerial mycelia than that of dikaryons of '505 Ho'.

Mycelial growth on different culture media was variable in two strains. The culture media influenced colony growth rate, which ranged from 24.2–58.7 mm/17day (Fig. 4). Two strains showed higher growth rate on CDA followed by PDA and MCM. Strain 'Seolgang' was higher on CDA and PDA but slower on MCM.

As a secondary decomposer, fruiting of *A. bisporus* depends on a complex set of variables including atmospheric factors such as carbon dioxide concentration, temperature, humidity and pH and on the nutritional status of the substrate (Flegg and Wood, 1985). The mushroom crop grows in repeating 3– to 5–day cycles called 'flushes'. These flushes are followed by a few days when no mushrooms are available to harvest. The individual flushes tend to produce progressively fewer mushrooms. In commercial practice, three to five flushes are picked before the crop is removed to make room for the next. Most strains of *A. bisporus* are picked before the veil breaks and the stem elongates.

In the cultivation strain 'Seolgang' was shorter in primordial formation and fruit body development as

Table 3. Cultivation period of *A. bisporus* strain 'Seolgang' and '505 Ho'.

Strain	Period(days)		
	Primordial formation	Development of fruit body	Total
Seolgang	27	7	34
505 Ho	29	8	37

Table 4. Morphological characteristics of fruiting bodies of *A. bisporus* strain 'Seolgang' compared to '505 Ho'.

Strain	Pileus		Stipe	
	Diameter (mm)	Thickness (mm)	Length (mm)	Thickness (mm)
Seolgang	41.2	25.1	31.2	14.9
505 Ho	40.4	23.5	29.1	15.6

Table 5. Determination of color value of *A. bisporus* strain 'Seolgang' compared to '505 Ho'.

Strain	L[a]	a	b	ΔE
Seolgang	93.15	− 0.43	9.75	8.71a
505 Ho	91.23	0.21	12.69	12.19b

[a]L : lightness, a : redness, b : yellowness; ΔE(color difference) = $\sqrt{(L-L')^2+(a-a')^2+(b-b')^2}$, DMRT 5%

Table 6. Texture profile analysis on pileus of *A. bisporus* strain 'Seolgang' compared with '505 Ho'.

Strain	Springiness(mm)	Chewiness(mJ)	Adhesiveness(N)	Cohesiveness(g.s)	Gumminess(N)
Seolgang	0.891	46.5	0.186	0.162	49.3
505 Ho	0.880	45.4	0.177	0.169	42.1

27 and 7 days, respectively (Table 3). As a result, total period was 34 days in 'Seolgang' compared to 37 days in '505 Ho'. As a new cultivar, this could be an advantage for the commercial cultivation.

At the time of harvest, morphological characteristics of fruiting bodies was different between two strains (Table 4). New strain showed 41.2 and 25.1 mm in diameter and thickness of pileus and 32.2 and 14.9 mm in the length and thickness. When it was compared with '505 Ho', 'Seolgang' had bigger and rounder in pileus and thinner and longer in stipe. Therefore, new hybrid strain 'Seolgang' looked oblate spheroid in the shape and '505 Ho' looked rather flat.

When the surface color value was determined by a Chroma meter, the color difference of 'Seolgang' showed 8.71 compared to 12.19 in '505 Ho' resulting significant ΔE, where ΔE is degree of overall color differences in comparison with color values of L (brightness)=97, a (greenness) =0 and b (yellowness) =1.95 (Table 5). This result shows that 'Seolgang' is whiter compared to '505 Ho'.

Texture is one of important factors to evaluate quality of mushrooms. Undesirably, the stability of texture can be only maintained very short period of storage, i.e. it is changed quickly after harvest (Nichol 1985). Springiness, chewiness, adhesiveness, cohesiveness and gumminess are considerable characteristics in the texture profile analysis of fruit bodies (Table 6). There were some relations between textural properties and quality of fruit body (Beelman *et al.*, 1987; Mc Garry and Burton, 1994). Springiness, chewiness, adhesiveness, and gumminess were higher except cohesiveness

Table 7. Hardness, weight, and yield of *A. bisporus* strain 'Seolgang' compared to '505 Ho'.

Strain	Hardness(kg/ø5mm)	Individual weight(g/ea)	Yield(kg/m^2)	Yield index
'Seolgang'	1.12	9.6	11.1a[a]	109
505 Ho	0.94	9.3	10.2b	100

[a]LSD(5%)=0.669

Table 8. Proximate constituents of nutrients in *A. bisporus* strain 'Seolgang' compared with '505 Ho'.

Strain	Calorie (kcal/100g)	Moisture (%)	Crude protein (%)	Crude lipid (%)	Carbohydrate (%)	Ash (%)
Seolgang	19.54	92.21	5.39	0.07	1.39	0.94
505 Ho	19.91	92.14	5.25	0.07	1.60	0.94

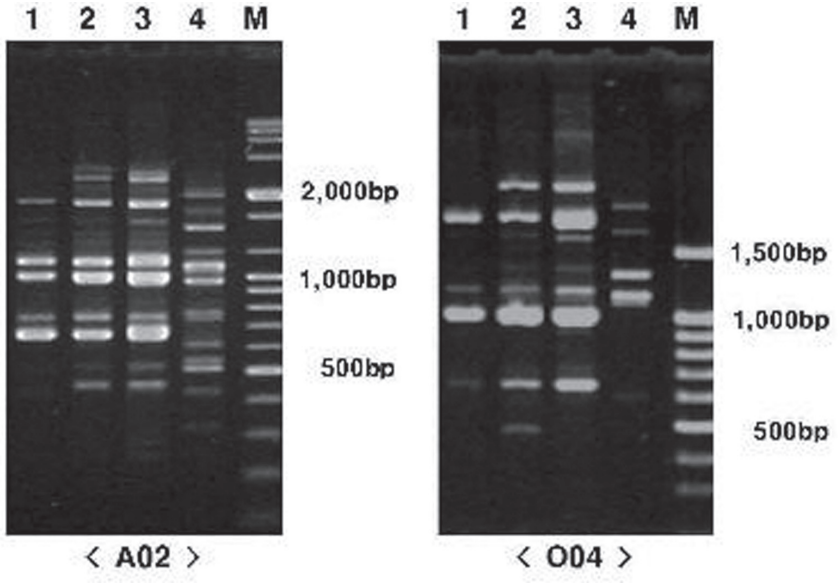

Fig. 5. RAPD profiles (1: Seolgang, 2: 505 Ho, 3: CM02913-27, 4: SSU413-31, M: 100bp DNA ladder.

in 'Seolgang', suggesting better quality in general.

Especially hardness of harvested fruit bodies were considered important to evaluate the quality and shelf life of mushrooms (Mohapatra *et al.* 2010; Singh *et al.*, 2010). The weight of fruit body also could be affected by hardness in *A. bisporus*. 'Seolgang' had heavier fruit body with 1.12 kg/ø5mm compared to '505 Ho' with 0.94 kg/ø5mm (Table 7). The yield of "Seolgang" was significantly high with 11.1 kg/m^2 compared to 10.2 kg/m^2. Analyses of correlations between yield components showed that the average weight of fruiting boies decreased when the yield and the number of mushrooms increased (Foulongne-Oriol *et al.*, 2012; Rodie *et al.* 2000). Given these relationships, the development of high yield producing mushrooms with a high weight may be challenging.

The proximate composition of 'Seolgang' were compared with '505 Ho' shown in Table 8. 'Seolgang' was low in calorie and carbohydrate in comparison to '505 Ho', while crude lipid and ash contents of both species were comparable. The crude protein content was 5.39 in 'Seolgang' and 5.25 in '505 Ho'. In general, two strains were similar in the nutrient contents for the same mushroom species.

Cultivars of the white button mushroom *A. bisporus* are difficult to differentiate, which has made strain identification problematic for this crop species. Molecular markers have been used for identifying different strains in *A. bisprous* (Guan *et al.*, 2008; Malekzadeh *et al.* 2011). The RAPD approach is convenient for the easy generation of molecular markers linked to genes of interest. RAPD analysis was used to discriminate different strains, and to characterize the genetic relatedness of these strains (Khush *et al.*, 1992; Moore *et al.*, 2001; Ramirez *et al.*, 2001). Genetic analysis of the new strain 'Seolgang' showed different profiles compared to '505 Ho', CM02913-27, SSU413-31, when RAPD primers A02 and O04 were used (Fig. 5). In the use of primer A02 and O04, there were specific band at 400bp and 1,300 bp respectively suggesting they are different strains each other.

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