Journal of Mushroom Science and Production
Vol. 10, No 3, p101-108 September 2012
Printed in S. Korea

Mating Relationship between the parent and the mutant strains in *Pleurotus ostreatus*

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(Received august 12, 2012, Revised september 13, 2012, Accepted september 15, 2012)

ABSTRACT: *Pleurotus ostreatus* 'Miso' is a mutant strain showing white color in pileus from the known parent strain 'Wonhyeong 1'. Shape and several other characters also vary with culture conditions. Mating experiments were performed to understand interstrain mating relationship using monokaryons of the parent and the mutant strains. All monokaryons were grown from single spores isolated from freshly collected fruit bodies. Pairings were performed in 90 mm petri dishes on PDA. They were allowed to grow at 25 until two fronts of the advancing mycelia met and developed a conspicuous contact zone. The contact zone and the outer edges of paired colonies on each plate were examined for clamp connections. The parent and the mutant resulted in tetrapolar incompatibility in intrastrain crosses. In interstrain crosses, each monokaryotic tester strain of the parent strain was out-crossed to monokaryotic tester strains of the mutant. As a result of these crosses it was found that both strains share the same A and B incompatibility factors yielding 25% compatibility.

KEYWORDS : Incompatibility, Interstrain cross, Mating, Mutant strain, *Pleurotus ostreatus*, Monokaryon

INTRODUCTION

Pleurotus ostreatus is a commercially important edible mushroom commonly known as the oyster mushroom comprising about 25% of world's production of cultivated mushrooms. *It is an efficient low-fat protein source*, cultivated on a variety of substrates and has many industrial and medical applications (Bobek *et al.*, 1998; Larraya *et al.*, 2001; Vyas *et al.*, 1994; Wang *et al.*, 2000). In the life cycle of *Pleurotus ostreatus*, two different vegetative states can be identified as the monokaryon and the dikaryon. The monokaryon is the primary mycelium which develops on germination of a single haploid basidiospore and the dikaryon is the secondary mycelium which develops the fruit bodies given the right environmental conditions.

When two compatible monokaryotic hyphae mate, a dikaryon is generally derived from the interaction. After mating of two compatible monokaryons, a dikaryon is formed containing two haploid nuclei, one from each monokaryon, in every cell. The two parental nuclei of dikaryons remain independent throughout vegetative growth and fruiting body development. Only in the basidia karyogamy occurs in the fruiting bodies within the specialized cells, the basidia and it is directly followed by meiosis (Larraya *et al.*, 2001).

Pleurotus ostreatus was composed of 11 chromosomes that account for a total genomic size of about 35.1 Mbp per haploid genome and the A mating locus was on chromosome III and the B locus on chromosome IX in the construction of a genetic linkage map (Larraya *et al.*, 2000; Larraya *et al.*, 1999). In the tetrapolar system there are two unlinked mating type genes, A and B, each with multiple alleles. The existence of at least 63 A and 190 B alleles has been calculated in the natural population of *Pleurotus ostreatus* (Eugenio and Anderson, 1968). They determine sexual compatibility and control the different events involved in dikaryon morphogenesis. A genes are responsible for nuclear pairing, clamp cell formation, co-ordinate nuclear division, and clamp cell septation, while B genes are responsible for nuclear migration, septal dissolution, and clamp cell fusion. Therefore, dikaryotic mycelium occurs when it is crossed between two monokaryons with different alleles in both A and B mating type genes.

Four types of heterokaryons could be possible in terms

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of combinations of alleles of the mating type genes when the monokaryons are mated with each other: the dikaryon (A≠B≠), the common A (A=B≠), the common B (A≠B=), and the common AB (A=B=)(Raper, 1966).

A≠B≠ has a different A and B, which is usually called a dikaryon leading to development of clamp connections and to the formation of fruiting bodies. A=B≠ has a common A and different B, leading to septal disruption, and reciprocal nuclear exchange and migration (Raudaskoski, 1998). In A≠B=, a common B and different A, pseudoclamps are formed in the contact zone, but no nuclear migration takes place (Raudaskoski, 1998). Finally, A=B= has a common A and B, showing no distinction in gross morphology from monokaryotic mycelium.

Strain improvement breeding programs help to produce high yield and high quality mushrooms. For such breeding programs it is important to understand mating type genes and then identify compatible mating types (Gharehaghaji *et al.*, 2007; Kothe, 2001). Therefore, this study examined the tetraporarity of *Pleurotus ostreatus* with the parent strain 'Wonhyeong 1' and its mutant strain 'Miso' and that may make an attempts at an efficient strain improvement.

MATERIALS AND METHODS

Conditions for fruiting bodies

Pleurotus ostreatus 'Miso' is a mutant strain showing white color in pileus from the parent stain 'Wonhyeong 1' (Kim *et al.*, 2008). Mycelia of them were cultured at 25℃ on potato dextrose agar (PDA) medium sterilized at 121℃ for 20 minutes, and then preserved at 4℃. Agar blocks taken from actively growing colonies on PDA plates were inoculated into 500 ㎖ of potato dextrose broth (PDB) liquid medium in flasks and incubated on a rotary shaker (120 rpm) at room temperature for seven to ten days. For fruiting, the substrate containing sawdust and wheat bran at 8:3 (v/v) with 65% water was put into 850 ㎖ polypropylene bottles and sterilized at 121℃ for 90 minutes and cooled to 20℃. Each bottle was inoculated with 10 ㎖ liquid spawn in a clean room. After inoculation, they were cultivated in a well aerated room at temperature 21~24℃ for 22~25 days until mycelia spread all over the media and then they were transferred to the growth room of 85~95% humidity, 16~17℃ under light conditions. Fruit bodies were collected just before sporulation.

Single basidiospore isolates and monokaryons

Single basidiospore isolates were obtained from freshly collected fruiting bodies of *Pleurotus ostreatus* 'Wonhyeong 1' and 'Miso'. To obtain them, an aqueous suspension was prepared from spore prints deposited in petri dishes. Suspensions were spread on 9cm petri dishes with PDA medium. After incubation for 2~3 days at 25℃ in dark, colony of each basidiospores were removed manually and transferred into the fresh PDA medium. One week later, monokaryons were checked under the microscope for absence of clamp connections to verify their haploid nature. Selected isolates were then transferred into test tubes with PDA medium.

Mating tests

Mating experiments were performed to provide mating compatibility data using 22 monokaryotic strains isolated from fruiting bodies of *Pleurotus ostreatus* 'Wonhyeong 1' and 'Miso'. Cultures were routinely maintained on PDA at 25℃ in dark. The pieces of agar blocks from PDA plates of the two monokaryons were placed in all possible combinations approximately 1 cm apart in the center of a 9 cm petri dish of PDA. They were allowed to grow for about 1~2 weeks until two fronts of the advancing mycelia from the agar pieces met and developed a conspicuous contact zone. From the contact zone and the outer edges of paired colonies on each plate a piece of mycelium was taken and examined under a microscope (×400) for clamp connections. When clamp connections were at the growing margin of either side of the interacting strains, the crosses were considered positive. As a result of mating tests, the four expected classes (A_xB_y, A_xB_y, A_yB_x, and A_yB_y) were identified and one monokaryotic strain of each class was used as a tester representing the four incompatibility types to determine the mating types of the remaining monokaryons.

Nuclear Staining

Culture disks were fixed in a 95% ethanol for 10 min and then hydrolyzed in 1N HCl for 15 min at 60℃. Af-

Table 1. Morphological characteristics and yield of the parent and the mutant in *Pleurotus ostreatus* 'Miso' and 'Wonhyeong No. 1'

Strain	Shape of pileus	Color of pileus	Size of pileus (mm)	Length of stipe (mm)	Yield (g/850ml)
Miso	Convex-umbonate	White	28.3	53.6	104.3
Wonhyeong 1	Semispherical	Gray	27.6	52.1	97.6

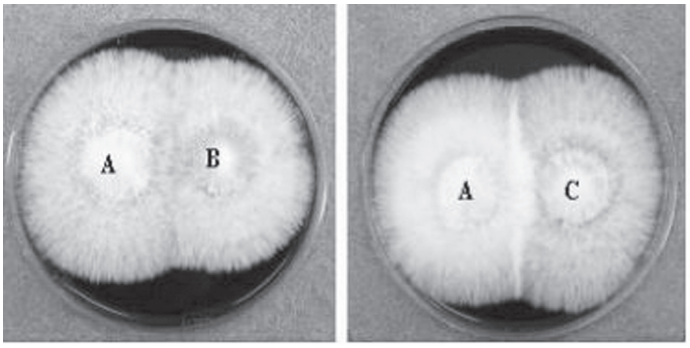

Fig. 1. Dual cultures of mycelium colony *Pleurotus ostreatus* 'Miso' (A), 'Wonhyeong 1' (B), and 'Suhan 1' (C).

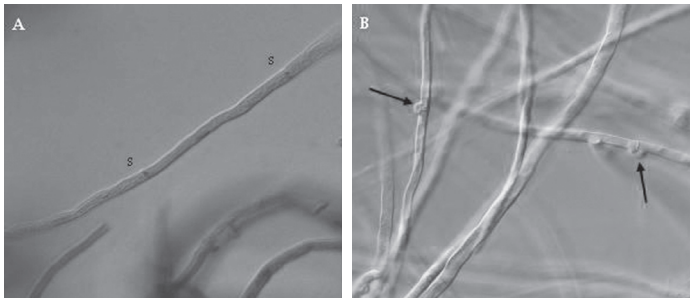

Fig. 2. DIC microscopy of monokaryotic mycelium without clamp connection (A) and dikaryotic mycelium having clamp connections (B) of *Pleurotus ostreatus*. Arrows point to clamp connections and the letter "s" marks septum.

ter hydrolysis, the disks were placed in three changes of distilled water and 95% ethanol alternatively, 5 min per change, and then disks were stained with 3~4 drops of Giemsa's stain solution (Azur-Eosin-Mehylene blue solution, Muto Pure Chemicals Co.) for 1 hour. Tissue carriers were then placed in 4~5㎖ water. Culture disks were transferred slides with a section lifter, and free liquid was absorbed with bibulous paper. Then the disks were mounted and examined microscopically.

RESULTS AND DISCUSSION

Pleurotus ostreatus 'Miso' is a mutant strain showing white color in pileus from the known parent strain 'Wonhyeong 1' with gray color in pileus. Shape of pileus is convex-umbonate in 'Miso' and semispherical in 'Wonhyeong 1'. Several other characters also vary slightly with culture conditions (Table 1), suggesting that their characteristics of morphological and yield traits are mutated considerably. However, the results of interaction of mycelia between 'Miso' and 'Wonhyeong 1' presented in Fig. 1 showed the absence of demarcation line compared with the interaction of mycelia between 'Miso' and 'Suhan 1'. The presence or absence of demarcation line in paired cultures frequently constitutes a reliable basis for distinguishing genetically distinct mycelia. The demarcation lines as well as line intensity depends upon genetic

Table 2. Characteristics of monokaryotic mycelial growth in *Pleurotus ostreatus* 'Wonhyeong 1' on PDA medium.

Monokaryotic mycelia	Mycelial growth (mm/day)	Mycelia density	Morphology of mycelial colony
1	4.9	+++[a]	P[b]
2	4.1	++	P
3	5.1	+++	F
4	2.7	+	P
5	4.6	++	F
6	5.2	++	Fl
7	4.3	++	P
8	5.0	++	Co
9	4.5	++	P
10	4.1	++	S
11	3.9	++	Fl
12	5.4	+++	P
13	6.1	+++	C
14	4.8	++	P
15	4.2	++	Fl
16	3.9	++	Fl
17	2.5	+	Co
18	5.7	+++	P
19	4.6	++	F
20	4.2	++	S
21	5.2	++	P
22	4.6	++	Fl
23	4.4	++	P

[a]+, very weak; ++, weak; +++, high; ++++, very high
[b]C, Cumulous; F, Feathery; P, Puffy; Co, Concentric; Fl, Fluffy; S, Streak

relationship of the paired cultures and is independent of colony appearance. Therefore, the absence of demarcation line in dual culture between 'Miso' and 'Wonhyeong' may be caused by their genetically close relationship. In the life cycle of *Pleurotus ostreatus*, single basidiospore germinates to produce monokaryotic mycelia. The monokaryon is self-sterile and capable of indefinite vegetative growth (Fig. 2A) but when it mates with compatible one, they produce dikaryotic mycelium, which has clamp connections (Fig. 2B). Two complex mating type factors control sexual compatibility in the monokaryons and regulate the maintenance of the dikaryotic state. The hyphae of dikaryons develop clamp connections at each septum, while the hyphae of monokaryons do not. The monokaryotic and dikaryotic mycelia are capable of indefinite growth, allowing for the maintenance and duplication of the genotype of each ploidy state. In monokaryotic mycelia they consist of uninucleate cells with genetically uniform haploid nuclei (Fig. 3A) and in dikaryotic mycelia they consists of binucleate cells, each of which contains the two haploid gametic nuclear types in paired association (Fig. 3B). Although the dikaryon resembles a diploid in that two haploid genomes reside in each cell with full opportunity for genetic complementation, the dikaryon differs from a diploid in that the two haploid genomes remain separated in different nuclei.

Monokaryons produced less dense and fluffy colony in aerial mycelia than that of dikaryons and colonies generally remained white on PDA plates. Monokaryotic mycelial growth of *Pleurotus ostreatus* 'Wonhyeong 1' was from 2.5 to 6.1 mm/day (Table 2). Mycelia density varied from very weak to high. In general, the component

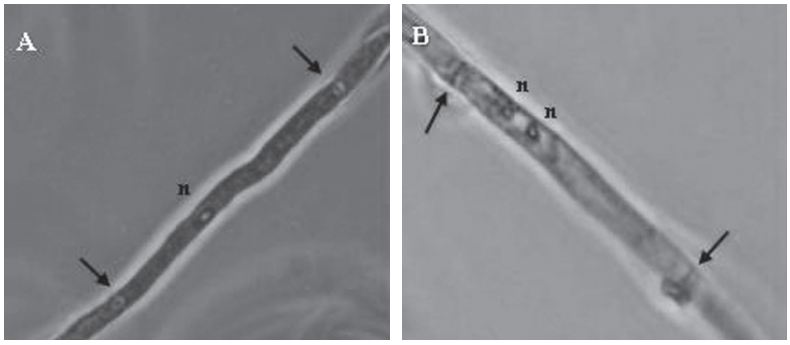

Fig. 3. Monokaryotic hypha with one nucleus (A) and dikaryotic hypha with two nuclei (B) stained by HCl-Giemsa. Arrows point to septaand the letter "n" marks nucleus.

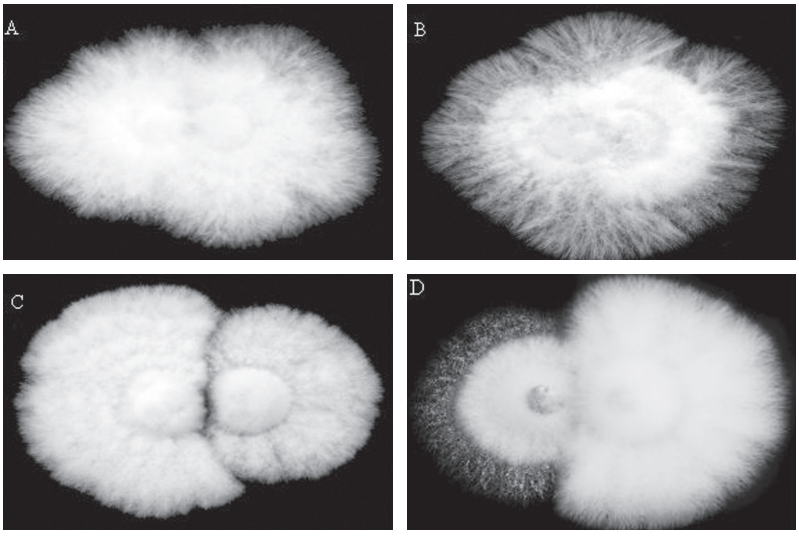

Fig. 4. Representative mating interactions of *Pleurotus ostreatus* 'Wonhyeng 1' in four types of matings between progeny from a fruitbody. A, Identical A and B factors (A=B=); B, different A and B factors resulting in fertile dikaryon mycelium (A≠B≠); C, Common B (A≠B=); D, Common A (A=B≠).

monokaryotic mycelial growth and density were slower and weaker compared to those of parental dikaryon. Similar relationship has also been reported by others (Eillott, 1993; Gharehaghaji *et al.*, 2007). The morphology of mycelial colony was fluffy in parental dikaryon. However, colony morphology of monokaryons was varied fluffy, concentric, puffy, feathery, streak to cumulous. Interestingly, the crosses between fluffy monokaryons were incompatible and the fluffy mycelium was not considered as a desirable monokaryon for breeding program (Kligman, 1943; Gharehaghaji *et a*l., 2007). Therefore, the morphological characteristics may affect the compatibility between monokaryons. However, this experiment did not show that relationship.

Matings were made by placing two small agar blocks containing monokaryotic mycelia approximately 1 cm apart on agar plates. In those mating experiments, two monokaryons grew into contact and fusions occurred between the hyhae. The mating type of each strain can be determined by the four basic reactions between different A and B factors. As a result, four types of typical morphology could be observed after 7~14 days of cultivation, showing compatible ($A_xB_x \times A_yB_y$ or $A_xB_y \times A_yB_x$), common A ($A_xB_x \times A_xB_y$ or $A_yB_y \times A_yB_x$), common B ($A_xB_x \times A_yB_x$ or $A_xB_y \times A_yB_y$), and common AB ($A_xB_x \times A_xB_x$ or $A_yB_y \times A_yB_y$) reactions (Fig. 4). Compatible matings resulting dikaryons could be recognized macroscopically by the rapid growth and its gross morphology to some extent. They

Table 3. Intrastrain matings of 16 progeny of *Pleurotus ostreatus* 'Wonhyeng No. 1'.

Mating types of monokaryons		I			II			III				IV					
		12	13	15	5	8	9	1	10	14	16	2	3	4	6	7	11
I	12	–[a]	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
	13	–	–	–	–	–	–	–	–	–	–	+	+	+	+	$+_1$	+
	15	–	–	–	–	–	–	–	–	–	(+)	+	+	+	+	+	+
II	5	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–
	8	–	–	–	–	–	–	$+_1$	+	+	+	–	(+)	–	–	–	–
	9	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–
III	1	–	–	–	+	+1	+	–	–	–	–	–	–	–	–	–	–
	10	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–
	14	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–
	16	–	–	(+)	+	+	+	–	–	–	–	–	–	–	–	–	–
IV	2	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	3	+	+	+	–	(+)	–	–	–	–	–	–	–	–	–	–	–
	4	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	6	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	7	+	+1	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	11	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]+, clamp connections formed at the contact zone and both side of parental monokaryons; –, no clamp connections formed; (+), clamp connections formed only at the contact zone; $+^1$, clamp connections at the contact zone and one side of parental monokaryons.

Table 4. Intrastrain matings of 7 progeny of *Pleurotus ostreatus* 'Wonhyeng1' using tester strains.

Testers	A_xB_x	A_xB_y	A_yB_x	A_yBy	Mating types
	12	5	1	2	
17	+[a]	–	–	–	A_yB_y
18	–	–	+	–	A_xB_y
19	–	+	–	–	A_yBx
20	–	–	–	+	A_xBx
21	–	–	+	–	A_xB_y
22	–	–	+	–	A_xB_y
23	+	–	–	–	AyB_y

[a]+, clamp connections formed at the contact zone and both side of parental monokaryons; –, no clamp connections formed.

Table 5. Assignment of mating types to monokaryons of *Pleurotus ostreatus* 'Wonhyeng 1' based on intrastrain crosses.

Mating Group	Mating type	No of monokaryons
I	A_xB_x	12, 13, 15, 20
II	A_xB_y	5, 8, 9, 18, 21, 22
III	A_yB_x	1, 10, 14, 16, 19
IV	A_yB_y	2, 3, 4, 6, 7, 11, 17, 23

produced a significant amount of aerial mycelium that resulted in a puffy appearance of the colony. Common B mycelia developed a 'barrage' showing demarcation line at the contact zone. However, 'barrage' reaction was not reliable to distinguish common B matings from common A because sometimes such reaction was absent in common B and could be observed even in common A matings. False clamp connections also could or could not be observed at the contact zone depending on the mated monokaryon. The morphology of common A mycelia developed aerial hyphae in the central region of the colony but they were characterized by reduced aerial hyphae in the margin, showing depressed growth in general. The hyphae were gnarled and knobbed. The so called 'flat' reaction was reported in other species showing cytoplasmic extrusions, frequent hyphal fusion, variable numbers of nuclear per cell, and disrupted septa. In common A matings only the B-regulated developmental pathway is activated resulting nuclear migration without clamp connections (Casselton and Olesnicky, 1998). On the other hand, common AB matings with similar both A and B, showed no discernible appearance or overgrowth of the two monokaryons each other.

Intrastrain matings of monokaryons from the same progeny were carried out with 16 monokaryotic strains in all possible combinations. They showed that *Pleurotus ostreatus* 'Wonhyeong 1' has a tetrapolar mating system (Table 3). Compatible reactions were observed between two compatible monokaryons through hyphal fusion, resulting in the formation of clamp connections. The dikaryotic hyphae, bearing abundant clamp connections, arose only from areas where the parental monokaryons met at the contact zones and clamp connections were progressed to the rest part of the colonies as time goes on. These mating reactions were consistent in all the dikaryons tested but sometimes clamp connections could be found only at the margin of one side. This meant that dikaryotization could be unidirectional. Unidirectional matings were not unusual because it could be found in *Pleurotus ostreatus* and other fungi (Eger, 1978; Takenaru, 1961). *Clamp* connection was a useful indicator for identifying dikaryon formation. The nuclear division and septum formation after compatible mating are possible through clamp connections (Larsen *et al.*, 1992). When only the contact zone between two mycelia was examined for clamp connections, it could be misled between compatible and common B. Therefore, it was important to examine clamp connections at the contact zone and periphery of the paired colonies (Aschan, 1954; Terakawa, 1960). In the common B matings, examination of clamp connections is laborious, and may not practical for the large samples. Nevertheless, when identifying clamp connections only at the contact zone gave important information, that is, it is the one of the evidence for detecting the common B mating.

After finding four different mating types of monokaryons, one strain from each of the four reaction groups was then chosen as the fur testers for mating type analysis. They represented the four different mating types and mated with a number of additional monokaryons. Using the four testers, it was possible to determine the mating types of rest monokaryotic strains from 17 to 23 (Table 4). As a result of intrastrain mating tests with 23 monokaryons in this study, four mating types were confirmed within the same progeny in oyster mushroom 'Wonhyeong 1' (Table 5).

To understand mating relationship between the parent strain 'Wonhyeong 1' and its mutant strain 'Miso', interstrain crosses were made with respective monokaryotic tester strains. For 'Wonhyeong 1', tester strains were

Table 6. Interstrain matings of tester strains between 'Miso' and 'Wonhyeng No. 1'.

4 incompatibility groups of 'Wonhyeong No. 1'		Testers of 'Miso'				Mating types
		A^1B^1	A^1B^2	A^2B^1	A^2B^2	
		1	6	10	8	
I	12	–[a]	–	–	+	A^1B^1
II	5	–	–	+	–	A^1B^2
III	1	–	+	–	–	A^2B^1
IV	2	+	–	–	–	A^2B^2

selected as follows: group I = monokaryon 12; group II = monokaryon 5; group III = monokaryon 1; group IV = monokaryon 2. For 'Miso', tester strains were used as follows: A_1B_1 = monokaryon 1, A_1B_2 = monokaryon 6, A_2B_1 = monokaryon 10, and A_2B_2 = monokaryon 8, which were identified in the other mating study (Lee et al., 2009). In interstrain crosses, each monokaryotic tester strain of the parent strain was out-crossed to monokaryotic tester strains of the mutant. As a result of these crosses it was found that both strains share the same A and B incompatibility factors yielding 25% compatibility (Table 6). These results indicate that the parent strain 'Wonhyeong 1' and its mutant 'Miso' have same mating type genes although they show different color in pileus and several other characteristics.

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