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Studies on the Inheritance of fruitbody color in *Flammulina velutipes*

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팽이버섯 자실체 색택의 유전연구

변명옥* · 공원식 · 김영호 · 유창현 · 차동열 · 이두형[1]

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ABSTRACT: Esterase isozyme band patterns were compared between the wild strain and commercial strain of *Flammulina velutipes*. Monospores were isolated from wild strain. ASI4019 and their mating types were determined. We investigated the relationship between pigmentation on the plate and fruitbody color to understand genetic relationship among *F. velutipes* strains. Dikaryotic strains mated between nonpigmenting strains produced white fruitbodies. However dikaryon obtained from mating between nonpigmenting monokaryon and brown pigmenting monokaryon produced brown fruitbody as the dikaryon obtained from mating of brown pigmenting monokaryons. The white fruitbody from wild strain was distinguished from that of commercial strain. When the nonpigmenting wild monokaryon was mated with commercial monokaryon, pale brown mushroom was produced. The BC1F1 was obtained by mating the above mentioned F_1 with commercial monokaryon. Fruitbody color of BC1F1 shared two types; one strain with all pale brown fruitbodies, and the other strain with separated eight pale brown and two mixed type involving pale brown and white fruitbodies.

KEYWORDS: *Flammulina velutipes*, Esterase, Mating type, Pigmentation, Fruitbody color

Winter mushroom, *Flammulina velutipes*, has been grown commercially by mechanized bottle cultivation system under controlled environment condition since the late 1980s. Its production is being rapidly increased and estimated about 3,867 M/T followed by oyster mushroom, oak mushroom and button mushroom in Korea in 1995('95 Production of Industrial Crops, 1996).

F. velutipes belongs to white rot fungi and forms clusters on the dead trunks or stumps of broad-leaved trees such as mulberry, persimmon and poplar trees in early spring or late autumn, in Korea.

Fruitbody color of wild type shows brown cap and dark brown stipe in wild habitat. Illumination is one of the most important environmental factors that affect the fruitbody formation. Fruitbody turns dark brown with increased light intensity during fruitbody formation. Brown fruitbody was not popular in the market as consummers prefer white color. Therefore it is important to develop new variants producing white fruitbody under the dim light. However, basic studies on the inheritance of fruitbody color are lacking, al-

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though detailed information on these aspects is crucial to effective mushroom breeding. The objective of this study was to elucidate the genetic basis for color determination of fruitbody in *F. velutipes*.

Materials and method

Strains, growth conditions and mating type determination

Strains of *Flammulina velutipes* were collected from wild flora in Korea and commercial strain in Japan(Table 1). Cultures were grown on potato dextrose agar (PDA) or mushroom complete medium at 25°C. Basidiospores were obtained from fruitbodies. Single spore isolates were obtained from spore prints by serial dilution of basidiospores in distilled water. Spore suspension was plated in petri dishes containing approximately 30 ml potato dextrose agar (PDA) and incubated for 3-6 days at 25-28°C. Single colony was transferred to new PDA plate and confirmed as monokaryon by observation of hyphae lacking clamp connection under the microscope.

Mating types of the monosporus isolates were identified by mating between them in all possible combinations, or by mating them with appropriate tester strains of known mating types. When two monokaryons were combined as a pair, each inoculum was placed about 10 mm apart from its neighbor. After 7-12 days incubation at 25°C, the appearance of clamp connections in the mycelium taken from the contact zone between two paired mycelia and from either side of the zone was examined to score whether monokaryotic or dikaryotic or heterokaryotic. A mycelium was regarded as monokaryotic when its hyphae had simple septa, and as dikaryotic or heterokaryotic when clamp connections were observed.

Electrophoresis

Each Strain was grown in 250 ml Erlenmyer flask containing 50 ml of potato dextrose medium for two weeks at 25°C. Mycelia were harvested by filtration through Whatman 3MM filter paper and ground in liquid nitrogen. Sample solution was centrifuged at 12000 rpm for 30 min and the supernatant was loaded into the sample wells of gel.

Polyacrylamide gel electrophoresis (PAGE) was conducted on 14×16 cm vertical slab gels according to manufacturer's procedures (Hoefer). Acrylamide and bisacrylamide concentrations were adjusted 10% T and 5% C in separating gel, and 3.125% T and 20% C in stacking gel. The discontinuous Tris glycine HCl buffer was used.

Electrophoresis was performed at 4 C at a current of 80 V (20 mA) until the sample had passed through the stacking gel and then at

Table 1. *Flammulina velutipes* used in this experiment

Strains	Collection			Remark
	Origin	Habitat	Year	
ASl 4006	Koea	–	1976	Brown type
ASl 4016	Opo, Kyunggi, Korea	Persimmon tree	1982	Brown type
ASl 4017	Opo, Kyunggi, Korea	Mulberry tree	1982	Brown type
ASl 4019	Pusan, Korea	Poplar tree	1992	Brown type
ASl 4021	Japan	–	1984	Commercial strain, white type
ASl 4038	Japan	–	1990	Commercial strain, novel white type
ASl 4040	Japan	–	–	Commercial strain, novel white type
ASl 4041	Japan	–	–	Commercial strain, novel white type
ASl 4045	Japan	–	–	Commercial strain, novel white type

400 V (50 mA) until the tracking dye had traveled before 1 cm from the bottom line.

Isoelectric focusing (PAGIF) was carried out on horizontal slab gel (7 cm × 22 cm × 0.75 cm) by the modification of Stegemann *et al* (1985). Gel mixture contained 6% polyacrylamide and pharmalyte pH 4- 6.5 as carrier. Electrode buffers were as follows: cathode - saturated $Ca(OH)_2$ solution; anode - 0.1 mole phosphoric acid. Electrofocusing was performd at 100 V for an hour. and then continued for 3 hours at 200-500 V.

Following electrophoresis, gels were immediately stained for esterases in 100 ml 0.2 M Tris HCl buffer (pH 7.2) containing 20 mg α-naphtylacetate, 2 ml ethylene glycol monomethyl ether, 20 mg fast blue RR salt under the dark condition for 30 min. at 37°C.

Fruitbody formation

Dikaryotic strains obtained by mating between compatible monokaryons were grown on PDA, and then the mycelia were transferred to sawdust medium. Cultivation process of the mated dikaryon strain was shown as Fig. 1.

Results and discussion

Cultures of *Flammulina velutipes* collected from wild flora and commercial strain showed two types. Wild strains pigmented brown color of media on agar plate such as panel B of 4019-6 in Fig. 3 and produced brown fruitbody. On the other hand, commercial strain did not pigment and showed white color of media on PDA plate and produced novel white fruitbody like fruitbody of 4031 strain in Fig. 4.

Among commercial strains, ASI 4021 showed brownish stipe toward base in fruitbody in growing room with the dim light. In ASI 4038 stipes were adhered together at the base, whereby in ASI 4021, 4040, 4041, and 4045 stipes were separated at the base as well as the upper part. Therefore strain ASI 4021 and 4038 are distinguished from three other commercial strains by the morphology of fruitbody. By esterase isozyme band pattern, four wild strains showed different band pattern but the commercial strains showed similar band pattern. Therefore wild strains may be distinguished by esterase band pattern even if those strains are not identified by the morphology of the mycelia or fruitbody. It was difficult to identify the commercial strains by native gel electrophoresis. However, fifteen different bands were identified for the strains studied in IEF. The strains classified

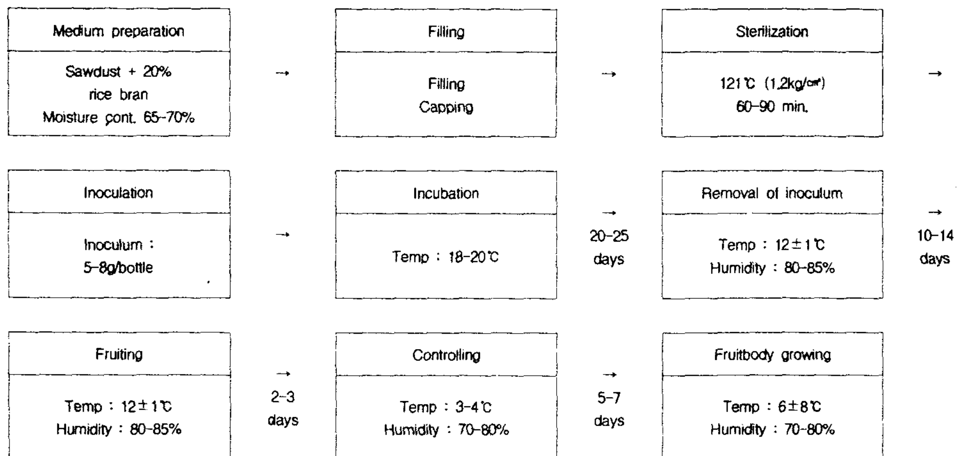


Fig. 1. Cultivation process of winter mushroom *Flammulina velutipes*.

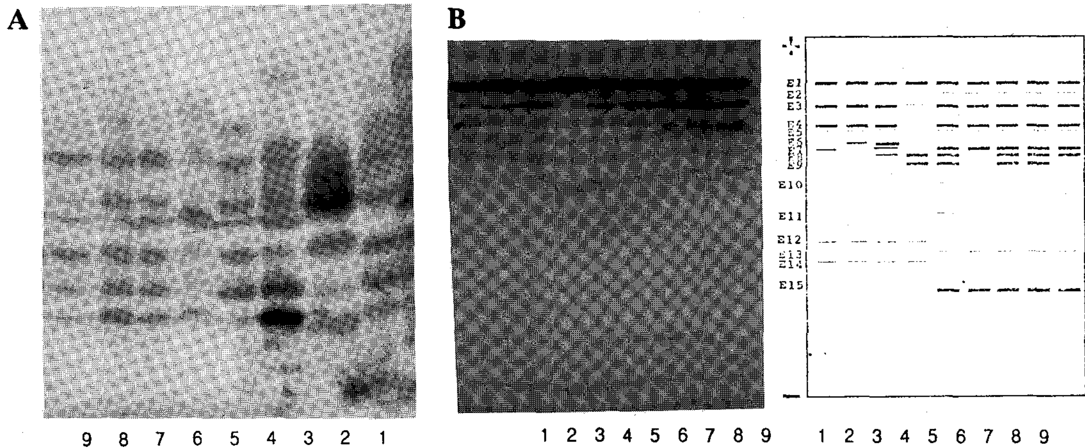


Fig. 2. Esterase isozyme band pattern of *Flammulina. velutipes* wild strains and commercial strains.
A: Slab gel electrophoresis B: Isoelectric focusing
1: ASl 4006 2: ASl 4016 3: ASl 4017 4: ASl 4019
5: ASl 4021(commercial strain; winter mushroom #1)
6: ASl 4038(commercial strain) 7: ASl 4040(commercial strain)
8: ASl 4041(commercial strain) 9: ASl 4045(commercial strain)

into two groups; one group is commercial strain group with E2, E13 and E15 bands, the other group is wild strain group with E12 and E14 bands. Commercial strains could be distributed into four types. No differences between #4040 and 4041 were observed. All wild type strains showed characteristically different banding patterns at the acidic end of the focusing gel (Fig 2).

Ten monokaryons of ASI 4019 were mated each other in all possible combinations. They were sorted into four mating types, three A 3B3, three A4B4, three A4B3 and one A3B4 on the basis of the clamp-connections. As all four types were compatible with tester strain S2(A1B1), S5(A2B2), S8(A1B2), and S1(A2B1) (Masuda,1995), they were designated to incompatible factor A3B3, A4B4, A3B4, and A 4B3. The results of the matings are represented in Table 2, in which plus signs(+)indicate the presence of clamp connections, which mean compatible pairs and a minus sign(-) their absence. A factor controls nuclear pairing and clamp formation. B factor controls nuclear migration and clamp fusion. Therefore, a difference of alleles at both factors (A≠B≠ e.g. A3B3×A4B4) is fully compatible and results in the formation of truly clamped dikaryon indicated with + in Table 2. The clamp of A≠B= heterokaryon (e.g. A3B3×A 4B3), because of the lack of nuclear migration, is restricted to contact jone of mated mycelia and it was designated as (+). A=B≠ heterokaryon lacking clamp and involving nuclear migration results in the formation of infertile heterokaryon(Raper, 1978) In Basidiomycetes, there are two basic breeding systems referred to bipolar and tetrapolar. In the bipolar system, there is a single mating type gene A with multiple alleles. Two different alleles (A1A2) give compatibility. From a single fruitbody the progenies fall into two cross-compatible groups, hence there is 50% compatibility on selfing. In tetrapolar system, there are two genes, A and B, with multiple alleles. A and B are in different chromosome and assort independently at meiosis. Therefore four mating types are derived from a sin-

Table 2. Mating between monokaryons from *Flammulina. velutipes ASl 4019*

Mating type		A_3B_3			A_4B_4			A_4B_3			A_3B_4
		11	15	27	18	22	29	9	20	21	24
A_3B_3	11	−	−	−	+	+	+	−	−	−	−
	15	−	−	−	+	+	+	−	−	−	−
	27	−	−	−	+	+	+	−	−	−	−
A_4B_4	18	+	+	+	−	−	−	−	−	−	(+)
	29	+	+	+	−	−	−	−	−	−	(+)
	22	+	+	+	−	−	−	−	−	−	(+)
A_4B_3	9	−	−	−	−	−	−	−	−	−	+
	20	−	−	−	−	−	−	−	−	−	+
	21	−	−	−	−	−	−	−	−	−	+
A_3B_4	24	−	−	−	(+)	(+)	(+)	+	+	+	−

*− : No clamp on contact area, +: clamp

gle fruitbody. This allows inbreeding compatibility to be reduced to 25 %(Casselton 1985). As 25% pairs in tested ASI 4019 strains are showed compatible, It was concluded that the *F. velutipes* is heterothallic and tetrapolar like the results of Takemaru(1954, 1961) (Table 2).

When the monokaryon was grown on PDA plate, pigmentation on the medium was classified into 3 types, white, yellow and brown. Only white and brown type were shown in Fig. 3. The strain showing non- pigmentation on media produced white fruitbody in case of mating with white or yellow type, whereas it produced brown fruitbody in case of mated with brown type. However brown types produced only brown fruitbodies in all mating combinations such as white, yellow and brown types in Table 4. Some blanks mean that mating was not accomplished due to the incompatibility. Only compatible mating types produced fruitbody eventhough mating was tried among all monokaryon. The results of fruitbody color was similiar to the results of Kinugawa (1983) that dark color and pale color gave F1 progenies producing fruitbodies of intermediate color between two parents. Therefore he suggested the a major gene F and recessive allele of f of a commercial var-

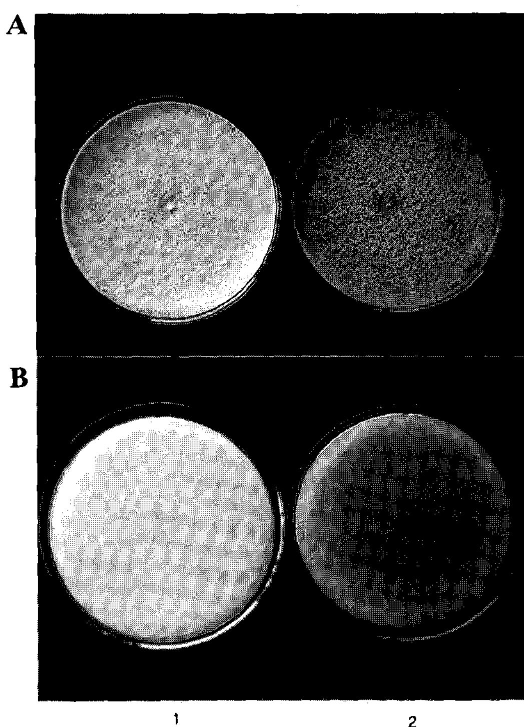


Fig. 3. White colored and brown colored of mycelial colonies obtained from monokaryon of wild mushroom ASl 4019.
A: Mycelial mat
B: Pigmentation color of medium
1: 4019-18(white colored)
2: 4019-6(brown colored).

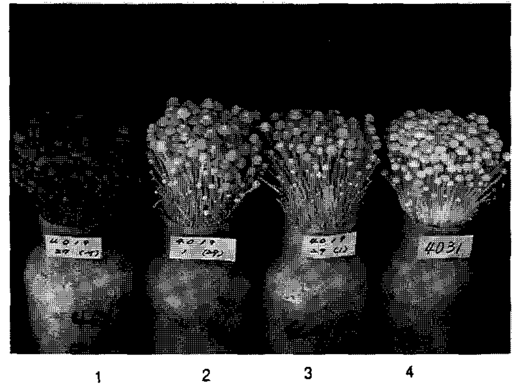
iety, Maruei. The genotype of f/F exhibited features which were intermediate between

the f/f and F/F. However, degree of brown color was variable in brown fruitbody in this experiment and it may suggest that several genetic factors are involved.

Brodie(1936) suggested that the inheritance of the brown color of the mycelium is determined by two pairs of factors (R, r, V and v) which are quite independent of the two pairs of sex factors. The presence of either of the two dominant factors R and V causes a mycelium to possess a brown coloration, but R gives a more intense color than V ; RV haplophytes are intense brown, Rv and rV haplophytes are light brown of different intensities, while rv haplophytes are pure white.

To determine the effect of cytoplasm on the fruitbody color, monokaryons were mated on PDA plates. Fukuda (1995) and Casselton (1985) identified the origin of cytoplasm during crossing by restriction fragment length polymorphisms (RFLPs), and mitochondrial gene and restriction pattern, respectively. Newly established dikaryons appeared bidirectionally at the periphery of the mated colonies after contact between paired monokaryons. Sample plugs with the dikaryon were removed from peripheral points on both sides of mating colonies(Kinugawa, 1974). The dikaryon of one side was designated for as 1(× 29) in Table 3. This formula indicates that the dikaryon possesses the acceptor nucleus #1, the donor nucleus #29, and cytoplasm #1. The culture of 29(×1) (The reciprocal dikaryon of 1(×29)) was obtained. These dikaryons are characterized by the identical nuclear components(#1 and #29) and different cytoplasm(#1 or #29) (Fig 4).

However, the cytoplasms of the dikaryons produced in this experiment must strictly be identical, because all the homokaryotic stocks are originated from a single fruitbody. Reciprocal dikaryons were compared with each other on the fruitbody color. All of the dikaryons examined were found that fruitbody color has a tendency to follow their nucleus, showing no cytoplasmic effect on the fruitbody color(Table 3).



1 2 3 4

Fig. 4. Color and shape of fruitbidies obtained from mating between monokaryons in *Flammulina. velutipes* ASl 4019.
1: Brown type(dikaryon mated between white monokaryon and brown monokaryon)
2, 3: White type(dikaryon mated between white monokaryons)
4: Commercial strain; ASI 4031

Table 3. Fruitbody color of reciprocal dikaryons brown pigmenting monokaryon and non pigmenting monokaryon on PDA

Group	Mated monokaryons	Color of fruitbody*
Brown×Brown	B3 (×B27*)	B
	B27 (×B 3)	B
	B13 (×B 3)	B
	B3 (×B13)	B
Brown×White	W17 (×B27)	B
	B27 (×W17)	B
	W18 (×B13)	B
	B13 (×W18)	B
White×White	W1 (×W29)	W
	W29 (×W1)	W
	W15 (×W17)	W
	W17 (×W15)	W

*Pigmentation of medium: B; brown, W; none
**Color of Fruitbody: W; white, B; brown

White fruitbody produced from mating of wild type monokaryons showed a little bit

Table 4. Fruitbody color of dikaryon mated between monokaryon showing different pigmentation on the media

Pigmentation on the medium		White							Yellow		Brown		
		1	5	10	17	18	24	29	2	15	3	13	27
White	1							W			B		
	5			W			W						
	10						W						
	17									W		B	B
	18												
	24											B	B
	29												
Yellow	2										B		
	15											B	
Brown	3												B
	13												
	27												

*Fruitbody color: W; White, B; Brown

Table 5. Backcross between wild strain and commercial strain

BC1F1 #18 (A×B)×A	Yield (g/bottle)	Fruitbody Color	BC1F1 #29 (A×C)×A	Yield (g/bottle)	Fruitbody color
AB1	81.7	pale brown	AC1	57.3	pale brown
AB2	84.3	pale brown	AC2	44.0	white and pale brown
AB3	64.0	pale brown	AC3	49.7	pale brown
AB4	74.0	pale brown	AC4	77.1	pale brown
AB5	94.4	pale brown	AC5	63.7	pale brown
AB6	70.4	pale brown	AC6	85.1	white and pale brown
AB7	84.7	pale brown	AC7	65.7	pale brown
AB8	37.0	pale brown	AC8	63.8	pale brown
AB9	63.7	pale brown	AC9	50.3	pale brown
AB10	65.3	pale brown	AC10	77.1	pale brown
A×B(F1; control)	51.9	pale brown	A×C(F1; control)	73.3	pale brown
Commercial strain(control)	-**	white	Commercial strain(control)	-**	white

*(A×B)×A: (4045-9×4019-18)×4045-9
(A×C)×A: (4045-9×4019-29)×4045-9
**Commercial strain was infected by *Cladobotryum* sp.

grayish color and its fruitbody type was flat pilus and short stipe. Commercial white fruitbody showed ivory white color and its feature is convex pileus with inrolled margin when young. It's stipe was broad and long.

Therefore intermating between white fruitbody from wild type and commercial strain was made in order to investigate the genetic relationship between monokaryon with white color of commercial strain and wild strain which is non-pigmenting white monokaryon, #18 or #29 of wild type was mated with a monokaryon #9 of commercial strain. Fruitbody color of both two F1 showed pale brown (Table 5). Spores of F1 were collected and monokaryons were germinated from the spore suspension. Ten monokaryons of each F1 were mated with #9 of commercial strain

for making BC1F1. F1 produced pale brown fruitbody in two combination but backcross of F1 with #9 of commercial strain produced different phenomenon. BC1F1 of #18 was produced one type of pale brown fruitbody but BC1F1 of #29 produced two types; one is only pale brown fruitbody and the other is mixed fruitbody containing pale brown fruitbody and white fruitbody. In that case, white fruitbody was similar to the color of hybrid between white monokaryons from wild type. As the commercial strain producing white fruitbody was susceptible to pathogenic fungus *Cladobotryum* sp., we could not obtain any fruitbody of ASI 4031 in that experiment(Table5).

White variety of commercial strain in Japan is originated by recessive mutation involving simultaneous inactivation of a series of complementary genes. Therefore, color of mycelium and fruitbody was controlled by one single major gene with unlinked multiple genes having additive effects (Kinugawa, 1983). Genetic analysis suggests that there are several nuclear genes that control the color of mycelia and fruitbody . Among the color related genes, the gene on white color seems to be inherited as recessive factor and the gene on brown color is dominant. However, further genetic analysis is required for the determination of fruitbody color relationship between commercial strain and wild type strain.

적 요

팽이버섯 야생종과 재배종들의 esterase isozyme 밴드패턴을 비교하고 그 중 특정 야생종 ASI 4019균을 단포자 분리후 교배형을 결정하였다. 단포자의 PDA plate상에 색소 집적과 자실체 형성관계를 비교한 결과, 갈색 색소를 집적 시킨 단포자와 색소를 집적시키지 않은 단포자간 교배시 갈색 자실체를 형성하였으며 색소를 집적 시키지 않은 백색 단포자간 교배한 결과 백색 자실체를 형성하였다. 백색 자실체는 재배종의 백색과 색택이 다르게 나타났다. 백색 균사체를 나타내는 단포자와 재배종 단포자를 교배한 결과 연갈색 자실체가 나타났으며 이 F1의 단포자를 재배종 단포자와 여교배하여 BC1F1을 비교한 결과 1계통은 연갈색 자실체만 형성하고 또 다른 한계통은 연갈색 자실체 8개, 연갈색과 백색 자실체가 혼합된 것이 2개 있었다.

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