Mycobiology 33(3): 125-130 (2005)
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Genetic Analysis of Pigmentation in *Cordyceps militaris*

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(*Received March 9, 2005*)

Pigmentation of ascospore-derived isolates from seven different natural specimens of *Cordyceps militaris* EFCC C-5888, EFCC C-7159, EFCC C-7833, EFCC C-7991, EFCC C-8021, EFCC C-8023 and EFCC C-8179 was observed on the plates of Sabouraud Dextrose agar plus Yeast Extract at 25°C under continuous illumination (500 lux). Pigmentation of the wild-type isolates of *C. militaris* was diverse ranging from yellowish white to orange, while white color was believed as a mutant. Inheritance of pigmentation was found to be controlled by both parental isolates when F1 progeny were analyzed. Pigmentation and mating type were shown to be either independent or distantly linked each other due to the high percentage of non-parental phenotypes among F1 progeny. Crosses between white mutant isolates of *C. militaris* yielded progeny with wild type pigmentations, indicating that the albino mutations in the parents were unlinked to each other.

KEYWORDS: Ascospore progenies, Complementary effects, Linkage, Pigmentation

Fungi produce different types of pigments such as orange, yellow, black, green, blue, etc. In case of *Cordyceps militaris*, stromata produce orange to bright orange colors (Kobayasi, 1941; Mains, 1958; Seaver, 1911; Sung, 1996). However, *in vitro* culture of ascospore-derived isolates of *C. militaris* shows different types of pigmentation such as deep orange or yellow depending upon media and culture age (Brown and Smith, 1957; Pettit, 1895; Shanor, 1936; Sung, 1996; Wang, 1989). The pigmentation of fungal colonies has used as a genetic marker for identification and isolation of a large number of mutants (Fincham *et al.*, 1979). Good examples are provided by the albino mutants of *Neurospora crassa* in contrast to the normal orange, and the white and yellow mutants of *Aspergillus nidulans* in contrast to dark green of the wild types. However, there has not been any study on pigmentation of *C. militaris* isolates and their mutant phenotypes. Moreover, due to recent study showing heterothallism in *C. militaris* (Shrestha *et al.*, 2004a), genetic analysis of pigmentation of isolates is possible in this fungus by sexual crosses. The objectives of this study are i) to determine pigmentation types of *C. militaris* isolates, ii) to determine whether pigmentation of *C. militaris* is controlled by a single genetic locus, iii) to characterize albino mutations in *C. militaris* and iv) to determine the genetic linkage between pigmentation and mating type in *C. militaris*.

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Materials and Methods

Wild-type pigmentation of *Cordyceps militaris* through single ascospore isolation. *Cordyceps militaris* specimens EFCC C-5888, EFCC C-7159, EFCC C-7833, EFCC C-7991, EFCC C-8021, EFCC C-8023 and EFCC C-8179, preserved in Entomopathogenic Fungal Culture Collection (EFCC), Kangwon National University, Korea, were collected from different parts of Korea (Table 1). A large number of single ascospores were isolated from those wild specimens of *C. militaris* and grown on the plates of nutritionally rich Sabouraud Dextrose agar plus Yeast Extract (SDAY) (dextrose 40 g, peptone 10 g, yeast extract 10 g, agar 15 g per 1000 *ml*; pH 5.6) to observe their pigmentation. For single ascospore isolation, freshly collected wild specimens were attached to the inner side of the lid of Petri dish containing 2% water agar (WA) and regularly observed for the discharge of ascospores over WA surface. Precaution was taken not to make the density of ascospore discharged on WA surface high in order to isolate as many single ascospores as possible. High density of ascospores hinders the isolation of single ascospores. Single ascospores, after isolation from the WA surface through a Zeiss dissecting microscope Stemi SV11 using a sterile insect pin, were inoculated onto SDAY plates and incubated at 25°C under continuous white fluorescent light (500 lux). Pigmentation of the ascospore-derived isolates was observed after three weeks of myce-

Table 1. *Cordyceps militaris* specimens used in this study

Specimen[a]	Site of collection	Date of collection	Host
EFCC C-5888	Yangyang-Gun, Kangwon-Do	August 2000	*Lepidopterous* pupae
EFCC C-7159	Mai Mt., Jeonla-Do	July 2001	*Lepidopterous* pupae
EFCC C-7833	Hanla Mt., Jeju-Do	August 2001	*Lepidopterous* pupae
EFCC C-7991	Gyaeryong Mt., Chungcheong-Do	August 2001	*Lepidopterous* pupae
EFCC C-8021	Sogri Mt., Chungcheong-Do	August 2001	*Lepidopterous* pupae
EFCC C-8023	Sogri Mt. Chungcheong-Do	August 2001	*Lepidopterous* pupae
EFCC C-8179	Seolag Mt., Kangwon-Do	August 2001	*Lepidopterous* pupae

[a]EFCC, Entomopathogenic Fungal Culture Collection, Kangwon National University, Chuncheon 200-701, Korea.

Table 2. Pigmentation of ascospore-derived isolates of different *Cordyceps militaris* specimens

Pigmentation[a]	EFCC specimens						
	C-8179	C-7991	C-8023	C-5888	C-8021	C-7159	C-7833
Yellowish white	39 (78%)	12 (24%)	1 (1.7%)	1 (5.6%)	18 (28.1%)	19 (38%)	
Pale yellow	10 (20%)	33 (66%)	33 (55%)	5 (27.8%)	19 (29.7%)	17 (34%)	2 (9.5%)
Light yellow		3 (6%)	23 (38.3%)	11 (61.1%)	11 (17.2%)	1 (2%)	
Orange white			1 (1.7%)		5 (7.8%)	1 (2%)	2 (9.5%)
Pale orange		1 (2%)			9 (14.1%)	7 (14%)	13 (61.9%)
Light orange		1 (2%)	2 (3.4%)	1 (5.6%)	2 (3.1%)	4 (8%)	4 (19.1%)
Orange						1 (2%)	
White	1 (2%)						
Total	50[b]	50	60	18	64	50	21

[a]Pigmentation descriptions based on Kornerup and Wanscher (1978): yellowish white (2A2, 3A2), pale yellow (2A3, 3A3), light yellow (3A4, 5), orange white (5A2), pale orange (5A3), light orange (5A4, 5), orange (5A6,7) and white (1A1).
[b]Total number of single ascospore isolates observed.

lial growth and identified based on Kornerup and Wanscher (1978). The ratio of each pigmentation type was counted among the isolates (Table 2).

Sexual crosses among single ascospore isolates. Single ascospore isolates obtained from specimens EFCC C-7159 and EFCC C-8179 were previously determined for their mating type (Shrestha *et al.*, 2004a; Table 3). The isolates differed on types of pigmentation on SDAY plate as shown in Table 3. Isolates showing similar pigmentations such as EFCC C-7159-21 and EFCC C-7159-43, and EFCC C-7159-2 and EFCC C-7159-17 were crossed in brown rice medium to produce *in vitro* perithecial stromata. Similarly, isolates showing contrasting pigmentations such as EFCC C-7159-5 and EFCC C-7159-33, EFCC C-7159-17 and EFCC C-7159-21, and EFCC C-7159-41 and EFCC C-8179-1-1-7 were also crossed for the production of *in vitro* perithecial stromata. Liquid inoculum of each isolate was first prepared by inoculating mycelial agar discs (4 mm in diameter) in SDAY broth and culturing for 4~5 days at room temperature with occasional manual shaking. Fruiting medium for stromata production was prepared by mixing 60 g of brown rice, 10 g of silkworm pupae and 90 *ml* of distilled water in 1000 *ml* Polypropylene (PP) mushroom fruiting bottle, which was sterilized at 121°C for 20 min, as previously reported (Shrestha *et al.*, 2004b; Sung *et al.*, 2002). For sexual cross, liquid inocula of both isolates of each cross (about 10 *ml* of each isolate) were simultaneously inoculated in bottle containing brown rice medium. After inoculation, bottles were incubated at 20±1°C under continuous light (500~1000 lux) and high humidity (70~90%) for 50~60 days until mature perithecial stromata developed. Previous study showed that no perithecial stromata developed when the isolates were inoculated in single without combination with the other isolate in fruiting medium (Shrestha *et al.*, 2004a). F1 progeny ascospores were isolated from mature *in vitro* perithecial stromata of all the crosses through a Zeiss dissecting microscope Stemi SV11 using a sterile insect pin as mentioned earlier and inoculated on SDAY plates. Pigmentation of F1 progeny isolates was observed after 3 wks of growth under continuous white fluorescent light (500 lux) and identified based on Kornerup and Wanscher (1978).

Crossing between white isolates. A large number of progeny ascospores, which were isolated from *in vitro* perithecial stromata of a self-fertile isolate EFCC C-8179-1, produced white color on SDAY plates (Table 3). Mating type of the white isolates was previously determined (Shrestha

Table 3. *Cordyceps militaris* isolates used in this study

Isolate No.	Pigmentation[a]	Mating type	Origin
EFCC C-7159-2	yellowish white (3A2)	B	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-7159-5	yellowish white (3A2)	A	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-7159-17	yellowish white (3A2)	A	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-7159-21	pale orange (5A3)	B	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-7159-33	orange (5A7)	B	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-7159-41	light orange (5A5)	A	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-7159-43	light orange (5A5)	A	Single ascospore isolate obtained from specimen EFCC C-7159
EFCC C-8179-1	pale yellow (3A3)	Self-fertile	Single ascospore isolate obtained from specimen EFCC C-8179
EFCC C-8179-1-1-7	white (1A1)	B	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-2-1	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-12	white (1A1)	B	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-19	white (1A1)	B	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-42	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-44	white (1A1)	B	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-47	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-50	white (1A1)	B	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-55	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-59	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-3-62	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-4-4	white (1A1)	B	Single ascospore progeny of isolate EFCC C-8179-1
EFCC C-8179-1-4-16	white (1A1)	A	Single ascospore progeny of isolate EFCC C-8179-1

[a]Pigmentation on SDAY plate, based on Kornerup and Wanscher (1978).

Table 4. Pigmentation of progeny isolates of crosses between white mutant isolates of EFCC C-8179-1

Pigmentation[a]	Crosses[b]							
	3-62 × 4-4	3-44 × 3-47	2-1 × 3-19	3-50 × 4-16	3-12 × 3-42	3-55 × 4-4	3-19 × 3-59	3-19 × 4-16
White	90 (42.1%)	42 (30.4%)	29 (27.6%)	18 (17.1%)	7 (16.3%)	3 (7.3%)		
Yellowish white	70 (32.7%)	20 (14.5%)	47 (44.8%)	38 (36.2%)	11 (25.6%)	12 (29.3%)	38 (32.8%)	20 (10.3%)
Pale yellow	24 (11.2%)	48 (34.8%)	24 (22.9%)	41 (39.1%)	12 (27.9%)	13 (31.7%)	57 (49.2%)	107 (54.9%)
Light yellow	15 (7.0%)	14 (10.2%)	5 (4.8%)	8 (7.6%)	8 (18.6%)	9 (22.0%)	20 (17.2%)	64 (32.8%)
Orange white	13 (6.1%)	4 (2.9%)			2 (4.7%)	3 (7.3%)		1 (0.5%)
Pale orange	2 (0.9%)	5 (3.6%)			1 (2.3%)	1 (2.4%)	1 (0.9%)	1 (0.5%)
Light orange		5 (3.6%)			2 (4.7%)			2 (1.0%)
Total	214[c]	138	105	105	43	41	116	195

[a]Pigmentation descriptions based on Kornerup and Wanscher (1978): yellowish white (2A2, 3A2), pale yellow (2A3, 3A3), light yellow (3A4, 5), orange white (5A2), pale orange (5A3), light orange (5A4, 5) and orange (5A6,7), white (1A1).
[b]Cross, for example, 3-62 × 4-4 denotes EFCC C-8179-1-3-62 × EFCC C-8179-1-4-4.
[c]Number of single ascospore isolates observed.

et al., 2004a; Table 3). Following crossings between white isolates EFCC C-8179-1-3-62 × EFCC C-8179-1-4-4, EFCC C-8179-1-3-44 × EFCC C-8179-1-3-47, EFCC C-8179-1-2-1 × EFCC C-8179-1-3-19, EFCC C-8179-1-3-50 × EFCC C-8179-1-4-16, EFCC C-8179-1-3-12 × EFCC C-8179-1-3-42, EFCC C-8179-1-3-55 × EFCC C-8179-1-4-4, EFCC C-8179-1-3-19 × EFCC C-8179-1-3-59, and EFCC C-8179-1-3-19 × EFCC C-8179-1-4-16 were made in brown rice medium as mentioned above and incubated for the production of *in vitro* perithecial stromata (Table 4). F1 progeny ascospores were isolated from mature *in vitro* perithecial stromata of all the crosses and inoculated on SDAY plates. Pigmentation of F1 progeny isolates was observed after three wks of growth under continuous white fluorescent light (500 lux) (Table 4) and identified based on Kornerup and Wanscher (1978).

Back-cross of F1 progeny with parental isolates. F1 progeny isolates from three crosses EFCC C-7159-5 × EFCC C-7159-33, EFCC C-7159-17 × EFCC C-7159-21 and EFCC C-7159-41 × EFCC C-8179-1-1-7 were back-crossed with their respective parental isolates and number of parental and non-parental types was counted. Liquid inocula of parental as well as progeny isolates were prepared in SDAY broth, as mentioned above. Progeny isolates were inoculated in brown rice medium in combination

with each parental isolate at a time and incubated at 20±1°C under continuous light (500~1000 lux) and high humidity (70~90%) for 50~60 days to observe *in vitro* perithecial stromata formation. Mating type of the progeny was determined by observing fertile perithecia formation on *in vitro* stromata when inoculated in combination with parental isolates.

Results and Discussion

Pigmentation of wild-type isolates of *Cordyceps militaris*. The number of each pigmentation type among single ascospore isolates from seven different wild specimens of *C. militaris* is given in Table 2. In this study, seven different types of pigmentation were identified in wild-type isolates of *C. militaris*, namely, yellowish white (2A2, 3A2), pale yellow (2A3, 3A3), light yellow (3A4, 5), orange white (5A2), pale orange (5A3), light orange (5A4, 5) and orange (5A6, 7), except only one white isolate (1A1), that was believed to be an albino mutant (Table 2). Thus, we report here that wild-type pigmentation of *C. militaris* isolates on SDAY plate under continuous illumination is wide and ranges from yellowish white to orange; white color being a color mutant. Among wild-type pigmentation, yellowish white to light yellow tended to be much more frequent than orange white to orange, except in case of EFCC C-7833 isolates that were mostly pale orange to light orange. Thus, the pigmentation of *C. militaris* isolates could be broadly divided into two types, yellow and orange, based on intensity and darkness, and are reported here as the two main types of pigmentation in wild-type *C. militaris* isolates.

Although pigmentation pattern is complicated, it has been used as a genetic marker in this study due to easy observation. Other characters of *C. militaris* isolates such as mycelial texture (cottony or non-cottony) or growth rate were not found to be suitable since most of the isolates were non-cottony and uniform in growth rate. The orange pigmentation of *C. militaris* has been reported to be due to the presence of carotenoid (Friederichsen and Engel, 1958). A number of fungal mutants affected in carotenoid biosynthesis have been isolated, with those of *Phycomyces blakesleeanus* being the most widely studied (Fraser *et al.*, 1996). The *P. blakesleeanus* mutants typically accumulate phytoene or lycopene, or have regulatory mutations which cause either the over-accumulation or negligible production of carotenoids. Variation of pigmentation in *C. militaris* isolates could be due to low or over-production of carotenoid.

Genetic analysis of pigmentation. In the sexual cross between two orange-colored isolates EFCC C-7159-43 × EFCC C-7159-21, 93.64% of progeny were orange white to orange; the rest produced yellowish white to light yellow pigmentation. It showed that orange color is controlled by a single genetic locus or closely linked loci and does not complement with each other to produce yellow color in progeny. However, the reason for the low percentage of yellowish white to light yellow progeny in the above cross could not be understood. None of one hundred six progeny from the cross between yellowish white isolates EFCC C-7159-2 × EFCC C-7159-17 showed orange white to orange colors; all were only yellowish white to light yellow, except two that were white. This result also showed that yellow color is controlled by a single genetic locus or closely linked loci and do not complement with each other to produce orange color. In another cross between isolates showing contrasting colors EFCC C-7159-5 × EFCC C-7159-33, forty four out of one hundred progeny were yellowish white to light yellow, similar to one of the parents (EFCC C-7159-5), whereas the color of the other fifty six ranged from orange white to orange, similar to another parent (EFCC C-7159-33), showing nearly equal ratio of yellow- and orange-like colors among the progeny. Out of one hundred twenty five progeny from second cross between isolates with contrasting pigmentations EFCC C-7159-17 × EFCC C-7159-21, seventy eight (62.4%) developed yellowish white to light yellow pigmentations (similar to the parental isolate EFCC C-7159-17), while the other forty seven (37.6%) produced orange white to light orange pigmentations (similar to another parental isolate EFCC C-7159-21), showing inheritance of both parental colors to the progeny. Based on the above results, it can be speculated that pigmentation in *C. militaris* isolates is controlled by single genetic locus or closely linked loci. It was further shown that neither yellow color complemented with each other to give orange color, nor vise versa, showing that yellow and orange colors are alleles of the same genetic locus or closely linked genetic loci. But, to the surprise of the above results, out of two hundred twenty five progenies from the cross of an isolate with light orange to a white mutant (EFCC C-7159-41 × EFCC C-8179-1-1-7), one hundred eighty four progenies (81.78%) developed orange white to orange pigmentations (similar to the parental isolate EFCC C-7159-41), thirty nine appeared from yellowish white to light yellow in pigmentation (intermediate colors, dissimilar to both parental isolates), only two (0.89%) were white (similar to another parental isolate EFCC C-8179-1-1-7). The low frequency of white progeny from the above cross is not consistent with the hypothesis that wild type color (light orange) is controlled by a single genetic locus. Expression of intermediate colors in the progeny of the above cross also showed that white mutant complemented with light orange color to produce other wild type colors.

Complementary effects of pigmentation character. To observe whether mating compatible white mutant isolates

could complement each other to produce progeny with wild type pigmentations, they were excessively crossed among themselves. In the cross EFCC C-8179-1-3-62 × EFCC C-8179-1-4-4, about 42% of the progeny remained white, the rest showing different wild-type colors (Table 4), indicating that pigmentation is a polygenic character, and is expressed through a large number of linkages. In other crosses EFCC C-8179-1-3-44 × EFCC C-8179-1-3-47 (30.4%), EFCC C-8179-1-2-1 × EFCC C-8179-1-3-19 (27.6%), EFCC C-8179-1-3-50 × EFCC C-8179-1-4-16 (17.1%), EFCC C-8179-1-3-12 × EFCC C-8179-1-3-42 (16.3%) and EFCC C-8179-1-3-55 × EFCC C-8179-1-4-4 (7.3%), less and less white mutants were produced in the progeny (Table 4). Still, in other crosses EFCC C-8179-1-3-19 × EFCC C-8179-1-3-59 and EFCC C-8179-1-3-19 × EFCC C-8179-1-4-16, no white mutant progeny was produced at all (Table 4).

In a cross between two mutants, wild recombinants can result from two types of events, via., crossing-over giving reciprocal recombinants, and conversion yielding non-reciprocal recombinants (Lissouba *et al.*, 1962). In *Neurospora*, pseudowild ascospores occur among the progeny of a cross between closely linked auxotrophic mutants. In *C. militaris*, low percentages of white mutant progeny could be due to either excessive crossing-over or conversion or both. Conversely, high occurrence of wild type pigmentation in progeny of white mutant parents could be due to polygenic nature of fungal pigmentation, causing high chance of complementation.

Linkage between pigmentation and mating type. Parental and non-parental types among F1 progeny were counted with regard to mating type and pigmentation by back-crossing progeny with both parents, following the method of Fincham *et al.* (1979). Progenies similar to one or the other parent in both mating type and pigmentation were counted as parental types, otherwise non-parental. Out of ninety five progeny from the cross EFCC C-7159-5 × EFCC C-7159-33, twenty eight and thirty two showed the parental types of EFCC C-7159-5 and EFCC C-7159-33, respectively, i.e., twenty eight progeny were same to EFCC C-7159-5 in both color and mating type, while thirty two were same to EFCC C-7159-33 in both color and mating type. The remaining of the progeny (36.84%) showed non-parental types, i.e., they were similar to parental isolates only in mating type or color, but not in both. In another cross EFCC C-7159-17 × EFCC C-7159-21, forty nine and twenty nine progenies showed parental phenotypes of EFCC C-7159-17 and EFCC C-7159-21, respectively, while thirty seven (32.17%) were of non-parental type. In another cross EFCC C-7159-41 × EFCC C-8179-1-1-7, twenty-three out of fifty two progeny showed parental type of EFCC C-7159-41, while none were parental type of the other parent EFCC C-8179-1-1-7. Twenty-nine progeny (55.77%) of this cross were non-parental type. High frequency of non-parental types in all the three crosses showed that mating type and pigmentation are not linked with each other.

In this paper, wild type color of *C. militaris* isolates and its white mutant was described. Yellow and orange colors were found as alleles of single genetic locus or closely linked genetic loci. However, the number of genetic locus controlling pigmentation in *C. militaris* could not be determined. The pigmentation in *C. militaris* was shown to be more complicated due to low frequency of white mutant progeny in the cross between a white mutant and a wild-type isolate. Polygenic nature of pigmentation in *C. militaris* could be further shown by high ratio of wild type progeny in the crosses between white mutant isolates. In *C. militaris*, pigmentation and mating type were found distantly linked from each other due to high frequency of non-parental types among the progeny. This was proved further by the successful production of perithecial stromata from crosses between white isolates suggesting that mating type was stable in white mutants also.

Acknowledgement

This work was financially supported by Strategic National R&D Program of Genetic Resources and Information Network Center of Ministry of Science and Technology and Regional Strategic Industry Program of Ministry of Commerce, Industry and Energy. Institute for Cordyceps Research of Kangwon National University is thanked for providing facilities to carry out this research

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