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Distribution and *in vitro* Fruiting of *Cordyceps militaris* in Korea

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Cordyceps militaris **specimens were continuously collected by Entomopathogenic Fungal Culture Collection (EFCC), Kangwon National University from different mountains, national parks and recreation parks of Korea from 1986 to 2002, mainly from late May to October of each year. Dry specimens of *C. militaris* along with their isolates have been preserved in EFCC. Fruiting of *C. militaris* was induced from single ascospore isolates as well as their combinations in brown rice medium. Fruiting experiments showed that combinations of single ascospore isolates produced fertile fruiting bodies, but single isolates could not produce any fruiting bodies. It was shown that two isolates of the opposite mating types were required to produce fertile stromata. However, combinations of the same mating type isolates produced no fruiting body, showing that *C. militaris* is a bipolar, heterothallic fungus.**

KEYWORDS: Artificial fruiting, *Cordyceps militaris*, Mating type, Entomopathogenic fungi

There are more than 1,200 entomopathogenic fungi in the world (Humber, 2000). Most of them are classified in the Ascomycota, including Deuteromycetes; the rest are in the Zygomycota, Chytridiomycota, Oomycota, and few in the Basidiomycota. *Cordyceps* (Clavicipitaceae, Hypocreales, Ascomycota) is one of the large entomopathogenic genera, comprising about 300~500 species and varieties (Kobayasi, 1982; Hodge *et al*., 1998; Hywel-Jones, 2002; Liu *et al*., 2002). Its members are mostly pathogenic to insects, including spiders, with few growing on hypogeal mushroom *Elaphomyces* species. *C. militaris* is the type species of *Cordyceps* (Seaver, 1911). It is widely distributed in North America, South America, Europe and Asia (Mains, 1958), from sub-tropical to temperate regions around the world. It mainly infects and grows upon lepidopteran larvae and pupae, but occasionally on coleopteran, hymenopteran and dipteran insects too. *Cordyceps* species have been used to cure diseases related to heart, liver, kidney, respiratory system, immune system, etc. (Jones, 1997; Halpern, 1999; McKenna *et al*., 2002). In Korea, *Cordyceps* species are well-known as a tonic for preserving health after sickness and long fatigue and as a secret medicine for eternal youth of life. Recent clinical studies of *C. militaris* have shown their high anticancer effects (Yoo *et al*., 2004).

Annual collections of *Cordyceps* species and other entomopathogenic fungi have been conducted in Korea by Entomopathogenic Fungal Culture Collection (EFCC, www.mushtech.org), Kangwon National University since 1984. *C. militaris* is one of the most commonly found *Cordyceps* species in Korea. *In vitro*-mycelial growth and fruiting of *Cordyceps* species have attracted interest of several mycologists and industrialists due to their medicinal values. Kobayasi (1941), among others, first mentioned artificial fruiting of *C. militaris* and Sung (1996) developed a simple liquid inoculum method for large scale fruiting of *C. militaris* in rice medium. Several *Cordyceps* species such as *C. militaris*, *C. pruinosa*, and *Paecilomyces* spp. are at present being cultivated in Korea for health food productions. Sung (1996) also reported variation of fruiting of *C. militaris* among different isolates and their subcultures *in vitro*. It was the main problem for its mass cultivation. Only recently, bipolar heterothallism has been shown in *C. militaris* (Sung and Shrestha, 2002; Shrestha *et al*., 2004). But, Sato and Shimazu (2002) showed that *C. militaris* behaves as a homothallic fungus. This present work intends to summarize the distribution of *C. militaris* in Korea and to fix up the artificial cultivation of *C. militaris*.

Materials and Methods

Collection of *C. militaris* specimens. Specimens of *C. militaris* were continuously collected from different parts of Korea from 1986 to 2002 and have been preserved in Entomopathogenic Fungal Culture Collection (EFCC), Kangwon National University (www.mushtech.org). From the initial years of 1986 to 1992, collections were done only in the Kangwon Province, where EFCC is located. Since 1993, collections were regularly made in other provinces as well. Specimens of *C. militaris* were identified following the keys of Kobayasi (1982) and Sung (1996). Soon after the collection, different macroscopic and microscopic characteristics were determined.

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Isolation of single ascospores. Single ascospores were isolated from three different specimens of *C. militaris* EFCC C-7833, EFCC C-8021 and EFCC C-8023. Specimen EFCC C-7833 was collected from Hanla Mt. of Jeju Province on August 1, 2001, whereas specimens EFCC C-8021 and EFCC C-8023 were collected from Sogri Mt. of Chungcheong Province on August 11, 2001. Fresh specimens of *C. militaris* were attached to the inner side of Petri-dish lid and left over 2% WA plate until ascospores were discharged in large numbers. The lid of Petri dish was moved every few minutes so that the concentration of discharged ascospores could be minimized. Single ascospores were isolated from the WA plate through the Zeiss dissecting microscope Stemi SV11 using a sterile insect pin and were inoculated on SDAY (dextrose 40 g, peptone 10 g, yeast extract 10 g and agar 15 g per 1000 *ml*) agar plates. Single ascospores were grown on SDAY agar for three to four weeks and used for artificial fruiting experiment of *C. militaris*.

***In vitro* fruiting.** Five single ascospore isolates of each specimen of *C. militaris* were used for artificial fruiting. Single ascospore isolates of specimens EFCC C-7833, EFCC C-8021 and EFCC C-8023 were numbered from C-7833-1~C-7833-5, C-8021-1~C-8021-5 and C-8023-1~C-8023-5, respectively. Each isolate was first individually grown in 100 *ml* of SDAY broth (SDAY without agar) in a shaker (120 rpm) for five days at 25°C by inoculating five pieces of mycelial discs (4 mm) of each isolate. Fruiting medium was prepared by mixing brown rice (60 g), silkworm pupae (10 g) and 90 *ml* of distilled water in 1000 *ml* polypropylene (PP) bottle and autoclaving for 20 min at 121°C. For artificial fruiting of *C. militaris*, broth cultures of all isolates were inoculated in PP bottles containing fruiting medium in single as well as in pair-wise combinations among isolates of the same specimen. For example, five isolates C-7833-1~C-7833-5 of specimen EFCC C-7833 were inoculated in single as well as in pair-wise combinations among themselves. Similarly, five isolates of each EFCC C-8021 and EFCC C-8023 specimens were inoculated in single and pair-wise combinations among themselves, but not between EFCC C-8021 and EFCC C-8023 isolates. The inoculated bottles were incubated at 20±1°C under high humidity (70~90%) and light condition (1000 lx) with occasional air circulation for 60 days. Artificial fruiting body production was observed regularly in all inoculated bottles. Production of fertile fruiting body was marked as (+) and no production of fruiting body was marked as (−).

Results

Morphological characteristics. The length of stromata of fresh specimens of *C. militaris* ranged between 35~60 mm. The apical fertile portion of stromata was 8~30 mm in length and 4~12 mm in width. Similarly, the stalk portion was 17~45 mm in length and 4~12 mm in width. The number of stromata per host varied from single to several. Its color varied from yellow, red to orange red and shape was club, clavate or cylindrical. The size of perithecium was 420~700 × 180~400 μm. Asci were narrowly cylindric in shape and 390~425 × 3~4 μm in size. Ascospores were filamentous, septate, 360~415 × 1 μm in size. The size range of part-spores was 2.5~3.7 × 1 μm.

Distribution. Table 1 shows the number of *C. militaris* specimens collected from different provinces of Korea from 1986 to 2002. More than half of the total specimens (56.0%) were collected from Kangwon Province alone. The highest number of specimens is mainly due to continuous collections from 1986 to 2002. From the initial years of 1986 to 1992, total numbers of specimens collected were very low compared to later years, although the number is fluctuating. The main reason for that might be due to the difficulty in searching for the appropriate habitat of *C. militaris*. The highest number of specimens was collected in 1999, after which the number was in the decreasing tendency. Second highest number of *C. militaris* specimens (22.5%) was collected from Jeju Province within two years of collections, in 2001 and 2002. Com-

Table 1. Number of *C. militaris* specimens collected from different provinces of Korea (1986 to 2002)

Province	Collection year																	Total	%
	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02		
Kangwon	4	5	1	4	9	24	14	39	25	15	5	13	43	324	97	14	9	645	56.0
Jeju									2			5				150	102	259	22.5
Gyeonggi								1		9	1	2	29	14	1	2	1	60	5.2
Gyeongbuk													2	8	43	6		59	5.1
Jeonbhuk									1							53		54	4.7
Chungbuk																26	6	32	2.8
Chungnam								8					6	1	7	5		27	2.3
Gyeongnam																16		16	1.4
Total	4	5	1	4	9	24	14	48	28	24	6	20	80	347	148	272	118	1152	100

Table 2. Number of *C. militaris* specimens collected from different mountains of Kangwon Province (1986 to 2002)

Site	Collection year																	Total	%
	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02		
Mt. Seolak					3	22		7		5			14	175	57	2	1	286	44.3
Mt. Chilsung													6	134	5			145	22.5
Mt. Chiag			1	1			3	14	7	6	2	3	1	4	21	5	4	72	11.2
Mt. Gujeol				1	1	1		14	9		1	2	7	9			1	46	7.1
Mt. Eungbong					3			2		2	2	3	9		12	6		39	6.1
Mt. Odae	1	1		1	1		1	2	2			3	6	2	1	1	2	24	3.7
Mt. Daeryong	3	2			1		8		3	1					1			19	3.0
Mt. Obong		2		1		1	2		4	1		2						13	2.0
Mt. Taegi																	1	1	0.2
Total	4	5	1	4	9	24	14	39	25	15	5	13	43	324	97	14	9	645	100

paratively, less number of *C. militaris* specimens were collected from Gyeonggi Province, the nearest province from Kangwon, although the collections were made continuously from 1993 to 2002, except in 1994. In other provinces, the numbers of *C. militaris* specimens collected were low, mainly because the collections were made only for few years (Table 1). In general, four years from 1999~2002 were very favorable for *C. militaris* collection, although the number varied from year to year and province to province. *C. militaris* was collected from 9 different mountains of Kangwon Province (Table 2). Nearly half of the specimens (44.3%) were collected from Mt. Seolak., the most popular tourist site in Korea. But, the number was in the decreasing tendency after 1999, which affected the total number of specimens collected from Kangwon Province as a whole. A total number of 134 specimens were collected from Mt. Chilsung only in 1999, but very few in other years. Except Mt. Taegi, *C. militaris* was collected frequently during the whole collection period (Table 2).

Table 3. Fruiting body production from single and pair-wise combinations of EFCC C-7833 isolates

Isolate No.	C-7833-1	C-7833-3	C-7833-5	C-7833-2	C-7833-4
C-7833-1	–	–	–	+	+
C-7833-3	–	–	–	+	+
C-7833-5	–	–	–	+	+
C-7833-2	+	+	+	–	–
C-7833-4	+	+	+	–	–

Table 4. Fruiting body production from single and pair-wise combinations of EFCC C-8021 isolates

Isolate No.	C-8021-1	C-8021-2	C-8021-3	C-8021-4	C-8021-5
C-8021-1	–	–	+	+	+
C-8021-2	–	–	+	+	+
C-8021-3	+	+	–	–	–
C-8021-4	+	+	–	–	–
C-8021-5	+	+	–	–	–

Table 5. Fruiting body production from single and pair-wise combinations of EFCC C-8023 isolates

Isolate No.	C-8023-1	C-8023-2	C-8023-3	C-8023-4	C-8023-5
C-8023-1	-	+	+	+	+
C-8023-2	+	–	–	–	–
C-8023-3	+	–	–	–	–
C-8023-4	+	–	–	–	–
C-8023-5	+	–	–	–	–

***In vitro* fruiting.** Artificial fruiting body production from various isolates of *C. militaris* is shown in Tables 3~5. In case of EFCC C-7833, three isolates C-7833-1, C-7833-3 and C-7833-5 did not produce fruiting body either from single inoculation or combination of two, but produced fruiting body when inoculated together in pair-wise combinations with remaining two isolates C-7833-2 and C-7833-4 (Table 3). Similarly, out of five EFCC C-8021 isolates, two isolates C-8021-1 and C-8021-2 produced fruiting body in all pair-wise combinations with remaining three isolates C-8021-3, C-8021-4 and C-8021-5, but not among themselves (Table 4). In case of EFCC C-8023, four isolates C-8023-2, C-8023-3, C-8023-4 and C-8023-5 produced fruiting body in combination with C-8023-1, but not among themselves (Table 5).

Discussion

The macroscopic as well as microscopic morphological characters of Korean *C. militaris* were observed well within the descriptions given in earlier mycological literature. The length of the stromata was very similar to those described by Kobayasi (1941), Mains (1958) and Chen (1978), but was comparatively broad. The color and shape were also similar to the earlier descriptions. Similarly, sizes of perithecium, ascus and part-spores were also within the range of earlier descriptions.

The number of *C. militaris* specimens varied from province to province and year to year. In spite of regular

collection trips to different mountains of Kangwon Province, the number varied extremely from year to year. Highest number of specimens was collected in 1999, but the obvious reason could not be understood. After 1999, the number decreased sharply. It may be due to the dry season with low rainfall in the following years. Outside Kangwon Province, Jeju Province was found more favorable for *C. militaris* in recent years. Besides those provinces, collection was very few from other provinces.

Present study showed that fruiting body of *C. militaris* is produced by combination of two isolates, similar to observation of Sung and Shrestha (2002) and Shrestha *et al*. (2004). Out of five isolates of EFCC C-7833, three isolates produced fruiting body with other two isolates. It could be shown that the three isolates were of the same mating type and did not produce fruiting body when they were inoculated together in combination. Other two isolates were of the opposite mating types, hence they did not produce fruiting body when combined together. Similarly in EFCC C-8021 isolates, three isolates were identified as possessing the same mating types, hence producing no fruiting body among them, while the remaining two isolates were identified as having the opposite mating type. Surprisingly, four out of five isolates of EFCC C-8023 were identified as the same mating type, the remaining one as having the opposite mating type. The result of the present study has confirmed that *C. militaris* is a bipolar, heterothallic fungus, requiring two opposite mating type isolates to produce fruiting bodies. Further studies on mass production of artificial fruiting body of *C. militaris* using superior isolates could enhance both productivity and quality in future.

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