Biotechnology and Bioprocess Engineering 2004, 9: 440-446

Bipolar Heterothallism, a Principal Mating System of *Cordyceps militaris In Vitro*

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Abstract Interest in *in vitro* study of entomopathogenic fungi, including *Cordyceps* species, has been increasing due to their valuable bioactive compounds and biocontrol effects. Among *Cordyceps* species, *in vitro* stromata of *C. militaris* has been successfully produced and cultivated for industrial purposes. However, genetic study on *in vitro* stromata formation of *C. militaris* has not been carried out yet. Here, relationship between mating system and perithecial stromata formation of *C. militaris* is reported. Mating system was determined by observing perithecial stromata formation from mono-ascospore cultures and their pair-wise combinations. Certain combinations of mono-ascospore strains produced perithecial club-shaped stromata, whereas other combinations produced either no stromata or only abnormal non-perithecial stromata. Similarly, mono-ascospore cultures without combination produced either no stromata or only abnormal non-perithecial stromata. Despite obvious heterothallism, self-fertility was occasionally observed in few strains of *C. militaris*. These observations indicated that *C. militaris* behaves as a bipolar heterothallic fungus and requires two mating compatible strains in order to produce regular club-shaped perithecial stromata, a fundamental requirement for its industrial cultivation.

Keywords: artificial fruiting, bipolar heterothallism, hermaphroditic nature, mating system, self-fertility

INTRODUCTION

The genus *Cordyceps* (Fr.) Link (*Clavicipitaceae, Hypocreales, Ascomycota*) is a large, cosmopolitan genus, comprising of 300 to 500 species and varieties [1,2]. Most of its members are pathogenic to different insects, including spiders, while few grow on hypogeal fungi of *Elaphomyces* spp. They are mainly distributed in the sub-tropical to temperate regions of the world. *C. militaris* is the type species of the Genus *Cordyceps*. It infects and grows upon a wide range of insects, mainly lepidopteran larva and pupa such as those of *Bombyx pithyocampa, B. caja, B. rubi, Euprepia caja, Gastropacha rubi, G. quercus, Phalera bucephala* and *Syntypistis punctatella*. Besides lepidopterans, it infects colepteran *Tenebrio molitor*, hymenopteran *Cimbex similis* and dipteran *Tipula paludosa*.

A large number of fungi have been studied for the commercial production of bioactive compounds through their *in vitro* culture and cultivation [3-6]. Similarly, *in vitro* mycelium growth and fruiting of *Cordyceps* species and other entomopathogenic fungi have attracted interests of several mycologists, entomologists and biotechnologists due to their medicinal values and biocontrol properties. de Bary was the first to study the *in vitro* growth of *C. militaris* by inoculating the larva of *Sphinx euphorbiae* with its ascospores and obtained stroma with immature perithecia [7,8]. Since then, stromata of *C. militaris* have been successfully grown in artificial conditions on pupae of other lepidopterans such as *Basilona imperialis, Callosamia promethia, Hyloicus pinastri, Mamestra brasicae, Spodoptera litura, Bombyx mori* and coleoptreran *Tenebrio molitor*. Host specificity of *C. militaris* has been reported in various genera of insects during *in vitro* fruiting [9].

Besides insects, a stromatal clavae of *C. militaris* having rudimentary perithecia were formed in old cultures of potato medium [10]. Later, big orange stromata of *C. militaris* were demonstrated in other media, such as malt extract agar, agar and gelatine mixed with peptone, urine, extract of pea or soy-bean, or boiled meat or coagulated colostrums-milk [11]. Effects of various environmental and nutritional factors on *in vitro* fruiting of *C. militaris* were also observed [12]. According to them, abundant stromata of *C. militaris* could be obtained in media containing high concentrations of complex, organic nitrogen sources, such as peptone, hemoglobin, casein, *etc.*, whereas negligible or no stromata formed in the presence of various concentrations of individual amino acids. But, there have been reports of unsuccessful fruiting of *C. militaris* in other protein rich media such as fat pork, lean pork, lean

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beef, egg yolk, egg albumin or an agar media made from the substance obtained from boiled pupae and coagulated egg yolk, milk agar, Sabouraud glucose agar, meat extract-egg yolk agar, malt agar, malt-peptone agar and hen's eggs.

In addition to insects and other mycological media, *in vitro* stromata of *C. militaris* was reported by Kobayasi [13] in rice medium containing 10 g of rice and 25 mL of distilled water in 100-mL Erlenmeyer flask. Effects of different additives, such as organic nitrogen sources including yeast extract, on fruiting of *C. militaris* in rice medium were studied in 100 g of brown rice in 160 mL of water as the basal medium for fruiting [14,15].

In Korea, Sung [16] reported *in vitro* fruiting of *C. militaris* in brown rice medium supplemented with silkworm pupae and developed a simple liquid inoculum method for large scale fruiting of *C. militaris*. Effects of various nutritional and environmental factors on mycelial growth and fruiting of *C. militaris* were also studied [17-19]. It has been well documented by various authors that the lower temperature of 18~22°C is optimum for *in vitro* fruiting of *C. militaris*. In spite of successful fruiting of *C. militaris* in brown rice medium, too much variation was observed among *in vitro* fruiting of *C. militaris* from different isolates and their subcultures under the same environmental conditions, which was the major problem for the mass cultivation of *C. militaris* [16-18].

Species of graminicolous *Clavicipitaceae* genera such as *Atkinsonella*, *Balansia*, *Echinodothis* and *Epichloe* have been studied in detail for their cultural characteristics, anamorph stages, conidiogenesis, spermatization, stromata development, perithecium formation, mating system, *etc.*, in natural conditions. Many of them have been found to be bipolar heterothallic. But, there is no report of mating system in *Cordyceps* species, including *C. militaris*. However, conflicting types of mating system in *C. militaris* was reported recently [20,21]. The objectives of this study were to understand fruiting factor of *C. militaris* from mono-ascospore cultures as well as their combinations, and subsequently to control fruiting development for its mass cultivation. The present study reports bipolar heterothallism as the principal mating system in *C. militaris*, except occasional occurrences of self-fertility.

MATERIALS AND METHODS

Fungal Isolates

Single ascospores were randomly isolated from EFCC C-5888, EFCC C-7159, EFCC C-7991 and EFCC C-8179 specimens of *C. militaris*, preserved in Entomopathogenic Fungal Culture Collection (EFCC, Kangwon national university, Korea) and were used in this study. Specimens EFCC C-5888 and EFCC C-8179 were collected from Galchon and Seolag Mt. of Kangwon province on Aug 24, 2000 and Aug 21, 2001, respectively. Similarly, specimens EFCC C-7159 and EFCC C-7991 were collected from Mai Mt. of Jeonla province on July 19, 2001 and Gyaeryong Mt. of Chungcheong province on Aug 10, 2001, respectively. All the hosts were lepidopterous pupae. For single ascospore isolation, freshly collected stromata were attached to the inner side of the lid of petri dish containing 2% water agar and left at room temperature under light. They were regularly observed for ascospore discharge. Single ascospores were randomly isolated from petri dishes containing discharged ascospores through Zeiss dissecting microscope (Zeiss, West Germany) with the help of a sterile pin. After isolation, ascospores were inoculated in the center of SDAY agar plates (dextrose 40 g, peptone 10 g, yeast extract 10 g, agar 15 g per 1,000 mL; pH 5.6) of 87 × 15 mm size and incubated at 25°C under light (500 lux). Eighteen strains were isolated from EFCC C-5888 specimen and were numbered from EFCC C-5888-1 to EFCC C-5888-18. Similarly, each fifty strains isolated from EFCC C-7159, EFCC C-7991 and EFCC C-8179 specimens were numbered from EFCC C-7159-1 to EFCC C-7159-50, EFCC C-7991-1 to EFCC C-7991-50 and EFCC C-8179-1 to EFCC C-8179-50, respectively.

Inoculum Preparation and Fruiting

To prepare liquid inoculum for fruiting of *C. militaris*, mycelial discs (4 mm) from growing margins of mono-ascospore colonies were cultured in SDAY broth (SDAY without agar) in a shaker (120 rpm) for 3~5 days at 25°C. Fruiting medium of *C. militaris* was prepared by mixing 60~80 g of brown rice, 10 g of silkworm pupae pieces and 90~120 mL of distilled water in 1,000-mL polypropylene (pp) plastic bottle and were autoclaved for 20 min at 121°C. Each pp bottle containing fruiting medium was inoculated with 10~20 mL of liquid inoculum of *C. militaris* for *in vitro* fruiting. After inoculation, the bottles were incubated at low temperature (20 ± 1°C) under light (500~1,000 lux) and high humidity conditions (70~90%) for 50~60 days.

Mating Experiment

Two standard mating experiments given by Harris were followed in this study [22]. The first one was the simultaneous inoculation of two mono-ascospore strains in fruiting medium, while the second was the inoculation of two strains on opposite sides of medium. For the first experiment, liquid inocula of eighteen EFCC C-5888 mono-ascospore strains were inoculated in brown rice media, alone as well as in pair-wise combinations. For the determination of mating compatibility, fertile perithecium formation was observed on stromata grown from mono-ascospore cultures and their pair-wise inoculations. If a pair of two strains produced fertile perithecia when inoculated together, they were considered to be of opposite mating types; if not, they were considered as the same mating type strains.

To determine whether perithecium formation was stable or not, several generations of subcultures of mating compatible strains were used for fruiting. For this, four EFCC C-5888 strains (EFCC C-5888-1, EFCC C-5888-13, EFCC C-5888-17 and EFCC C-5888-18) were selected and subcultured for ten generations at the interval

of two weeks in SDAY agar plates. Fruiting was produced from each generation of subculture from single as well as pair-wise inoculations among them and was observed for fertile perithecial stromata formation.

Mating Ratio

EFCC C-7159, EFCC C-7991 and EFCC C-8179 strains were used to determine mating type ratios. Three pairs of strains EFCC C-7159-17 and EFCC C-7159-21, EFCC C-7991-1 and EFCC C-7991-3, and EFCC C-8179-2 and EFCC C-8179-8 were used as mating type tester strains to determine mating type ratios of EFCC C-7159, EFCC C-7991 and EFCC C 8179 strains, respectively. All the EFCC C-7159, EFCC C-7991 and EFCC C-8179 strains were crossed in pair-wise combinations with their respective mating type tester strains and observed for fertile perithecium formation on stromata.

For the second experiment, mycelial discs of mating compatible and incompatible strains were inoculated on opposite sides of SDAY agar plates and observed for mycelial growth and stromata initiation at the meeting line between the strains.

Mating Types of Progeny of Self-fertile Strain

Since few strains showed self-fertility without combination, single ascospore progeny were randomly isolated from *in vitro* stromata produced from one of the single ascospore strains, EFCC C-8179-1. Mating types of progeny strains were identified by crossing in pair-wise combinations with each mating type tester strains EFCC C-8179-2 and EFCC C-8179-8.

Reciprocal Crosses

In order to determine the ability of both parents to form perithecia on stromata, mating type tester strains EFCC C-7159-17 and EFCC C-7159-21 were inoculated on the opposite sides of brown rice medium and observed for the stromata initiation and perithecium formation at the meeting line between them. Similarly, reciprocal crosses were carried out between them by growing each strain first in separate brown rice media until the surfaces of the media were completely covered by their mycelia. Then, few drops of liquid inocula of the strains were inoculated on the mycelium mat of opposite mating type and observed for normal stromata growth and fertile perithecium formation. In further experiment, reciprocal crosses were made between mating compatible strains EFCC C-7159-5 and EFCC C-7159-33. Later, reciprocal crosses were made between them and their progeny strains to confirm hermaphroditic nature of each strain.

Designation of Mating Types

All the three pairs of mating type tester strains were out-crossed among themselves and were observed for their mating compatibility. Three out of six strains, one from each pair, having the same mating type were randomly designated as A, while the remaining three, with opposite mating type, were designated as B.

RESULTS AND DISCUSSION

Mating System of *C. militaris*

C. militaris showed bipolar type of heterothallism in most of the crosses. Among eighteen EFCC C-5888 strains, a group of ten strains produced perithecial stromata when inoculated in pair-wise combinations with the remaining group of 8 strains, but pair-wise inoculations within each group did not produce any perithecial stromata (Table 1, Figs. 1 and 2). All the eighteen strains produced various types of non-perithecial stromata when inoculated alone. It could be observed that two strains of a combination produced perithecial stromata when they were of opposite mating types and no stromata or non-perithecial stromata were formed if the two strains were of the same mating type. This shows single ascospore strains possess one out of two mating types, *i.e.*, bipolar heterothallism, which is a common mating characteristics of *Ascomycota*. All the subsequent subcultures of strain EFCC C-5888-1 produced perithecial stromata when inoculated together with subcultures of either EFCC C-5888-13 or EFCC C-5888-17, but not with those of EFCC C-5888-18. Similarly, the subcultures of EFCC C-5888-13 and EFCC C-5888-17 produced perithecial stromata when inoculated in pair-wise combination with EFCC C-5888-1 and EFCC C-5888-18, but not between them. Continuous perithecial stromata formation by crossing several generations of subcultures of opposite mating strains showed that the mating type character is stable for many generations of subculture.

Mating Ratio

Among fifty EFCC C-7159 strains, fifteen and twenty-three strains produced perithecial stromata with EFCC C-7159-17 and EFCC C-7159-21, respectively, but the remaining twelve produced perithecial stromata alone as well as with both EFCC C-7159-17 and EFCC C-7159-21. Similarly, thirty-five and eleven EFCC C-7991 strains produced perithecial stromata with EFCC C-7991-1 and EFCC C-7991-3, respectively, remaining four producing perithecia with both of them and alone. Among fifty EFCC C-8179 strains, twenty and twenty-one strains produced perithecial stromata with EFCC C-8179-2 and EFCC C-8179-8, respectively, remaining nine strains showing self-fertility. In aggregate, 36% and 47.33% of total one hundred fifty mono-ascospore strains of EFCC C-7159, EFCC C-7991 and EFCC C-8179 specimens were of one or the other mating type, respectively, whereas remaining 16.67% showed self-fertility. But, in subsequent experiments, the number of self-fertile strains was comparatively very low.

Stromata primordia were initiated at the meeting line between two mating compatible strains EFCC C-7159-21

Table 1. Fertile perithecium formation from pair-wise combinations of all eighteen EFCC C-5888 mono-ascospore strains (EFCC C-5888-1 to EFCC C-5888-18)

S. No.	1	2	3	6	7	8	10	12	15	18	4	5	9	11	13	14	16	17
1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
3	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
6	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
7	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
10	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
12	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
15	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
18	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
13	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
16	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
17	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	

Note: + indicates fertile perithecial stromata formation, - indicates no stromata or non-perithecial, abnormal shaped stromata formation

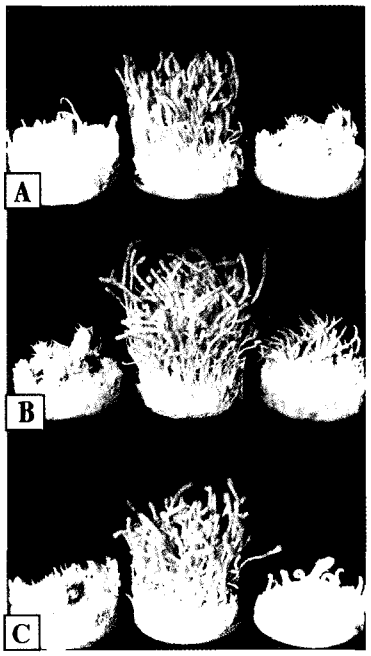

Fig. 1. Variation between stromata formed from mono-ascospore strains and their combinations on brown rice medium supplemented with pupae. Stromata on the left and right sides were formed from mono-ascospore strains (A, EFCC C-5888-4 and EFCC C-5888-1; B, EFCC C-5888-1 and EFCC C-5888-5; C, EFCC C-5888-1 and EFCC C-5888-9); they were non-perithecial, abnormal in shape and few in number. Stromata in the middle were formed from combinations of left and right strains (A, EFCC C-5888-1 × EFCC C-5888-4; B, EFCC C-5888-1 × EFCC C-5888-5; C, EFCC C-5888-1 × EFCC C-5888-9); they were perithecial, clavate or club-shaped and profuse.

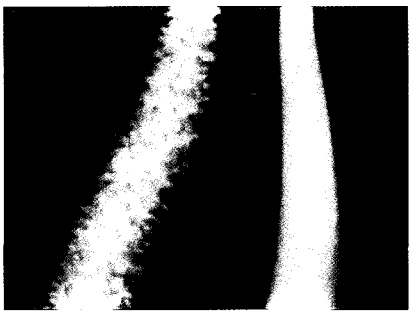

Fig. 2. Perithecial stromata (left) and non-perithecial stromata (right).

and EFCC C-7159-43 in SDAY agar (Fig. 3). Inoculating different strains on opposite sides of agar plates has been regularly practiced to study different phenomena such as mating system, vegetative compatibility, *etc.* In successful *in vitro* conditions, ascomycetous species without stromatal tissue can produce perithecia directly at the meeting line between two opposite mating type strains when inoculated in appropriate agar plate. But, in case of *C. militaris*, which produce stromata in addition to perithecia, stromata primordia could not fully develop into maturity in petri dish culture. It might be due to unfavorable condition inside petri dish, such as low nutrition availability, low oxygen availability, *etc.*

Mating Types of Progeny of Self-fertile Strains

Out of ninety-five progeny isolated from stromata produced by single culture of the self-fertile strain EFCC C-8179-1, forty seven produced fertile perithecia with EFCC C-8179-2 while other forty-two with EFCC C-8179-8.

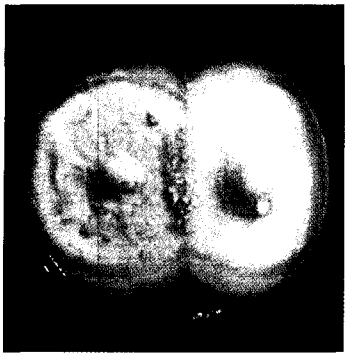

Fig. 3. Initiation of stromata primordia at the meeting line between EFCC C-7159-21 and EFCC C-7159-43 on SDAY agar plate.

Out of remaining six strains, two were self-fertile, whereas four were sterile, *i.e.*, they produced only mycelial growth or did not produce any perithecial stromata with one or the other tester strain. The progeny from a self-fertile strain EFCC C-8179-1 showed mostly heterothallism, except few self-fertile strains. It showed that self-fertile parent culture possessed both mating type loci, which were segregated among its progeny during ascospore formation.

The reason for high self-fertility rate of *C. militaris* might be that single ascospore strains were not pure and were probably mixtures of two or more than two ascospores due to poor recognition during ascospore isolations, although great care was taken. In future, single conidium strains must be tested to understand the possible reasons for self-fertility of *C. militaris*. The other reason might be due to mating heterokaryotic condition, in which single ascospores consisted of more than one nucleus with opposite mating type loci. Another reason might be the disomic (or diploid) condition, in which a nucleus (or nuclei) in single ascospores contained one extra chromosome of opposite mating type locus. Since *C. militaris* is a higher ascomycetous fungus, mating type switching or gene conversion cannot be a suitable reason for self-fertility.

Recently, mating type loci of *C. takaomontana* have been sequenced by Yokoyama *et al.* [23], although it's mating system is not known. Besides Clavicipitaceae family, mating systems of members of other orders and classes of filamentous *Ascomycetes* have been described and mating type loci have been sequenced. Till date, it has not been fully explained why certain single ascospore or conidial strains of heterothallic filamentous ascomycetous species behave as self-fertile. In *Neurospora crassa*, bisexuality has been reported, but most of the cases might be due to simple mixtures of ascospores during isolation and further growth. Occasional self-fertility has been observed in *Epichloe* species also. Mating type heterokaryosis and self-fertility have been recently reported in *Cryphonectria parasitica* [24]. Similarly, a mating system with multiple mating type alleles has been reported in the filamentous ascomycete *Glomerella cingulata*.

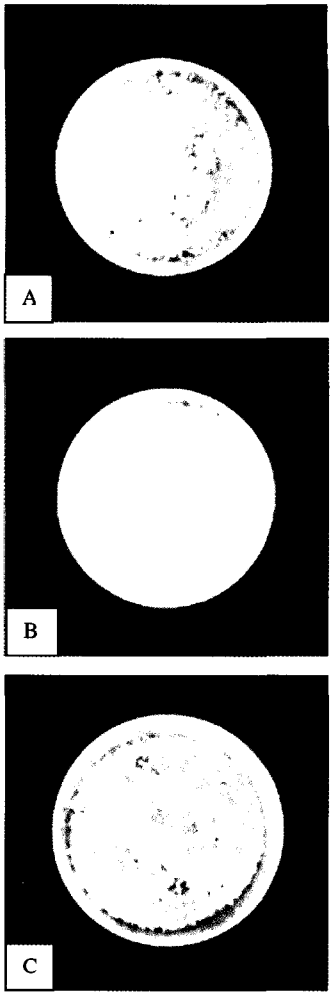

Fig. 4. Stromatal primordia formation at the contact line between two strains (A), stromatal primordia initiation at the contact line between EFCC C-7159-17 (left side) and EFCC C-7159-21 (right side) of the brown rice medium (B), no stromata induction in EFCC C-7159-17 culture, when inoculated by EFCC C-7159-21 (C), stromatal primordia formation in EFCC C-7159-21 culture, when inoculated by EFCC C-7159-17.

Reciprocal Crosses

Initiation of stromata primordia are shown in Fig. 4. Fig. 4A shows the formation of stromatal primordia at the contact line between mono-ascospore strains EFCC C-7159-17 (left, white mycelium) and EFCC C-7159-21 (right, orange mycelium) in brown rice medium. Fig. 4C shows the stromatal primordia formation when mono-culture of EFCC C-7159-21 was re-inoculated by EFCC C-7159-17. But, surprisingly, no stromata were formed when mono-culture of EFCC C-7159-17 was re-inoculated by EFCC C-7159-21 (Fig. 4B). It was speculated that strains EFCC C-7159-17 and EFCC C-7159-21 contrasted not only in mating type, but also in sexuality, the former behaving as male and the latter as female. But, it

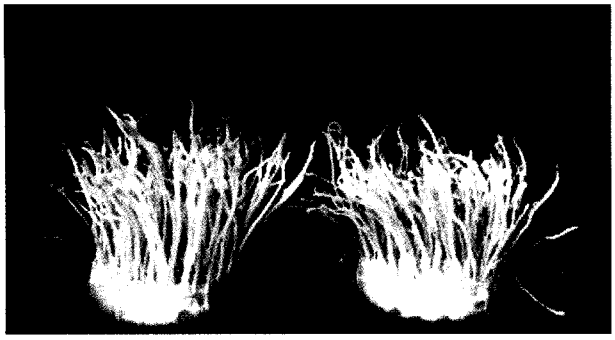

Fig. 5. Perithecial stromata formation from reciprocal crosses between EFCC C-7159-5 and EFCC C-7159-33 (left, stromatal culture of EFCC C-7159-5; right, stromatal culture of EFCC C-7159-33).

was observed from later experiments that each strain of a mating compatible pair is capable of producing fertile perithecial stromata, *i.e.*, they are hermaphrodite. Fig. 5 shows that both strains EFCC C-7159-5 and EFCC C-7159-33 were hermaphrodite and produced perithecial stromata when they were reciprocally crossed in brown rice media. When EFCC C-7159-5 (one of the parental strains) and 45 mating compatible progeny of the cross EFCC C-7159-5 × EFCC C-7159-33 were reciprocally crossed, perithecial stromata formed from both the parent and progeny strains. Similarly, when EFCC C-7159-33 (another parental strain) and fifty mating compatible progeny of the same cross were reciprocally crossed, all of them produced perithecial stromata. Three progeny showed self-fertility.

Designations of Mating Types

EFCC C-7159-17, EFCC C-7991-1 and EFCC C-8179-8 produced fertile perithecia on stromata, when out-crossed with non-sister strains EFCC C-7159-21, EFCC C-7991-3 and EFCC C-8179-2. Thus, the former three strains were randomly designated as mating type A strains, while the latter three strains as mating type B strains.

DNA sequencing of mating type loci has been effectively used to understand various mating systems of fungi. Turgeon and Yoder [25] have proposed a standard nomenclature system for mating type loci of filamentous *Ascomycetes*. They have proposed to name a strain as MAT 1-1 if its mating locus contains an open reading frame (ORF) encoding a protein with a motif called the α-box. The MAT 1-1 locus has been shown to be consisting of a single gene in case of *Loculoascomycetes*, but three genes in *Pyrenomycetes* and *Discomycetes*, which encode a protein with an α1 motif, an HMG motif-containing protein and a protein with an amphipathic δ-helix. The opposite strain has been proposed to be named as MAT 1-2, if the mating locus consists of a single ORF encoding a regulatory protein with a DNA –binding domain of the HMG type. Mating type loci of opposite mating types of *C. militaris* could be named according to Turgeon and Yoder [25] once their DNA sequences are complete. Similarly, the reason for self-fertility of *C. militaris* could be more clearly understood once the self-fertile strains are exposed to DNA sequencing.

Spawn preparation is one of the important steps in commercial mushroom cultivation [26]. The source of spawn may be tissue or spore (both sexual and asexual) of mushroom. Tissue isolation is comparatively easy, but cannot be assured of purity, while spore isolation is a time-consuming and complicated process but pure isolates can be obtained through single spore isolation method. Spawn culture should contain all necessary genetic materials of a mushroom for the regular development of fruit bodies in culture. Due to the lack of information of genetics of a mushroom, the spawn cultures prepared under the same condition give unstable fruiting causing great economic losses to mushroom growers. Hence, it should be ensured that the spawn culture for cultivation of mushroom such as *C. militaris* contain two compatible isolates, following the process described above.

CONCLUSION

The literature review showed various types of stromata formation of *C. militaris* in *in vitro* conditions such as mature and immature, normal and abnormal, perfect and imperfect, *etc.*, with ambiguous meanings. Similarly, unstable fruiting of *C. militaris* has been cited as the main problem for its mass cultivation. Here, we report that *C. militaris* is principally a bipolar heterothallic fungus, hermaphrodite in nature, except few self-fertile strains. In the present study, opposite mating type strains produced perithecial, club-shaped stromata, when grown together. Even single ascospore strains or combinations of same mating type strains produced stromata, but without perithecia and were abnormal in shape, showing that the vegetative stromata can be produced under appropriate environmental conditions in brown rice medium. Identification and crossing of mating compatible strains can solve the basic problems of cultivation of *Cordyceps* species including *C. militaris*. Further, mating compatible strains with enhanced biochemical properties can be selected for value-added production of *C. militaris* in future.

Acknowledgements 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.

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[Received July 19, 2004; accepted December 3, 2004]