Heterothallic Type of Mating System for *Cordyceps cardinalis*

Gi-Ho Sung¹, Bhushan Shrestha², Sang-Kuk Han³, Soo-Young Kim⁴ and Jae-Mo Sung^{5*}

¹Mushroom Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Suwon 441-707, Korea

²Green Energy Mission/Nepal, Anam Nagar, Kathmandu P.O. Box 10647, Nepal

³Division of Forest Biodiversity, Korea National Arboretum, Pocheon 487-820, Korea

⁴Donghae Agricultural Technology Center, Donghae 240-030, Korea

⁵Cordyceps Institute of Mushtech, Chuncheon 200-936, Korea

(Received August 17, 2010. Accepted November 2, 2010)

Cordyceps cardinalis successfully produced its fruiting bodies from multi-ascospore isolates. However, subcultures of multi-ascospore isolates could not produce fruiting bodies after few generations. Fruiting body production also differed from sector to sector of the same isolate. Single ascospore isolates were then co-inoculated in combinations of two to observe the fruiting characteristics. Combinations of certain isolates produced perithecial stromata formation, whereas other combinations did not produce any fruiting bodies. These results show that *C. cardinalis* is a heterothallic fungus, requiring two isolates of opposite mating types for fruiting body production. It was also shown that single ascospore isolates are hermaphrodites.

KEYWORDS : Heterothallism, Homothallism, Mating system, Perithecial stromata, Subculture

Cordyceps species are regarded as medicinal mushrooms in East Asian countries. Some of them, for example *Ophiocordyceps sinensis* (syn. *Cordyceps sinensis*), have played very important roles in the economic development of local communities [1, 2]. Recently, the mycelium growth and fruiting body formation of *Cordyceps* species have been studied with great interest with the objective of large-scale cultivation [3-11]. Mating system is an important genetic factor for fruiting body formation in fungi [12-14]. *Cordyceps* and other clavicipitaceous fungi have been studied in order to determine their mating systems in various environments, such as in nature and culture, or through gene sequencing [15-21]. Most of these *Cordyceps* species show heterothallism [17-20]. However, homothallic type behavior is also observed in *Cordyceps* despite apparent heterothallism, e.g., in *C. militaris* [17, 21]. Besides fruiting body formation, mating type can also help resolve biological species concepts in fungi [22]. In addition to ascospore-derived isolates, conidial isolates of asexual fungi have also led to the development of sexual states of *Cordyceps* in culture by proper combination of opposite mating types [23].

C. cardinalis is a recently reported fungus [24], and its *in vitro* fruiting bodies have been successfully produced in Korea [25]. However, the mating system of *C. cardinalis* is still unknown. In this study, the mating system of *C. cardinalis* was studied in culture in order for continuous cultivation of its fruiting bodies. Both multi-ascospore and single ascospore isolates were tested for fruiting body for-

mation. Different sectors from the same isolate were also employed for fruiting body formation.

Materials and Methods

Fungal isolates. *C. cardinalis* specimen CRI C-10735, preserved at the Cordyceps Research Institute (CRI) of Mushtech, Korea was used for multi-ascospore and single ascospore isolations. The specimen was collected from Mt. Dunryu in Jeollanam-do on August 13, 2003. Ascospores were discharged from the fresh specimen on 2% water agar. Multi-ascospore isolates were derived by transferring agar blocks containing numerous ascospores to Sabouraud dextrose agar plus yeast extract (SDAY; dextrose 20 g, yeast extract 5 g, peptone 5 g, and agar 15 g per 1,000 mL; pH 5.6) agar plates, followed by incubation at 25°C under white fluorescent light for 3 wk. Similarly, 38 single ascospore isolates were also derived, following the method of Shrestha *et al.* [17], and incubated as above. They were numbered from CRI C-10735-1 to CRI C-10735-38.

Fruiting body formation from multi-ascospore isolates. Liquid inocula of the isolates were prepared by inoculating five mycelial discs (4 mm) in 100 mL of SDAY broth (SDAY without agar). The inoculated SDAY broths were incubated at 25 ± 1°C for 5 days in a rotary shaker at 120 rpm. Fruiting medium was prepared by mixing 50 g of brown rice, 10 g of silkworm pupa, and 60 mL of distilled water in 1,000 mL of Polypropylene (PP) bottle, followed by sterilization at 121°C for 15 min. Between 15~

*Corresponding author <E-mail : cordyceps@hanmail.net>

20 mL of the liquid inocula was then inoculated into each PP bottle, followed by incubation at $25 \pm 1^\circ\text{C}$ for 60 days under white fluorescent light and humidity of 60~70%. After 60 days of incubation, the fruiting bodies were distinguished by observing perithecia on stromata, following the method of Shrestha *et al.* [17]. Fruiting bodies that developed perithecia were marked as (+) and those without perithecia as (-).

Fruiting body formation from subcultures and different sectors. Multi-ascospore isolates were subcultured every 3 wk on SDAY agar plates up to the sixth generation. Subcultures were also inoculated in fruiting medium for fruiting body formation. However, no fruiting bodies could be produced from subcultures after the fourth generation. To observe the effect of sectors on fruiting body formation, 21 different sectors from the same subculture of the fourth generation were inoculated into fruiting medium and observed for fruiting body formation. The sectors were numbered from CRI C-10735-1 to CRI C-10735-21. Six sectors, CRI C-10735-2, CRI C-10735-6, CRI C-10735-9, CRI C-10735-13, CRI C-10735-16, and CRI C-10735-20, which could not produce any fruiting bodies, were co-inoculated among themselves into fruiting medium in order to observe the effect of crossing on fruiting body formation.

Fruiting body formation from single ascospore isolates. All single ascospore isolates were separately inoculated into fruiting media, as described above, and observed for fruiting body formation. Four single ascospore isolates, CRI C-10735-1, CRI C-10735-2, CRI C-10735-3, and CRI C-10735-38, which could not produce perithecial fruiting bodies, were co-inoculated among themselves in order to observe the effect of crossing on fruiting body formation. A combination of isolates CRI C-10735-1 and CRI C-10735-38 produced the best perithecial fruiting bodies; hence, they were selected as tester isolates. These tester isolates were then co-inoculated with each of the remaining single ascospore isolates, and mating behavior was observed. The hermaphroditic nature of single ascospore isolates was also tested using isolates CRI C-10735-34 and CRI C-10735-38 by reciprocal inoculation, following the method of Shrestha *et al.* [17].

Results and Discussion

Fruiting body formation from multi-ascospore isolates and subcultures. The original isolates and subcultures up to the third generation produced perithecial fruiting bodies. Multi-ascospore isolates were, in fact, a mixture of genetically different ascospores. Moreover, they have been shown to produce unstable fruiting bodies as well as degenerating fruiting bodies in *C. militaris* [26]. In addition,

different sectors of the same agar culture produce various types of fruiting bodies [27]. Single ascospore isolates have been shown to possess distinct mating types and cultural characteristics [17, 28]. Usually, original isolates show better fruiting bodies, but the subcultures produce similar or inferior fruiting or no fruiting at all [26]. In this study as well, subcultures demonstrated reduced fruiting body production.

Fruiting body formation from different sectors of subcultures. Subcultures abruptly ceased producing fruiting bodies after the fourth generation. This could have been due to reduced vitality of the subcultures, or the fruiting ability could have differed from sector to sector of the same subculture. A second possibility was demonstrated when six out of 21 sectors from the same subculture of the fourth generation produced fruiting bodies. This phenomenon was again observed when non-fruiting sectors produced fruiting bodies after co-inoculation (Table 1). The combinations of sectors CRI C-10735-2 $\times$ CRI C-10735-6, CRI C-10735-2 $\times$ CRI C-10735-16, and CRI C-10735-9 $\times$ CRI C-10735-20 produced fruiting bodies (Table 1). This shows that the subcultures led to increased homogeneity in the genotypes, depending upon the sectors of the mother isolates that were used for subculturing. It was shown in this study that multi-ascospore isolates of *C. cardinalis* could not be used to understand mating behavior.

Fruiting body formation from single ascospore isolates.

Out of 38 single ascospore isolates, 16 produced fruiting bodies while the rest did not produce any. The fruiting bodies produced from single ascospore isolates were different from those produced from multi-ascospore isolates in that the former did not produce any perithecia on stromata.

Table 1. Fruiting body formation by different sectors of subculture of *Cordyceps cardinalis* isolate CRI C-10735

Sector No.	2	6	9	13	16	20
2	—	+	—	—	+	—
6		—	—	—	—	—
9			—	—	—	+
13				—	—	—
16					—	—
20						—

Table 2. Fruiting body formation by single ascospore isolates of *Cordyceps cardinalis* CRI C-10735

Isolate No.	1	2	3	38
1	—	—	—	+
2		—	—	+
3			—	+
38				—

Fig. 1. Fruiting body formation by combinations of single ascospore isolates of *Cordyceps cardinalis* CRI C-10735.

Fig. 2. Fruiting body formation by combinations of single ascospore isolates and tester isolates of *Cordyceps cardinalis* CRI C-10735.

Fig. 3. Fruiting body formation by reciprocal inoculations and co-inoculations of single ascospore isolates of *Cordyceps cardinalis* CRI C-10735-34 and CRI C-10735-38. A, E, Single inoculations; B, D, Reciprocal inoculations between two isolates; C, Co-inoculation of two isolates.

mata. Combinations of single ascospore isolates, however, did produce perithecial stromata. Combinations of CRI C-10735-38 $\times$ CRI C-10735-1, CRI C-10735-38 $\times$ CRI C-10735-2, and CRI C-10735-38 $\times$ CRI C-10735-3 produced perithecial fruiting bodies, whereas combinations of CRI C-10735-2 $\times$ CRI C-10735-1, CRI C-10735-3 $\times$ CRI C-10735-1, and CRI C-10735-3 $\times$ CRI C-10735-2 produced no perithecial fruiting bodies (Table 2, Fig. 1). Thus, isolates CRI C-10735-1, CRI C-10735-2, and CRI C-10735-3 were designated as type A and the other isolate CRI C-10735-38 as type B to indicate that they were of opposite mating type.

Most of the remaining single ascospore isolates produced perithecial fruiting bodies when co-inoculated with either CRI C-10735-1 or CRI C-10735-38 (Fig. 2). How-

ever, one of them produced fruiting bodies with both tester isolates, whereas the other two did not produce any fruiting bodies with either of the tester isolates. In summary, 23 isolates showed mating type A and 12 isolates showed mating type B. Hermaphroditic nature was shown by single ascospore isolates of opposite mating types CRI C-10735-34 and CRI C-10735-38 when inoculated reciprocally (Fig. 3).

Many *Cordyceps* species have been shown to be heterothallic by analysis of mating type genes [20]. Cultural studies should be conducted more critically to identify the mating system for *Cordyceps*. *C. bassiana* shows a very unstable mating system in culture [10, 11]. In nature, *Cordyceps* species generally show variation in their morphological characteristics. These variations could be due

to heterothallism in their mating systems. Since *C. cardinalis* has a wider distribution from North America to East Asia [24], heterothallism might be the reason for its adaptation to a wider ecological range.

This study showed *C. cardinalis* as a heterothallic fungus, similar to *C. militaris*, although the mating type of a few single ascospore isolates could not be determined. We also explained why fruiting body production varies from isolate to isolate of multi-ascospore origin. It might be possible that multi-ascospore isolates occasionally consist of ascospores of the same mating type, and hence do not produce any fruiting bodies. Further, continuous subcultures proliferate only a particular sector of the mother culture, leading to homogeneity in mating type after several subcultures.

Such studies can help accumulate more information about the mating systems of *Cordyceps* in general. Sung *et al.* [29] has shown that single ascospore isolates of *C. militaris* degenerate after several subcultures, but not as early as multi-ascospore isolates. Hence, it is necessary to select superior isolates for enhanced fruiting body production, as suggested by Sung *et al.* [30].

Acknowledgements

The authors wish to acknowledge the Cordyceps Research Institute for providing facilities to carry out this study.

References

1. Devkota S. Yarsagumba [*Cordyceps sinensis* (Berk.) Sacc.]; traditional utilization in Dolpa district, Western Nepal. *Our Nature* 2006;4:48-52.
2. Winkler D. Yartsa Gunbu (*Cordyceps sinensis*) and the fungal commodification of Tibet's rural economy. *Econ Bot* 2008;62:291-305.
3. Liang ZQ. Anamorph of *Cordyceps militaris* and artificial culture of its fruitbody. *Southwest China J Agric Sci* 1990;3:1-6.
4. Liu JL, Liang ZQ, Liu AY. Artificial culture of fruiting bodies of *Cordyceps gunnii*. *Southwest China J Agric Sci* 1990;3:6-10.
5. Liang ZQ. *Cordyceps* and its artificial culture. *J Guizhou Agric Coll* 1994;13:1-21.
6. Pen X. The cultivation of *Cordyceps militaris* fruitbody on artificial media and the determination of SOD activity. *Acta Edulis Fungi* 1995;2:25-8.
7. Sung JM. The insects-born fungus of Korea in color. Seoul: Kyohak Publishing Co., Ltd.; 1996.
8. Shrestha B, Han SK, Lee WH, Choi SK, Lee JO, Sung JM. Distribution and *in vitro* fruiting of *Cordyceps militaris* in Korea. *Mycobiology* 2005;33:178-81.
9. Li CR, Nam SH, Geng DG, Fan MZ, Li ZZ. Artificial culture of seventeen *Cordyceps* spp. *Mycosystema* 2006;25:639-45.
10. Lee JO, Shrestha B, Kim TW, Sung GH, Sung JM. Stable formation of fruiting body in *Cordyceps bassiana*. *Mycobiology* 2007;35:230-4.
11. Lee JO, Shrestha B, Sung GH, Han SK, Kim TW, Sung JM. Cultural characteristics and fruiting body production in *Cordyceps bassiana*. *Mycobiology* 2010;38:118-21.
12. Coppin E, Debuchy R, Arnaise S, Picard M. Mating types and sexual development in filamentous ascomycetes. *Microbiol Mol Biol Rev* 1997;61:411-28.
13. Kothe E. Mating-type genes for basidiomycete strain improvement in mushroom farming. *Appl Microbiol Biotechnol* 2001;56:602-12.
14. Pöggeler S. Mating-type genes for classical strain improvements of ascomycetes. *Appl Microbiol Biotechnol* 2001;56:589-601.
15. White JF Jr, Owens JR. Stromal development and mating system of *Balansia epichloë*, a leaf-colonizing endophyte of warm-season grasses. *Appl Environ Microbiol* 1992;58:513-9.
16. Yokoyama E, Yamagishi K, Hara A. Structures of the mating-type loci of *Cordyceps takaomontana*. *Appl Environ Microbiol* 2003;69:5019-22.
17. Shrestha B, Kim HK, Sung GH, Spatafora JW, Sung JM. Bipolar heterothallism, a principal mating system of *Cordyceps militaris* *in vitro*. *Biotechnol Bioprocess Engin* 2004;9:440-6.
18. Yokoyama E, Yamagishi K, Hara A. Development of a PCR-based mating-type assay for Clavicipitaceae. *FEMS Microbiol Lett* 2004;237:205-12.
19. Yokoyama E, Yamagishi K, Hara A. Heterothallism in *Cordyceps takaomontana*. *FEMS Microbiol Lett* 2005;250:145-50.
20. Yokoyama E, Arakawa M, Yamagishi K, Hara A. Phylogenetic and structural analyses of the mating-type loci in Clavicipitaceae. *FEMS Microbiol Lett* 2006;264:182-91.
21. Wen TC, Li MF, Kang JC, Lei BX. A molecular genetic study on the fruiting-body formation of *Cordyceps militaris*. *KSM Newsl* 2009;21(2):76-95.
22. Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. Phylogenetic species recognition and species concepts in fungi. *Fungal Genet Biol* 2000;31:21-32.
23. Lee JO, Shrestha B, Sung GH, Han SK, Sung JM. Successful development of *Cordyceps bassiana* stromata from *Beauveria bassiana*. *Mycobiology* 2010;38:13-6.
24. Sung GH, Spatafora JW. *Cordyceps cardinalis* sp. nov., a new species of *Cordyceps* with an east Asian-eastern North American distribution. *Mycologia* 2004;96:658-66.
25. Kim SY, Shrestha B, Sung GH, Han SK, Sung JM. Optimum conditions for artificial fruiting body formation of *Cordyceps cardinalis*. *Mycobiology* 2010;38:133-6.
26. Shrestha B, Park YJ, Han SK, Choi SK, Sung JM. Instability in *in vitro* fruiting of *Cordyceps militaris*. *J Mushroom Sci Prod* 2004;2:140-4.
27. Shrestha B, Nam IS, Kim HK, Sung JM. Effect of sectors of isolates on fruiting of *Cordyceps militaris*. *KSM Newsl* 2002;14:98.
28. Shrestha B, Lee WH, Han SK, Sung JM. Observations on some of the mycelial growth and pigmentation characteristics of *Cordyceps militaris* isolates. *Mycobiology* 2006;34:83-91.
29. Sung JM, Park YJ, Lee JO, Han SK, Lee WH, Choi SK, Shrestha B. Effect of preservation periods and subcultures on fruiting body formation of *Cordyceps militaris* *in vitro*. *Mycobiology* 2006;34:196-9.
30. Sung JM, Park YJ, Lee JO, Han SK, Lee WH, Choi SK, Shrestha B. Selection of superior strains of *Cordyceps militaris* with enhanced fruiting body productivity. *Mycobiology* 2006;34:131-7.