MOLECULAR IDENTIFICATION OF MATING TYPE GENES IN ASEXUAL SPORES OF *CORDYCEPS MILITARIS*

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ABSTRACT

Cordyceps species are important medicinal mushrooms widely used in traditional Chinese medicine for maintaining health and vitality. *C. sinensis* is the most highly sought-after species, but production of fruit bodies in artificial culture has yet to be achieved. However, strains of *C. militaris*, a closely related species, will fruit on artificial media although degeneration into non-fruiting strains often occurs following continued sub-culture. In order to better understand the molecular basis of fruiting in this species, we have examined the nature of *C. militaris* mating-type genes and their distribution in fruiting and non-fruiting strains. Two parental strains, CM-23B and CM-H07, which produce fruit bodies and abundant asexual spores in artificial culture, were selected for this purpose and 100 single spore isolates were obtained from each strain. Three specific primer pairs, designed according to the reference mating type genes of *C. militaris* deposited in the NCBI database, were used for PCR amplification of the mating type genes present in all the single spore isolates and their parental strains. Both parental strains contained two kinds of mating-type loci, MAT-HMG (MAT1-2-1 gene) and MAT-alpha (MAT1-1-1 and MAT1-1-2 genes). Of the 100 isolates derived from CM-23B, 40%, contained only MAT-alpha, 25% only MAT-HMG and 35% both MAT-alpha and MAT-HMG. Of the 100 isolates derived from CM-H07, 33%, contained only MAT-alpha, 30% only MAT-HMG and 37% both MAT-alpha and MAT-HMG. We infer from these data that, during the formation of asexual spores, some spores (heterokaryons) contain nuclei carrying both mating-type loci (MAT-HMG and MAT-alpha) while others (homokaryons) contain nuclei carrying only one type of mating-type locus, either MAT-HMG or MAT-alpha. Since both mating-type loci are essential for fruiting, it is important to confirm that both MAT-HMG and MAT-alpha are present in strains selected for artificial cultivation.

Keywords: *Cordyceps militaris*; asexual spore; mating type gene

INTRODUCTION

Cordyceps militaris (L.) Link, as a species similar to *Cordyceps sinensis* [1], has the same or similar medicinal value with *Cordyceps sinensis*. Since *C. militaris* is often used as a substitute for *Cordyceps sinensis*, the market of *C. militaris* has a good development prospect; in scientific research *Cordyceps* is also used as the type species of the genus *Cordyceps* [2].

Asexual reproduction is an important part of the life cycle of *C. militaris*. In the production of *C. militaris*, it is mainly through asexual reproduction to inoculation, propagation, conservation. According to our observation, *C. militaris* species produced large amounts of asexual spores in the process of asexual reproduction. These asexual spores were easy to germinate, and the mycelium after germination grew fast. Then we were interested in whether these mycelium germinated from asexual spores had the same genetic background with parent mycelium, which might be related to the phenomenon that *C. militaris* is prone to degradation.

There are two different sources mating type locus of *Cordyceps militaris*. One is mating type loci MAT-alpha, contains two kinds of mating type gene sequence, named MAT1-1-1 and MAT1-1-2, while the other mating type loci MAT-HMG contains only one mating type gene sequence, named MAT1-2-1 [3]. In the process of sexual reproduction in *C. militaris*, homocaryotic mycelium carrying distinct mating type genes are compatible with each other. After plasmogamy, these compatible homocaryotic mycelia form pairs, heterocaryotic mycelium, initiating the dikaryophase of the sexual cycle. Then after karyogamy and meiosis and the formation of ascospores, heterocaryons complete sexual life cycle. According to the life cycle of *Cordyceps militaris*, homocaryotic mycelia have one type of mating type genes, while heterocaryotic mycelia have two types of mating type genes. So we can identify the nuclear phase, homocaryon or heterocaryon, by indentifying the type of mating type genes of *Cordyceps militaris*.

This study isolated 200 strains germinated from asexual spores of two *Cordyceps militaris* strains. We indentified mating type genes of these 200 isolates by PCR.

MATERIALS AND METHODS

Strains. *Cordyceps militaris* strains, CM-23B and CM-H07, were obtained from Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences (SAAS). *Cordyceps militaris* mycelia cultured on potato dextrose agar medium (PDA).

Preparation of asexual spore suspension and single spore isolates. Strains were inoculated on PDA plates, 25 ℃ for 10 days dark culture. Then PDA plates covered with mycelium were washed by 1mL of sterile water to collect mycelium. Asexual spores were isolated by G-2 glass filter and suspended by sterile water and diluted to 10^5 ml^{-1}. 100μl spore suspension was poured onto a PDA plant. The plate was incubated at 25 ℃ in a dark place. When visible colonies appeared, these colonies were subcultured individually onto fresh PDA plates, and incubated at 25 ℃ in a dark place until they were covered with mycelium completely. Then these mycelium were scraped down and freeze-dried until use.

Genomic DNA extraction method of *Cordyceps militaris*. The genomic DNA was extracted from *Cordyceps militaris* with the improved CTAB method [4]. Methods briefly described as follows: freeze-dried *Cordyceps militaris* hyphae ground to powder was added to 65℃ preheated 2 x CTAB extraction liquid (2% CTAB; 1.4 M NaCl; 100mM Tris-HCl; 20mM EDTA, pH8.0), and was incubated at 65°C at least 30 min, then centrifuged at room temperature, 12000 rpm for 10 min. The supernatant was transferred into another fresh 1.5 ml tube，then equivalent volume of phenol-chloroform(1:1) was added, mixed gently but thoroughly about 1 min. This mixture centrifuged (12000 rpm) for 10min. The supernatant was transferred into another fresh 1.5 ml tube and equivalent volume of chloroform-isoamyl alcohol (24:1) was added, well mixed, then centrifuged (12000 rpm) for 10min. The supernatant was transferred into another fresh tube, and 1/10 volume 3M NaAc was added, then 2/3 volume -20°C precooled isopropanol was added, and then incubated at -20°C for 20 minutes, centrifuged at 4℃, 12 000 rpm for 10 min. The precipitation was washed with 75%ethanol for twice, air dried and resuspended in 30μl TE buffer. 1μl RNase(10mg/ml) was added to the DNA solution for 1 hour at 37°C to remove RNA. The final DNA extracts were stored at -20°C until use.

Specific primers amplification for identifying mating type genes of *Cordyceps militaris*. Three specific primer pairs, shown in Table 1, designed according to the reference mating type genes of *C. militaris* deposited in the NCBI database(registration number AB084257 and AB194982), were used for PCR amplification of the mating type genes MAT1-1-2, MAT1-1-1 and MAT 1-2-1.

Using single spore isolates' or their parental strains' mycelia total DNA as templates, mating type genes fragment was amplified under the following conditions: PCR reaction mixture(100 μl) contained 100 ng DNA, 1 x PCR buffer, 2 mmol/L Mg^{2+}, 0.2 mmol/L dNTPs, 0.25 μmol/L forward and reverse primer, and 3 U Ex *Taq* polymerase (Takara Bio Co., Dalian). Amplification conditions were: 1 cycle of 94 °C for 2min; 30 cycles of 94°C for 30 s, 50-55 °C (according to TM of primers) for 60 s and 72 °C for 40-60 s (according to the length of fragment), then a final extension at 72 °C for 5 min. All amplifications were carried out using a TP-600 Thermal Cycler Dice (Takara). PCR products were separated on 1% (w/v) agarose gels.

Table 1: Specific primers used for amplification of mating type genes of *Cordyceps militaris*

Types of mating type genes		Primers	Sequences
MAT-alpha	MAT1-1-2	MAT112F	5'- ATGGAACACAGATCGAGCGACAC -3'
		MAT112R	5'- ATATACCTTCGCGATCATTGCCCAG - 3'
	MAT1-1-1	MAT111F	5'- TTCAGCTTCAGTCCGTTCTGGACA -3'
		MAT111R	5'- GGCAGACATCGTACCTGGTCAAAT - 3'
MAT-HMG	MAT1-2-1	MAT121F	5'- ATGGATCTGCAACTGGATCGGACCA- 3'
		MAT121R	5'- CTACATGATTGACTCCGGGCTCATTG- 3'

RESULTS AND ANALYSIS

Asexual spores of *Cordyceps militaris* on PDA medium initiated germination easily. Generally spore germination could be observed in three days after plated, and in 5 days visible small colonies could be found. The speed of spore germination and hypha growth in each plate are about the same. We didn't find colonies formed from spores germinating slowly (Fig. 1). In this study, 100 single spore isolates were obtained from each strain, CM-23B and CM-H07. After subcultured individually onto fresh PDA plates, the hyphae grew about the same speed. We did not find the strains grew quickly or slowly obviously.

After DNA extraction from 200 asexual single spore isolates and their parent strains, their mating type genes were identified by PCR amplification. Fig. 2 shows the partial results of identification. The length of the mating type gene MAT 1-1-1 was 457bp, while the length of the mating type gene MAT1-1-2 and MAT1-2-1 were 1063bp and 839bp, respectively.

Statistical results of PCR identification showed that the parent strains were contained two kinds of mating-type loci, both MAT-alpha (MAT1-1-1 and 1-1-2) and MAT-HMG (MAT1-2-1). Of the 100 isolates derived from CM-23B, 40% , contained only MAT-alpha (MAT1-1-1 and 1-1-2), 25% only MAT-HMG (MAT1-2-1) and 35% both MAT-alpha(MAT1-1-1 and 1-1-2) and MAT-HMG (MAT1-2-1) as their parent strains. Of the 100 isolates derived from CM-H07, 33%, contained only MAT-alpha, 30% only MAT-HMG and 37% both MAT-alpha and MAT-HMG. The results are shown in Table 2.

The parent strains are heterokaryons; each hypha cell contains two kinds of the nucleus; each nucleus contains one type of mating type genes. So parent strains contain both two types of mating-type genes. For single spore isolates, 62 strains were the same as their parent strains

containing two types of mating-type genes. So these strains were believed to be heterokaryons containing two kinds of nucleus. The other 138 single spore isolate strains only contained one type of mating-type loci, which showed that their hypha cell just contain one kind of nucleus and they were homokaryons.

Table 2: The statistical results of the kind of mating type genes of asexual spore strains, isolated from *Cordyceps militaris*

Types of asexual spore strains	Kinds of mating type genes	strains derived from CM-23B	strains derived from CM-H07
Parental type	MAT1-1-1, MAT1-1-2, MAT1-2-1	35	37
Alpha type	MAT1-1-1, MAT1-1-2	40	33
HMG type	MAT1-2-1	25	30
Total		100	100

Figure 1. The germination status of asexual spore (The seventh day)

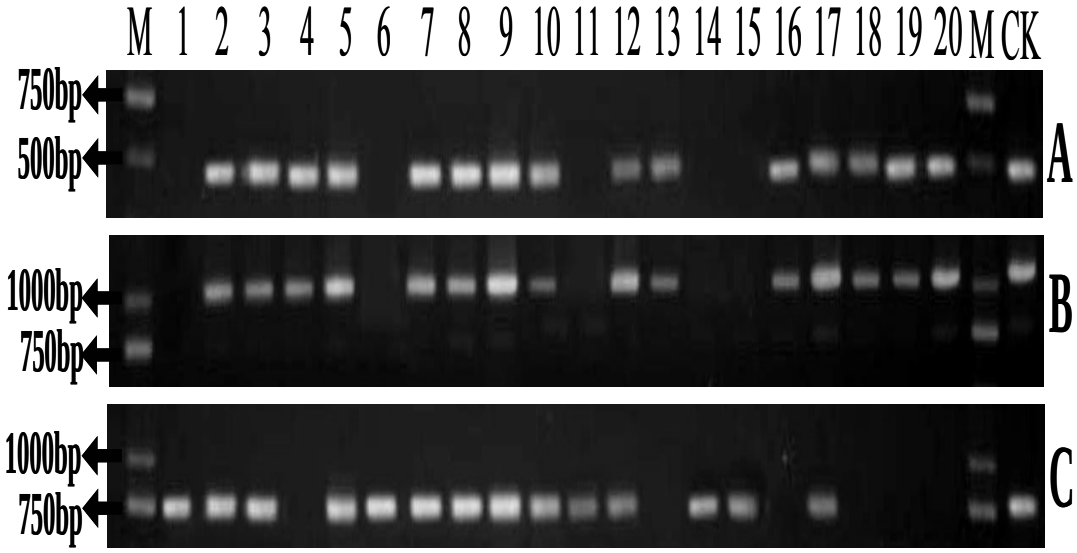

Figure 2: The partial amplification results of mating type genes fragment in single-spore isolates (M: D2000 DNA marker; 1-20: isolates No. 1-20 from CM-23B; CK：CM-23B；A：MAT1-1-1; B:MAT1-1-2; C:MAT1-2-1)

DISCUSSION

The study found that *Cordyceps militaris* strains, CM-23B and CM-H07, could produce a lot of asexual spores. We separated 200 single spore isolates and indentified their mating type genes. We found that a lot of single asexual spore isolates only contain MAT-alpha or MAT-HMG, while their parent strains contained both. Mating type genes regulate the genetic basis of sexual compatibility and sexual reproduction. *Cordyceps militaris* is a typical bipolar heterothallism mushroom [5]. It must grow from two sexual compatible pairs, which are germinated from two mono-ascospores, and then dicaryons grow to the fruiting bodies. The different degree absence of mating type genes of the mycelium grown from asexual spores may be the genetics reason why degeneration of Cordyceps militaris into non-fruiting strains often occurs following continued sub-culture. The question is worth further research. Since both mating-type loci are essential for fruiting, it is important to confirm that both MAT-HMG and MAT-alpha are present in strains selected for artificial cultivation.

According to the proportion of isolates which contained different kinds of mating type genes in this study, we supposed that the two nucleuses of *Cordyceps militaris* heterocaryons, containing MAT-alpha and MAT-HMG respectively, would be packed by plasma membrane with same probability when they formed the asexual spores. Then three kinds of asexual spores would appear, containing MAT-alpha genes only and containing MAT-HMG only and containing them both. Asexual spores only containing MAT-alpha or MAT-HMG would grow to homocaryotic mycelia, while asexual spores containing both would grow to heterocaryotic mycelia. The speculation should be further affirmed by morphological observation.

REFERENCES

[1] Wei Wu et al. (2000). Review on Studies and Applications of *Cordyceps militaris*. *Acta Agriculturae Shanghai*. 16 (s1): 99-104.

[2] Shuqian Lin et al. (1997). Study on and Development of *Cordyceps (Fr.) Link*. *Edible Fungi of China*. (1):12

[3] Minfeng Li (2007). Molecular Biology Studies on the Different Phenotypes of Fruiting-body Forming of *Cordyceps Militaris*. *Dissertation for Master Degree of Guizhou University*.

[4] Lukens L. et al. (1996). Correlation of genetic and physical maps at the A mating type locus of *Coprinus cinereus*. *Genetics.* 144:1471-1477.

[5] Xinhua GAO.(2008). Mating types of *Cordyceps militaris*. *Acta Edulis Fungi.*15(1):1-5.

[6] Meina Li et al. (2003). Molecular analysis of degeneration of artificial planted *Cordyceps militaris*. *Mycosystema.* 22(2): 277-282.