African Journal of Microbiology Research Vol.6 (24), pp. 5215-5221, 28 June, 2012
Available online at http://www.academicjournals.org/AJMR
DOI:10.5897/AJMR12.522
ISSN 1993-8233 ©2012 Academic Journals

Full Length Research Paper

A molecular genetic study on fruiting-body formation of *Cordyceps militaris*

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Accepted 9 June, 2102

In the fungal genus *Cordyceps,* the type species *C. militaris* produces bioactive ingredients and exhibits medicinal effects as a Traditional Chinese Medicine (TCM). Currently, the fruiting-body of *C. militaris* has been artificially mass-produced as functional food and medicine in China. The unstable variation in forming fruiting-body is however a restriction in the production. The genetic study on perithecial stromata (fruiting-body) formation *in vitro* of C. *militaris* has not yet been reported. In this study, we report the effect of genetic variation including possession of the mating system on perithecial stromata formation of *C. militaris*. The results showed that the mono-conidial isolates with both MAT1-1-1 and MAT1-2-1 (genotype MAT1-1/2) produced stromata. While the isolates having only either MAT1-1-1 or MAT1-2-1 failed to produce stromata. Despite obvious heterothallism, homothallism was occasionally observed in a few isolates of *C. militaris*. Genetic variation was observed amongst the different mono-conidial isolates of *C. militaris*. The unstable variation or loss of fruiting-body formation was caused by the inner-specific genetic variation.

Key words: *Cordyceps militaris*, molecular genetics, genetic variation, homothallism, heterothallism, fruiting-body.

INTRODUCTION

The old genus *Cordyceps* (Fr.) Link (now belonging to the family of *Cordycipitaceae*, *Ophiocordycipitaceae* and *Clavicipitaceae*) is a large, cosmopolitan family, comprising of 460 to 500 species and varieties (Liu, 1999; Sung et al., 2007). Most of its members are pathogenic to different insects, spiders, and few grow on hypogeal fungi of *Elaphomyces* spp. They are mainly distributed in sub-tropical to temperate regions of the world. In China and East Asia, many species of *Cordyceps* have been utilized as medicinal mushrooms for thousands of years. *C. militaris,* the type species of the genus, has been recently used as functional food and medicine in China. The pharmaceutical component namely cordycepin produced by *C. militaris* has been found to be effective in antitumor (Overgaard-Hansen, 1964), antivirus (De Julian-Ortiz et al., 1999), antileukemic (Kodama et al., 2000), preventing and treating of obesity (Kim et al., 2008) and hypolipidemic (Zhu et al., 2003).

C. militaris has been studied for the commercial production of bioactive compounds through their *in vitro* culture (Basith and Madelin, 1968; Pen, 1995; Wen et al., 2008; 2009). Artificial culturing of *C. militaris* is a good way to solve the insufficient resource from the nature. However, in the process of artificial culturing, the isolates of *C. militaris* showed unstable variation in forming fruiting-body. Most of the isolates failed to produce fruiting-body or produced only few deformed ones. Meanwhile other isolates, which initially produced good fruiting-body, could not produce fruiting-body with the same quality in subsequent subcultures. The degenerating of the isolates in forming fruiting-body has become a key restrictive factor in industrial production. Up-to-date there were few reports on molecular genetic

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study of variation in fruiting-body formation and mating system of *C. militaris* (Shrestha et al., 2004; Yokoyama et al., 2005). Sato and Shimazu (2002) considered that *C. militaris* had homothallism based on the research on lepidopteran pupae. On the other hand, Shrestha et al. (2004) reported that *C. militaris* behaved as a bipolar heterothallic fungus and required two compatible mating strains in order to produce regular clubshaped perithecial stromata.

In this study, molecular genetics of *in vitro* stromata formation of *C. militaris* has been carried out based on mono-conidial isolates and their offspring. Mating system was studied via observation of perithecial stromata formation and PCR assay. Herewith, we report the effects of genetic variation including the mating system on perithecial stromata formation and both heterothallic and homothallic sexual behavior of *C. militaris.*

MATERIALS AND METHODS

Fungal culture

C. militaris isolate CGMCC2459 (namely Ori-S) were originally isolated from Sichuan province of China. The isolate was isolated from wild perithecial stromata and maintained on PDA medium at 25°C for 4 days.

22 mono-conidial isolates (named SSP1 to SSP22) were established from the original isolates (Liang and Fox, 1997). Ori-S was cultured until sporulation was evident (approximately 15 days). Conidia and the hyphae attached were removed with a loop and transferred to fresh medium. This was repeated for twelve successive *in vitro* sub-cultures (namely Deg-S). All cultures were stored at 2°C until use.

Inoculum preparation and fruiting

C. militaris was initially cultured on PDA in a Petri dish, and then transferred to the seed culture by punching out 5 mm of the agar disc with a sterilized self-designed cutter. The seed culture was in a 250 ml flask containing 50 ml of basal medium (20 g/L sucrose, 20 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 1 g/L K_2HPO_4 with 1000 ml distilled water), on a rotary shaking incubator, 26°C, 130 rev/min for 4 days. Fruiting medium of *C. militaris* was prepared by mixing 20 g of rice and 32 ml of liquid medium (20 g/L sucrose, 20 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 1 g/L K_2HPO_4 with 1000 ml distilled water) in cylindrical glass bottle and were autoclaved for 20 min at 121°C. Each glass bottle containing fruiting medium was inoculated with 5 ml of liquid inoculum of *C. militaris* for *in vitro* fruiting. After inoculation, the bottle were incubated at 20°C under dark for 10 days, then under 14:10 L:D (500 lux light) at 25°C and high humidity conditions (80 to 90%) for 50 days. All experiments were performed at least in duplicate (10 bottles as one treatment).

DNA extraction and reagents

Taq enzyme and dNTPs was purchased from Shanghai Sangon. An Agarose Gel DNA Purification kit ver 2.0 was purchased from TRKARA Company. Fresh, sporulating cultures on Czapek agar were used for DNA extraction following Tigano-Milani et al. (1995); the extracted DNA is stored at -20°C.

PCR amplification and determination of DNA sequences

In the first preliminary experiments, when different DNA extraction methods were compared, RAPD-PCR amplifications were performed with an Gene Amp PCR system 9700 (Bio-RAD) with a modified RAPD program (one cycle of 60 s at 95°C followed by 40 cycles of 20 s at 94°C, 60 s at 36°C and 60 s at 72°C) . 50 µl reaction system: 10× reaction buffer 5 µl, 10 mM dNTPs 0.66 µl, random primer 2 µl, 25 mM $MgC1_2$ 5 µl, 3 µl of template DNA (50 ng/µl), Taq DNA polymerase 0.66 µl, ddH_2O 33.68µl and 165 random 10-base oligonucleotide primers (Shanghai Sangon, Shanghai, China) were used in these experiments.

RESULTS AND DISCUSSION

Morphological polymorphism and fruiting

Amongst the 24 isolates, isolates SSP1 and SSP3 (mono-conidial) and ORI-S (original) and Deg-S (sub-culture) produced perithecial club-shaped stromata. The other twenty mono-conidial isolates SSP2 and SSP4 to SSP22 produced either no stromata or only abnormal nonperithecial stromata.

All the 24 isolates from the same original isolate differed significantly in their ability to form fruiting-body, morphological characteristics and mycelium growth rate. In particular, the difference in the 20 no fruiting mono-conidial isolates SSP2 and SSP4 to SSP22 were also significantly different. These results confirmed the poly-morphism in the anamorph of *C. militaris* (Liang and Fox, 1990, 1998).

RAPD

Initial screening of 165 RAPD primers resulted in the identification of 25 primers that yielded unambiguously scorable bands with high reproducibility. These primers amplified between 5 and 15 bands each. The molecular weight of the PCR products ranged between 200 to 2500 bp (Figure 1).

The 25 oligonucleotide primers produced a total of 247 bands from the 6 isolates including ORI-S, DEG-S, SSP2, SSP7, SSP19 and SSP21. Amongst them, 235 RAPD markers showed polymorphism (95.14%) (Table 1). The primers of S23, S30, S37, S46, S51, S61, S67, S80, S92, S103, S151, S153, S219 and S354 presented the highest polymorphism (100% of the bands).

Genetic distances

The genetic distances were calculated amongst the 6 isolates based on null allele frequencies within each isolate (Table 2). There was great variation in the genetic distances matrix. The longest genetic distance, 0.4890, was encountered between SSP2 and DEG-S, followed by SSP2 and SSP7 (0.4889), SSP19 and SSP7 (0.4777).

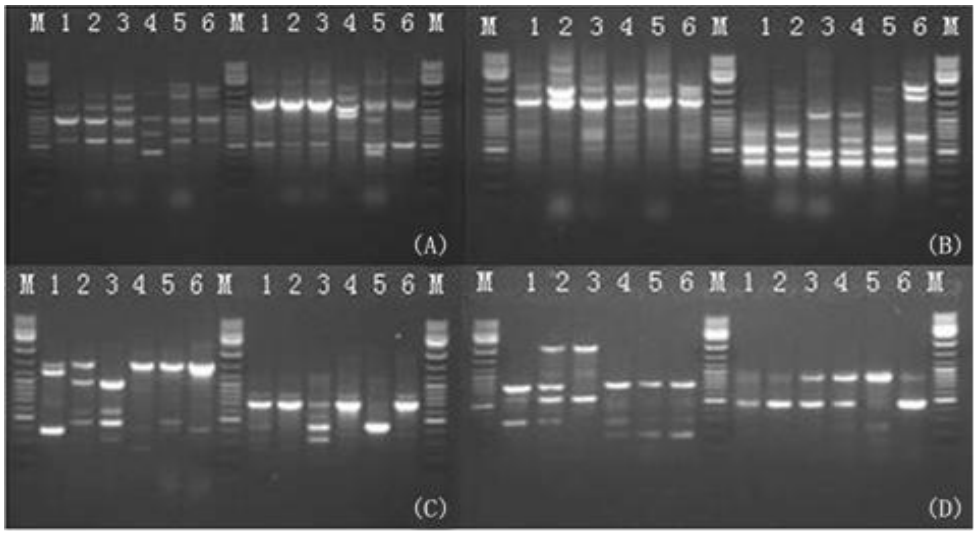

Figure 1. Comparison of amplification patterns obtained by random amplified polymorphic DNA (RAPD) with the 8 primers selected (A. S151 and S153; B. S140 and S301; C. S37 and S61; D. S23 and S20) from genomic DNA of the 6 isolates (from left to right): 1. ORI-S; 2. DEG-S; 3. SSP19; 4. SSP2; 5. SSP7; 6. SSP21; M: molecular weight marker (SM0331 mix DNA ladder, Fermentas, Burlington, Canada).

Table 1. Polymorphism provided by the RAPD primers.

Primers codes	Nucleotide sequence	Number of scorable PCR products	Number of polymorphic PCR products
S3	CATCCCCCTG	15	14
S6	TGCTCTGCCC	12	10
S20	GGACCCTTAC	5	4
S23	AGTCAGCCAC	8	8
S26	GGTCCCTGAC	7	6
S30	GTGATCGCAG	9	9
S37	GACCGCTTGT	10	10
S46	ACCTGAACGG	12	12
S51	AGCGCCATTG	11	11
S61	TTCGAGCCAG	8	8
S67	GTCCCGACGA	9	9
S79	GTTGCCAGCC	9	8
S80	ACTTCGCCAC	12	12
S90	AGGGCCGTCT	10	9
S92	CAGCTCACGA	10	10
S103	AGACGTCCAC	11	11
S136	GGAGTACTGG	11	10
S140	GGTCTAGAGG	12	10
S151	GAGTCTCAGG	11	11
S153	CCCGATTCGG	12	12
S216	GGTGAACGCT	8	7
S219	GTCCGTATGG	13	13
S301	CTGGGCACGA	14	13
S354	CACCCGGATG	8	8
S360	AAGCGGCCTC	10	9
Total		247	235

Table 2. Estimation of matrix genetic distances between the 6 isolates with enset clones studied using RAPD.

	ORI-S	DEG-S	SSP19	SSP2	SSP7	SSP21
ORI-S	0.0000					
DEG-S	0.3026	0.0000				
SSP19	0.3765	0.2834	0.0000			
SSP2	0.4656	0.4890	0.4534	0.0000		
SSP7	0.3865	0.4374	0.4777	0.4889	0.0000	
SSP21	0.3906	0.4009	0.4656	0.3684	0.4089	0.0000

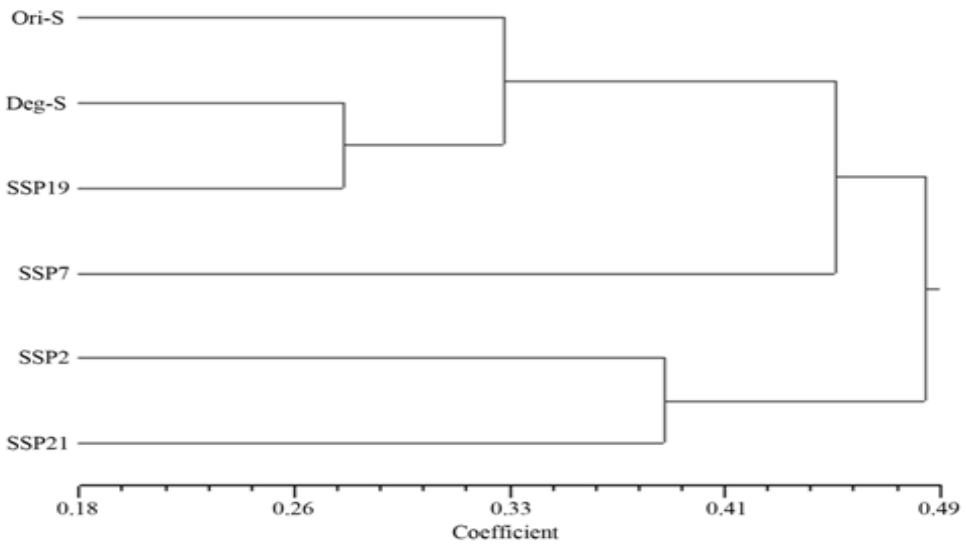

Figure 2. UPGMA dendrogram calculated from RAPD profiles in enset, based on matrix genetic distances among the 6 isolates.

The shortest distance, 0.2834, was between SSP19 and Deg-S, followed by ORI-S and DEG-S (0.3026). These genetic distances and phenotypes were positively correlated. For example, ORI-S and DEG-S (0.3026) have the same colony and both of them can produce perithecial stromata. SSP19 and ORI-S (0.3765), SSP19 and DEG-S (0.2834) also have shorter distance along with their same colony characteristics.

Based on the analysis of all primers, the average genetic distances between ORI-S and no fruiting isolates, DEG-S and no fruiting isolates, ORI-S and DEG-S, and the all no fruiting isolates were 0.4048, 0.4027, 0.3026, and 0.4438, respectively. The average distance of 0.4438 between no fruiting isolates showed higher diversity than those of others. These results also indicated that inner-species genetic diversity of *C. militaris* was indeed high.

Cluster analysis

A dendrogram (Figure 2) was constructed using UPGMA program based on the matrix of genetic distance among the 6 isolates in which ORI-S, DEG-S, SSP7 and SSP19 formed one cluster, while SSP2 and SSP21 formed another. Within these two clusters, SSP19 and DEG-S are more closely related isolates. This result correlated well with phenotype similarity coefficients.

PCR-based assay of the genotype MAT1-2 and fruiting

The primer sets of MAT1-1-1 and MAT1-2-1 used in this study (Yokoyama et al., 2004) could amplify the MAT1-1-1 gene of the 11 isolates and the MAT1-2-1 gene of the 13 isolates of *C. militaris* with a molecular weight of 220 to 250 bp respectively (Table 3, Figure 3). These results indicated that ORI-S, DEG-S, SSP1 and SSP3 which produced perithecial stromata possessed both MAT1-1-1 and MAT1-2-1 genes namely the genotype MAT1-1/2. While the other isolates which could not produce stromata possessed only either MAT1-1-1 or MAT1-2-1 namely the genotype MAT1-1 or MAT1-2.

Isolates ORI-S and DEG-S, which were not derived

Table 3. Fruiting-body formation and mating type test of C. *militaris.*

Strain	Mating type	Fruit body formation*	Fruit body dry weight (g/bottle) †
ORI-S	MAT1-1/2	10/10	2.37
DEG-S	MAT1-1/2	10/10	1.92
SSP1	MAT1-1/2	8/10	2.13
SSP3	MAT1-1/2	6/10	1.42
SSP5	MAT1-1	No fruiting	0
SSP6	MAT1-1	No fruiting	0
SSP8	MAT1-1	No fruiting	0
SSP9	MAT1-1	No fruiting	0
SSP10	MAT1-1	No fruiting	0
SSP12	MAT1-1	No fruiting	0
SSP14	MAT1-1	No fruiting	0
SSP2	MAT1-2	No fruiting	0
SSP4	MAT1-2	No fruiting	0
SSP7	MAT1-2	No fruiting	0
SSP11	MAT1-2	No fruiting	0
SSP13	MAT1-2	No fruiting	0
SSP15	MAT1-2	No fruiting	0
SSP16	MAT1-2	No fruiting	0
SSP19	MAT1-2	No fruiting	0
SSP21	MAT1-2	No fruiting	0

* Fruiting-body formation was examined in 10 trials. † Values are mean of all fruiting-body formation determinations.

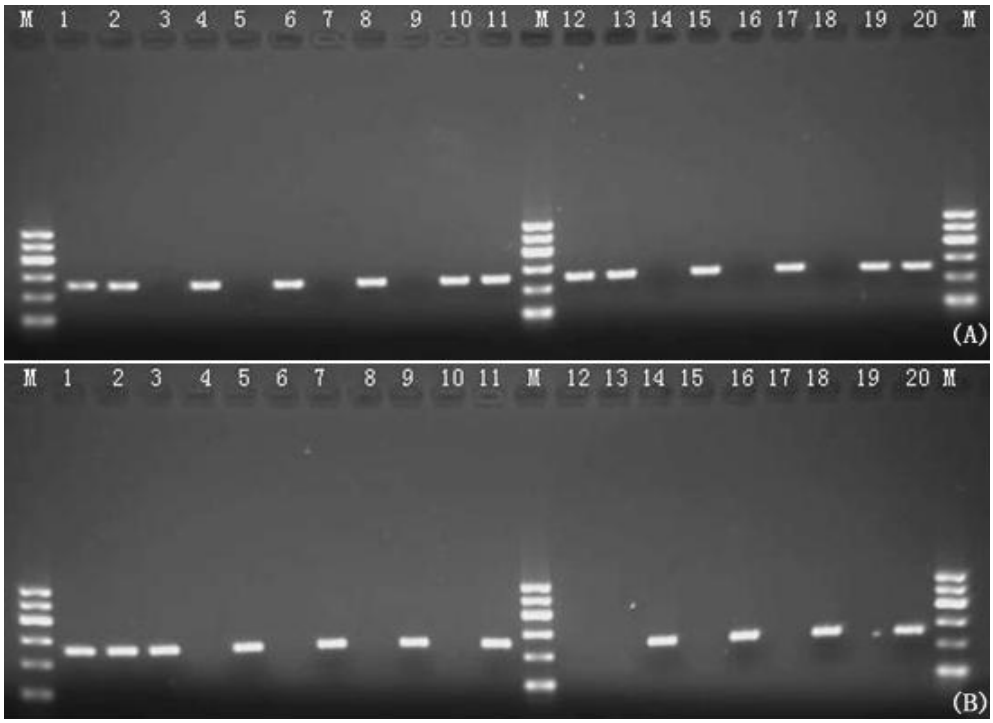

Figure 3. Results of PCR assay for the MAT1-2-1(A) and MAT1-1-1 (B) genes. The PCR products were electrophoresed on a 1.5% agarose gel. Lane M is a 100bp DNA ladder (Fermentas, Burlington, Canada). Lanes 1 to 20 show the PCR products of *MAT1-2-1*(A) and *MAT1-1-1* (B) genes from *C. militaris* isolates ORI-S, DEG-S, SSP9, SSP2, SSP6, SSP4, SSP5, SSP7, SSP10, SSP16, SSP1, SSP11, SSP13, SSP12, SSP15, SSP8, SSP19, SP14, SSP21 and SSP3, respectively.

from single conidium but a mass of conidium with a heterothallic mixture of MAT1-1 and MAT1-2 cells, possessed both MAT1-1-1 and MAT1-2-1 genes. However, isolates SSP1 and SSP3 which were derived from mono-conidium also possessed both MAT1-1-1 and MAT1-2-1 genes. One fungus that contained both homothallic and heterothallic mating system was reported in *Candida albicans* (Kevin, 2009), *Botrytinia fuckeliana* and *Chromocrea spinulosa* (Coppin et al., 1997). Our study demonstrated that *C. militaris* was both homothallic and heterothallic as isolates SSP1 and SSP3 were homothallic with the genotype MAT1-1/2, while the other isolates which possessed only either MAT1-1-1 or MAT1-2-1 with the genotype MAT1-1 or MAT1-2 were heterothallic.

Mating-type genes control sexual reproduction, which is the hub of the whole sexual reproduction process. Recently, heterothallism in *Cordyceps takaomontana* have been reported and the mating type loci of *C. takaomontana* have been sequenced (Yokoyama et al., 2005; Eiji et al., 2005). Besides *Clavicipitaceae* family, mating systems of other filamentous *Ascomycetes* have been described and mating type loci have been sequenced. Until now, it has not been fully understood why certain single ascospore or conidial strain of heterothallic filamentous *Ascomycetous* species behave as homothallic mating system. 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 (Shrestha et al., 2004). Mating type heterokaryosis and self-fertility have been recently reported in *Cryphonectria parasitica* (McGuire et al., 2004). Similarly, it has been reported that a mating system with multiple mating type alleles exist in the filamentous ascomycete *Glomerella cingulata* (Cisar and TeBeest, 1999).

Wang et al. (2008) reported that the genetic diversity of inter-species in *Cordyceps* was extremely small and did not correlate with geographical origins and types. In contrast, genetic variation from different monoconidial isolates and their serial sub-culture of inner-species in *C. militaris* was found in this study. It was therefore concluded that the reason for *C. militaris* showing unstable variation in fruiting-body formation was genetic variation of inner-species. Furthermore, the instability could reflect their loss of genotype of MAT1-1/2 in the same culture after serial sub-cultures of the same isolate (most common sub-culture method is streak plate method, there were only a few conidials random transferred to the next sub-culture as seed once a time).

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (NSFC No. 30870009), the Natural Science Foundation of Educational Council of Guizhou Province (No.[2009]0129) and the Agricultural Science and Technology Foundation of Guizhou Provincial Government of China (No. [2011]3054).

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