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Study of mating type ratio and colony sectorization of asexual spores on the progress of *Cordyceps militaris* degeneration

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ABSTRACT: *Cordyceps militaris* stands out as one of the limited species within the Cordyceps genus fungal that has the capability of being cultivated artificially, but the fruiting bodies yield was reduced along with subculture which have seriously hindered the development of the *C. militaris* industry. This paper primarily studied effect of asexual spores on fruiting bodies production of *C. militaris*. First, the mating type (MAT) and distribution ratio of individual asexual spores were investigated. Further, single asexual spores were randomly selected to subculture individually until visible colony sectorization produced; then, the fruiting bodies production and the genetic diversity by sequence-related amplified polymorphism (SRAP) polymerase chain reaction (PCR) of asexual spores from different colony morphologies were investigated. The results showed that *C. militaris* single asexual spores were homokaryotic. Asexual spores ratio of MAT-1-1:MAT-1-2 was about 1:2 in normal strain, which can produce fruiting bodies; but the mating type of single asexual spores from degenerated strains were the same. The strain of CMS1-3 (MAT-1-2) produced sectors after subculturing for 3 generations; asexual spores of sectorial colony could not produce fruiting bodies after cross-mating. The SRAP PCR results showed that the DNA of asexual spores isolated from different colonies have changed during subculturing. The results indicated that the imbalance of MAT ratio in individual asexual spores, along with the creation of mutants that fail to produce fruiting bodies, can significantly reduce the production of *C. militaris* fruiting bodies, potentially leading to a total loss of their ability to form fruiting bodies.

KEYWORDS: *Cordyceps militaris*, single asexual spores, degeneration, cross-mating, mating type

INTRODUCTION

Cordyceps militaris, classified under the family Cordycipitaceae within the order Hypocreales, class Sordariomycetes, and phylum Ascomycota, is listed on the Index Fungorum website (https://www.indexfungorum.org), accessed on December 21, 2023 [1]. It is edible and also used as medicinal fungus [2–4] contributed by various bioactive compounds, including cordycepin, cordycepic acid and polysaccharides [5–7]. Due to the scarcity of wild fruiting bodies and high market demands, artificially cultivated fruiting bodies, which offer higher production and enhanced chemical components, have been widely adopted as substitutes [8]. *C. militaris* fruiting bodies can be artificially cultivated on various substrates and insect pupae, as nature is an insufficient resource [9–12]. However, the ability to produce fruiting bodies will be reduced or even lost during preservation or subculturing, which is the degeneration of *C. militaris*. Strain degeneration is frequent, irreversible, and inheritable, which has become a major problem for industrial processes [13, 14]. *C. militaris* strain degeneration has been reported in previous studies, but the mechanism of degeneration is still unclear [15, 16]. Thus, the mechanism of strain degeneration is a topic of current interest in *C. militaris*.

C. militaris is a bipolar heterothallic fungus. and its sexual reproduction is regulated by mating type genes [17]. *C. militaris* has two mating type (MAT) loci, *MAT-α* box containing two genes, *MAT1-1-1* and *MAT1-1-2*; and *MAT-HMG* box containing one gene, *MAT1-2-1*. Partial function of MAT genes had been revealed, and *MAT1-1-2* is essential for ascospore genesis [18]. Homokaryotes, which contain only one MAT gene (either *MAT-α* or *MAT-HMG*), are unable to complete their sexual life cycle [19]. Additionally, cross-mating is an effective method to rejuvenate strain [20–22]. MAT switch is thought to be a cause of strain degeneration, but the reason remains unclear.

In history of fungal life, a large number of asexual spores are produced during mycelial growth [23]. Consequently, asexual spore production is an ideal reason for production decline and degeneration of fruiting bodies. To date, there have been limited studies on the impact of asexual spores in sub-cultured conditions. The aim of the present study was to investigate how the MAT and diversity of asexual spores affect the production and degeneration of fruiting bodies.

MATERIALS AND METHODS

Fungal material and medium

C. militaris normal strains were obtained from the Shihezi University of Life Science and Key Laboratory of Agricultural Biotechnology (Xinjiang, China; 44.3059° N latitude and 86.0419° E longitude). Modified potato dextrose agar medium (PDA) (200 g potato extract, 5 g peptone, 20 g glucose, 1.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.01 g vitamin B_1, 15 g/l agar in 1000 ml distilled water) and liquid medium have the same composition as modified PDA except without agar were used.

Screen of strains and isolation of single asexual spores

Parental strains were activated using a modified PDA medium at 25 °C in darkness. Subsequently, a colony was transferred to 50 ml of liquid medium and subjected to incubation at 25 °C × 120 rpm for 4 days in shaking incubation (Yiheng Scientific Inc., Shanghai, China). The fruiting bodies were cultivated in wheat medium as previously described [24]. Briefly, each box of substrates was inoculated with 5 ml spawn and, then, cultured at 20–25 °C for 10 days in darkness. Following this period, the boxes were moved to a culture room where they were exposed to scattered sunlight at 22–25 °C for 14 h and in darkness at 12 °C for 10 h in each day for a total 7 days. The culture room was then maintained at 22 °C with a 10 h (light):14 h (dark) and humidity of 70–80% for 45 days. The remaining culture was filtered through four layers of sterile gauze. The suspension was diluted to 10^5 spores/ml. A 100 µl suspension was spread on the 2% agar medium and incubated to obtain single colonies. Small agar blocks, each containing a single round microcolony, were carefully cut out and transferred onto modified PDA medium [25]. These samples were then incubated at a temperature of 25 °C for a duration of 14 days.

Identification of MAT in asexual spores

The hyphae of asexual spores were scraped from the surface of the medium, and genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, a widely used technique for DNA isolation [26]. Briefly, 0.2 g of fresh mycelium was placed into tissue grinder tube containing approximately 0.6 ml DNA extraction buffer (50 mm/l Tris-HCl (PH 8.0), 35 mmol/l EDTA, 0.1% PVP-K30) and crushed to disperse the mycelium. Then, 0.5 ml extracting solution buffer (10 mmol/l, Tris-HCl, pH 8.0, 1 mmol/l EDTA, 0.3 mol/l NaAc, 1.2% PVP-K30) was added to the tube and incubated at 65 °C for 90 min. After incubation, 0.4 ml of chloroform was added, and the tube was centrifuged (Eppendorf, Shanghai, China) at 14,000 rpm for 3 min. The supernatant was transferred into a new tube, and DNA was precipitated in a 0.6 vol of 2-propanol. The DNA was then washed with 70% ethanol and dissolved in TE buffer. MAT of asexual spores was detected by Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Primers were designed based on MAT1-1 and MAT1-2 (Table S1). PCR amplifications were: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s; and a final extension of 72 °C for 10 min. The PCR products were visualized using 1.2% agarose gel electrophoresis.

Asexual spores subculturing and characteristics of different colony morphology

Single spore of CMS1-2 (*MAT*-α) and CMS1-3 (*MAT-HMG*) were sub-cultured from the edge of the colony and incubated at 25 °C under scattered light for 14 days until sectorial colonies were detected. Sectorial and non-sectorial colonies were transferred onto fresh PDA plates, and single spores were isolated. The spores from different colonies were randomly selected to assess fruiting body by cross-mating. The mycelial growth, pigmentation, and fruiting body development of different combinations were recorded. DNA of asexual spores from CMS1-2-1, CMS1-2-4, CMS1-2-6, CMS1-2-8, CMS1-3N-1, CMS1-3N-2, CMS1-3S-8, CMS1-3S-11 were used to PCR. PCR amplification was carried out with EM1-EM9 and ME1-ME17 primers (Table S1) to finish SRAP (Random amplified polymorphic DNA) analyses. PCR amplification was carried out with an initial denaturation step of 94 °C for 5 min, followed by 8 cycles of 94 °C for 30 s, 35 °C for 30 s and 72 °C for 1 min; and 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min [26]. The samples were subjected to analysis with 2% agarose electrophoresis.

RESULTS

Distribution ratio of MAT among single asexual spores of different strains

The CMS1 strain is capable of producing fruiting bodies, while the CMZ1 and CMS5 strains have shown degenerative features. Moreover, each of these strains is characterized by being heterokaryotic. Asexual spores can easily germinate, and small colonies appeared after 4 days on 2% water agar. Asexual spores derived from the CMS1 strain exhibited a varied nuclear composition, with a ratio of MAT-α to MAT-HMG being 7:15, (Fig. 1A,B). Conversely, single asexual spores isolated from the CMZ1 strain were exclusively of the MAT-HMG type (Fig. 1C,D). Additionally, the asexual spores from the CMS5 strain were identified as MAT-α (Fig. 1E,F).

Fruiting bodies production of parental strain and its offspring by cross mating

The single asexual spore strains with opposite mating type from CMS1 were selected for crossing. The results

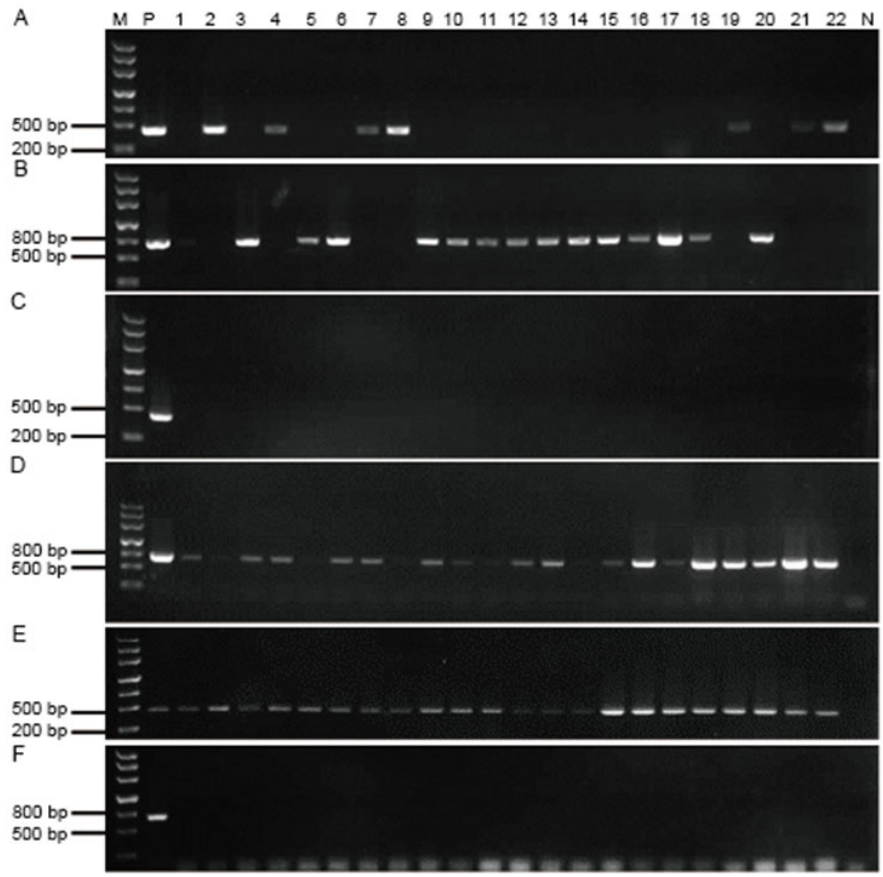

Fig. 1 PCR assay for MATs of single asexual spores. M: molecular III, P: parental strain; 1–23: single asexual spore strain; N: Negative control; A and B: asexual spores isolated from CMS1; C and D: asexual spores isolated from CMZ1; E and F: asexual spores isolated from CMS5; A, C, E: PCR products with MAT-α primers; B, D, F: PCR products with MAT-HMG primers.

showed that most single asexual spores strains could produce fruiting bodies by cross-mating. Within the various pairings, the combination of CMS1-2 (MAT-α) and CMS1-3 (MAT-HMG) resulted in a significantly higher yield of fruiting bodies, with a dry weight of 2.91 ± 0.12 g, and exhibited a greater number of perithecia compared to the parental strain, which had a dry weight of 1.73 ± 0.06 g, as shown in Fig. 2A and Fig. 2C, respectively. In contrast, the pairing of CMS1-3 (MAT-HMG) and CMS1-4 (MAT-α) led to the production of only a few undeveloped fruiting bodies (Fig. 2E). The fruiting bodies of CMS1-2 and CMS1-3 produced sexual perithecia (Fig. 2F).

Fungal colony of single spore during sub-culturing

The single asexual spores of CMS1-2 and CMS1-3 were sub-cultured on the modified PDA agar medium, there was no visible change after 3 generations in CMS1-2 (Fig. 3A), but visible sectorization was observed in CMS1-3 after 3 generations (Fig. 3B). The 3rd generation were named CMS1-2Z3 and CMS1-3Z3. The sectorization of CMS1-3Z3 in the colony exhibited a light-yellow color. There was a notable difference in the morphological characteristics of the asexual spores between CMS1-3Z3S (sectorized) and CMS1-3Z3N (non-sectorized). Additionally, the single spore strains derived from CMS1-3Z3N demonstrated the production of dense aerial mycelia (Fig. 3C).

Fruiting body assessment and genetic diversity

The single asexual spore strains from the third generation of CMS1-2Z3, CMS1-3Z3N, and CMS1-3Z3S were cross-mating on the wheat substrate. After incubation at 22 °C for 7 days, the mycelia thoroughly permeated the substrate, with no significant differences observed across various combinations. However, following a 7-day exposure to light, the mycelia of the combinations CMS1-2-1 × CMS1-3N-1, CMS1-2-4 × CMS1-3N-2, CMS1-2-4 × CMS1-3N-3, CMS1-2-6 × CMS1-3N-2, and CMS1-2-8 × CMS1-3N-3 remained white (Fig. 4A–E). After cultivation for 60 days, all combinations failed to produce fruiting bodies (Fig. 4I–M), and combi-

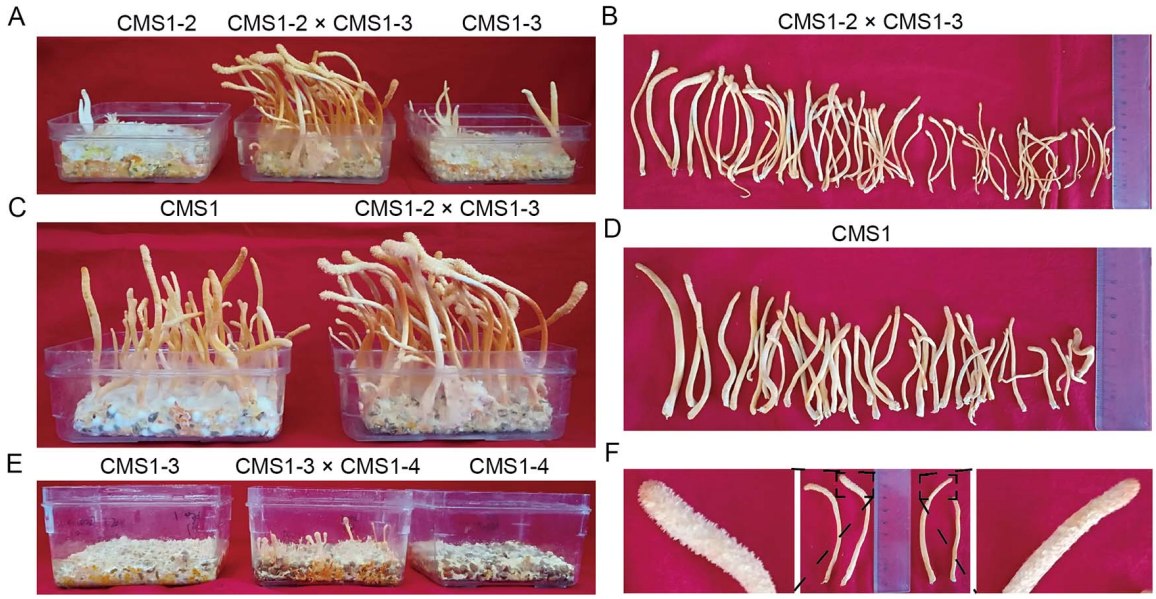

Fig. 2 Fruiting bodies produced from crossing of single spore strains. A: Fruiting bodies of CMS1-2, CMS1-3, CMS1-2 × CMS1-3; B: Fruiting bodies of CMS1-2 × CMS1-3; C: Fruiting bodies of CMS1 and CMS1-2 × CMS1-3; D: Fruiting bodies of CMS1; E: Fruiting bodies of CMS1-3, CMS1-4, CMS1-3 × CMS1-4; F: Fruiting bodies of CMS1-2 × CMS1-3 (left) and CMS1 (right) 20× magnification. Scale bars indicated 5 cm.

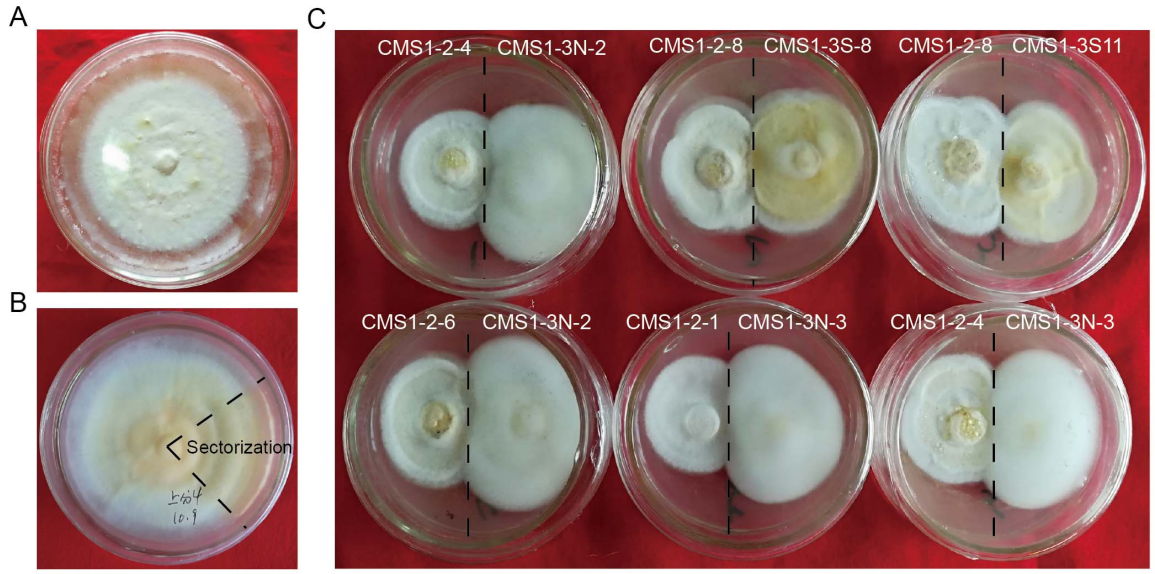

Fig. 3 Third generation colony features of single spores and asexual spore.s A: Colony features of CMS1-2Z3; B: Colony features of CMS1-3Z3; C: Colony features of single spore of CMS1-2Z3 and CMS1-3Z3. CMS1-2-1, CMS1-2-4, CMS1-2-6, CMS1-2-8 were isolated from CMS1-2Z3 (left of culture dish). CMS1-3N-2, CMS1-3N-3 were isolated from CMS1-3N (non-sectorization); CMS1-3S-8, CMS1-3S-11 were isolated from CMS1-3S (sectorization) (right of culture dish).

nation of CM1-2-1 × CMS1-3S-11, CMS1-2-8 × CMS1-3S-8, and CMS1-2-8 × CMS1-3S-11 became orange (Fig. 4F–H) and produced abundant fruiting bodies after cultivation for 60 days (Fig. 4N–P).

Twelve primer pairs, screened from the range of EM1-9 to ME1-17 SRAP primers, were successfully amplified, demonstrating stable amplification and rich polymorphism. These are listed in Table S1. Two

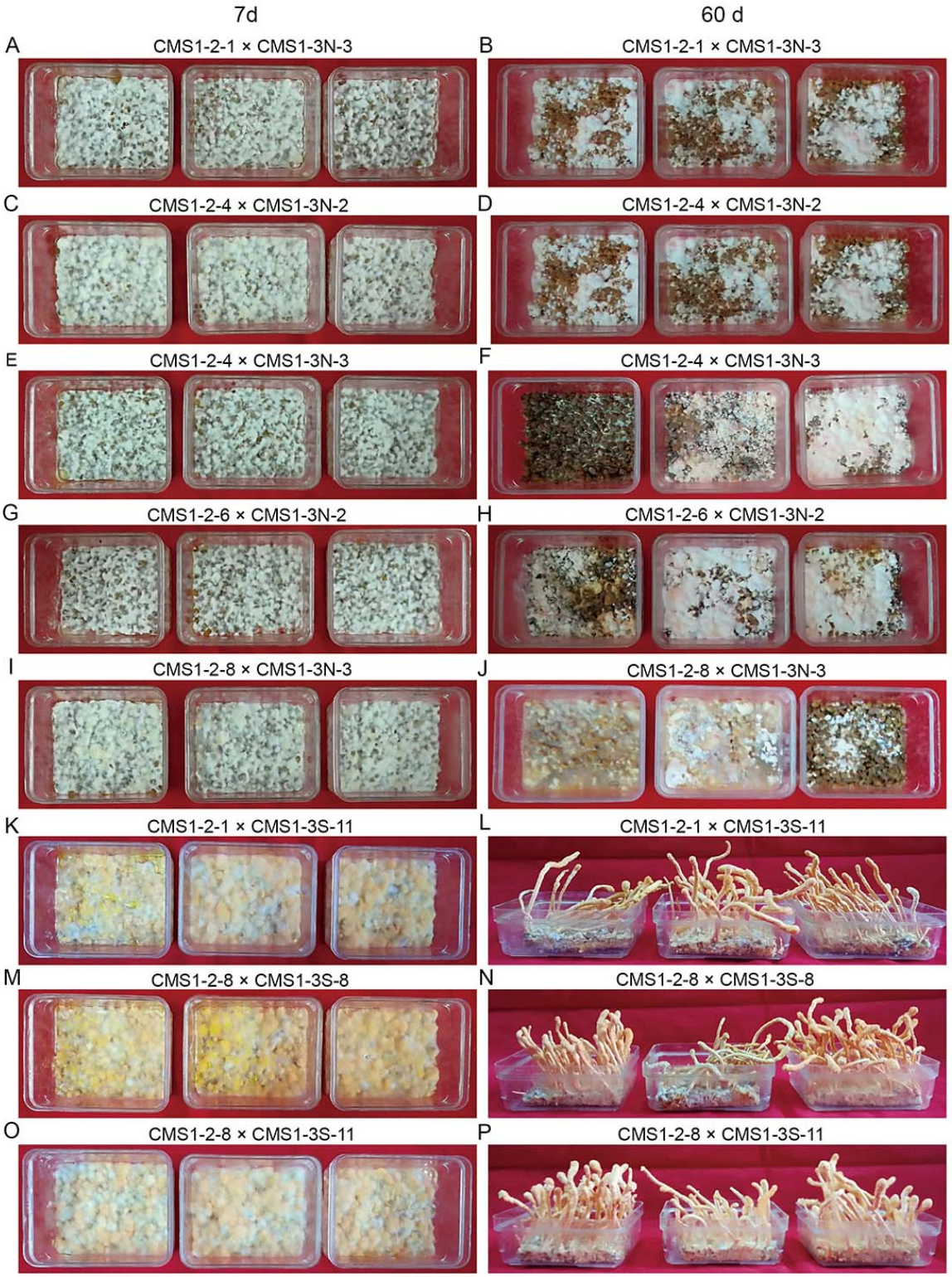

Fig. 4 Mycelium and fruiting body of crossing between different spores. A–H: Across various combinations of different single asexual spores from CMS1-2Z3 and CMS1-3Z3 strains exposed to light for 7 days; I–P: Across various combinations of different single asexual spores from CMS1-2Z3 and CMS1-3Z3 strains cultivated for 60 days. Scale bars indicate 5 cm.

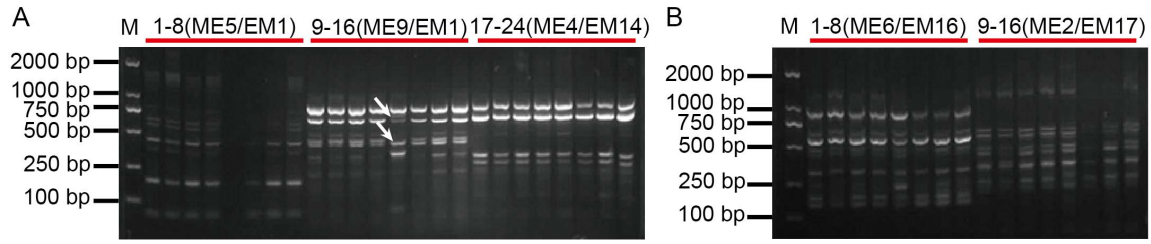

Fig. 5 SRAP analyses. M: Marker DS2000; 1, 9, 17: CMS1-2-1; 2, 10, 18: CMS1-2-4; 3, 11,19: CMS1-2-6; 4, 12, 20: CMS1-2-8; CMS1-3N-2: 5, 13, 21; CMS1-3N-3: 6, 14, 22; CMS1-3S-8: 7, 15, 23; CMS1-3S-11: 8, 16, 24.

polymorphic bands in CMS1-3N-2 were detected by the primer pair ME9/EM1 (Fig. 5A).

DISCUSSION

Fruiting bodies of *C. militaris* are edible and can be used for food and medicine, and it has been listed as a novel food by China. However, degenerated strains frequently occur during sub-culturing and result in great commercial losses. Degeneration of strains has been found in many edible fungi. As a bipolar heterothallic fungus, MAT switch could be one of the reasons causing degeneration [14, 20]. Asexual spores are produced during mycelial growth. Though lots of research found that *C. militaris* ascospores and asexual spores have three kinds of MAT: one of heterokaryon and two of homokaryon [13, 20, 24]. Conversely, our results showed that all single asexual spore strains were homokaryotic although the parental strains were heterokaryotic. Morphological characteristics and life periodic processes indicated single sexual spores are uninucleate [23], suggesting that heterokaryotic sexual spores could be due to false positives. The MAT ratio of single asexual spore was unequal in normal and degenerated strains. During subculturing, the mycelium generated a varying number of spores of two different MATs. It was observed that even in the normal strain, the ratio of the two types of asexual spores did not conform to a 1:1 distribution. Furthermore, the ratio of MATs in different sexual spores could be used to assess the extent of degradation.

Various studies have shown that cross-mating is an effective way to produce fruiting bodies [13, 20, 27]. The findings indicated that cross-mating between single spores isolated from the normal strain CMS1 resulted in the production of a greater number of fruiting bodies compared with the parental strain. However, it was noted that some combinations were unsuccessful in producing fruiting bodies. The results indicated that sterile single spore was one of the reasons that fruiting body production declined in the parental strain. As the number of sterile strains increased, the fruiting bodies production would decline until completely lost.

C. militaris strain was a mixture of original mycelia, and abundant spores were produced, leading to a complicated degeneration mechanism. Therefore, we focused on morphological and molecular changes of single spores during sub-culturing. The single spore of CMS1-3 formed a sectorization colony at the third generation. *C. militaris* mycelia produced carotenoid under light conditions and changed color from white to orange, which was necessary for fruiting body development [28]. The different pigmentation indicated a decrease in metabolic function of degenerated strains [29]. In *Metarhizium anisopliae*, sectorization experienced oxidative stress responses, a sign of aging [30]. Spores originating from non-sectorized sources were unable to undergo color change or produce fruiting bodies when cross-mated with spores of the opposite MAT. Additionally, mutations differentiating sectorization from non-sectorization were identified using SRAP technique, a method that aligns with findings from other research studies [31]. A detailed analysis of asexual spores might reveal the essence of strain degeneration. Fungal degeneration is not a rigid process, and it possibly enables the fungus to react to adverse conditions. In filamentous fungi, asexual spores are highly efficient for genome protection, survival, and propagation. Although research on the life cycle of *C. militaris* has been conducted for many years [32], the generation process and genetic diversity of asexual spores are still unclear. The present results showed that strain degeneration could be linked to DNA methylation, genetic mutations, harmful substance accumulation, and virus infections [33–35]. Asexual spores are produced by mitosis. Changing colony characteristics might be related to mutation or cytoplasmic genetic material [30], which also deserve further study.

CONCLUSION

Cordyceps miliatris fruiting bodies are important functional food and traditional medicine, but a high frequency of strain degeneration occurs during subculturing. The ratio of single asexual spore MAT was associated with fruiting bodies production. Sectorization was a sign of mutation, and sterile mutations

could occur during sub-culturing. Despite the observation of degeneration in single spores during sub-culturing, it was possible to obtain normal asexual spores capable of producing fruiting bodies through cross-mating. The degeneration of *C. militaris* was linked to the degeneration of asexual spores. Consequently, it was crucial to differentiate and selectively screen for non-degenerated asexual spore strains in the process of artificial sub-culturing. Changes in colonial morphology were indicative of strain mutation. Fertile asexual spores could be screened from parental strains based on MAT and colony characteristics of the asexual spores. However, strategies to slow down or prevent strain degeneration continue to pose a challenge. The simple genetic background of asexual spores could be advantageous in unraveling the mechanisms of degeneration in *C. militaris* and other fungi.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.074.

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Appendix A. Supplementary data

Table S1 List of SRAP primers.

Forward primer	Sequence (5′ → 3′)	Reverse primer	Sequence (5′ → 3′)
MAT1-1	GAGCCTACTATGGAACCC	MAT1-1	CAGGACTGATACCAGCAAA
MAT1-2	GCATCAACCCATTTGTGAAAGTTCT	MAT1-2	CCTGTCATAATGGTGCTGT
ME2	TGAGTCCAAACCGGAGC	EM1	GACTGCGTACGAATTAAT
ME4	TGAGTCCAAACCGGACC	EM 14	GACTGCGTACGAATTCAG
ME5	TGAGTCCAAACCGGAAG	EM 15	GACTGCGTACGAATTCTG
ME6	TGAGTCCAAACCGGTAG	EM 16	GACTGCGTACGAATTCGG
ME9	TGAGTCCAAACCGGTCA	EM 17	GACTGCGTACGAATTCCA