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Research article

Effect of successive subculture of *Cordyceps militaris* on growth, metabolites production and stability of *Rhf1* gene

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Abstract

Degeneration of *Cordyceps militaris* has been progressively studied because it is a highly valued fungus with strong market demand. The current study investigated mycelial growth, metabolite (adenosine and cordycepin) production and variations in the *Rhf1* gene (filamentous protein) during successive subcultures for five generations (G1–G5). The results indicated that the mycelial growth rate and biomass were initially consistent during G1–G2 and the degeneration initiated at G3 progressed until G5. The decrease was 22% (from 0.35 ± 0.01 cm/d to 0.27 ± 0.02 cm/d) and 42% (from 0.95 ± 0.04 g dry weight (DW) and 0.55 ± 0.01 g DW), respectively. There was a significant reduction in the adenosine and cordycepin production in G1 (1.97 ± 0.02 µg/g DW and 5.49 ± 0.11 µg/g DW) compared with G5 (0.03 ± 0.001 µg/g DW and 1.63 ± 0.02 µg/g DW), which was much for adenosine than cordycepin. Next, the gene copy number and gene expression of the *Rhf1* gene were compared among the five generations using quantitative real-time polymerase chain reaction, revealing that it significantly ($p < 0.05$) decreased after G3 compared to G1. Consistently, the percentage of DNA methylation of *Rhf1* was greater in G3 (more than 50% compared to G1). These results suggested that methylation may be associated with the stability of the *Rhf1* gene during subculture degeneration, thereby affecting *Rhf1* gene expression. Hence, the number of *Rhf1* gene copies may be useful in the determination of the quality of *C. militaris* before cultivation.

Introduction

Cordyceps militaris, an entomopathogenic fungus in the phylum *Ascomycota*, is a highly-valued, edible mushroom with important biological and pharmacological properties such as being immunomodulatory, antioxidant, anti-tumor, anti-inflammatory and hypotensive (Das et al., 2010). The fungus contains a series of bioactive metabolites, including cordycepin (3′-deoxyadenosine), adenosine, pentostatin and carotenoids (Zhou et al., 2009). In recent years, the market

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demand for artificially cultivated *C. militaris* has increased (Lou et al., 2019). Though the mushroom can be grown in culture, large-scale production is still a challenge.

Previous studies on the artificial cultivation of *C. militaris* discovered that its degeneration is caused by environmental and genetic variation, but is not associated with geographical origins (Wang et al., 2008). Optimization of fungal growth conditions and supplementing minerals to the growth media can delay the degeneration; furthermore, appropriate preservation can maintain fungal viability and reduce a reduction in fruiting body production (Liu et al., 2018b). Accumulation of cellular reactive oxygen species (ROS) in mycelia is another reason for the fungal growth degeneration (Xiong et al., 2013). Genetic variation studies have shown that *C. militaris* undergoes sexual or asexual reproduction (Lu et al., 2016; Yin et al., 2018), where the sexual reproduction is either heterothallic or homothallic and is controlled by the mating-type (MAT) loci in heterothallic mating-type genes (Wang et al., 2015a; Chen et al., 2017). Other genes also play roles in mycelial and fruiting body formation in *C. militaris*. The *Cmwc-1*, blue light receptor gene responds to light and is involved in fruiting body production (Jiao et al., 2018). The *Dash* gene is required for primordia during fruiting body development (Dong et al., 2013; Yang et al., 2016). The *Rhf1* gene was identified in the genome sequence of *C. militaris* (Zheng et al., 2011a), which is involved in the hyphal branching and fruiting body production of *C. militaris* (Jiang and Han, 2015).

Degeneration of *C. militaris* during subculturing has been reported with subcultured *C. militaris* strains initiating the degeneration in the third generation and having significant degeneration by the fourth generation (Yin et al., 2017), but the underlying mechanism is not yet known. The subculture degeneration effect has been assessed using genome, transcriptome and metabolome analysis (Chen et al., 2018a, 2019a). The transcriptome data of subcultured *C. militaris* suggested that the mechanism of strain degeneration is associated with toxin biosynthesis, energy metabolism, DNA methylation and chromosome remodeling (Yin et al., 2017). DNA methylation also impaired fungal growth and development, but to a different degree form distribution, and the function of DNA methylation varied greatly among fungal species (Zemach et al., 2010). Recently, it was reported that degenerative *C. militaris* strains have a higher DNA methylation level (0.56%) than normal strains (0.48%), which might be related to DNA replication and cell metabolism (Xin et al., 2019). It has been shown that DNA methylation is a dynamic process during sexual development in *C. militaris* (Wang et al., 2015b). however, there is only limited information regarding the degeneration of *C. militaris* during subculturing. Consequently, the current study, investigated alteration of the *Rhf1* gene and analyzed the bioactive compounds associated with the genetic instability of *C. militaris* during the progression of subculture. This investigation may provide useful information that can help to maintain the quality of *C. militaris*, which is important for its large-scale cultivation in industrial biotechnology (Shrestha et al., 2012; Lou et al., 2019).

Materials and Methods

Fungal subculture and growth determination

The *C. militaris* strain ATCC 34165 used in this study was maintained on potato dextrose agar (PDA) with 1% peptone at 4°C as stock. The fungal strain was grown on the same medium in a Petri dish at 20°C for 2 wk before being used. To study the subculturing effect, the fungal culture was prepared by blending at high speed for 10 s. The 10% of the inoculum was added to potato dextrose broth and incubated at 20°C for 3 wk. The process was repeated for five generations. *C. militaris* mycelia were grown on PDA and mycelia growth and dry weight were recorded every 5 d for 3 wk (10 replicates each generation), according to Sutton and Starzyk (1972), followed by freeze-drying and storage. The growth rates and biomass production levels of generations 1–5 (G1–G5) were determined using the total mycelial colony diameter (measured in centimeters) and the dry weight (in grams) by the cultivation time (in days). The percentage change from G1 to G5 was calculated was as: % Reduction = ((G1–G5)/G1) ×100), according to Stanbury et al. (2016).

Fungal genomic DNA extraction and gene amplification

Fungal genomic DNA was extracted using a Fungal DNA Mini Kit (Omega Biotek; USA) according to the manufacturer's protocol. The primers *Rhf1* (XM0066796.4) (216 bp) (Forward 5-CAACCCAACCAGCCACGCACCCTCC-3) and Reverse 5-GGAGTCTCGTCTGTTACCTGAATGG-3); *Actin* (XM00668935) (172bp) (Forward 5-GCCTTCTACGTCTCC ATCCA-3 and Reverse 5-TAG TCGGTAAGATCACGGCC-3) were used as described by Jiang and Han (2015). The oligonucleotides were synthesized by 1st BASE, Malaysia. The quality and quantity of DNA were estimated based on the optical density (OD) ration 260:280 using a spectrophotometer

(Nanodrop ND-1000; USA) as described by Sambrook and Russell (2001). Subsequently, fungal genomic DNA was diluted at 10 ng/µL. The polymerase chain reaction (PCR) mixture was composed of a DreamTaq Green PCR Master Mix (Thermo Scientific; USA) (2x), 1 µL of each 5µM of primer and 1 µL of genomic DNA adjusted with sterile distilled water. The PCR reaction was carried out in 20 µL volumes in an Mastercycler Nexus PCR Cycler (Eppendorf; Germany) using the following cycles: 95°C for 10 min, followed by 38 cycles of 95°C each15 s, 55°C each 10 s, 72°C each 1 min and 72°C for 10 min as modified from Jiang and Han (2015). The amplified products were examined using 2% agarose gel electrophoresis (Sambrook and Russell, 2001).

Fungal RNA extraction and cDNA synthesis

A sample (100 mg) of ground mycelia were used for extraction with liquid nitrogen. The total RNA preparation was carried out using a Fungal RNA extraction kit (Omega Biotek; USA), following the manufacturer's instructions. The RNA concentration was determined at 260 nm in a Nanodrop ND-1000 spectrophotometer (Sambrook and Russell, 2001) and stored at -80°C until further processing. Before cDNA synthesis, all RNA samples were treated with DNase (Bio-Rad; USA) to remove any traces of genomic DNA. A 0.5 µg sample of total RNA was prepared and digested with 1 unit/µL of DNase and incubated at room temperature for 1 hr before cleaning using an RNA purification kit (Thermo Scientific; USA). Total RNA (0.2 µg) was reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific; USA). A total volume of 20 µL mixture was made containing 500 ng of total RNA (each generation), 4 µL of 5x first strand buffer, 200 U/µL of RevertAid M-MuLV RT, 20 U/µL of RiboLock RNase Inhibitor, 1 µL 10 mM dNTP and oligo(dT)18 primer and then incubated for 60 min at 42°C. Finally, the reaction was terminated by heating at 70°C for 5 min.

Quantitation of a number of gene copies using quantitative polymerase chain reaction

Total genomic DNA (10 ng/µL) from each generation was used to analyze the number of gene copies (*Rhf1* and *Actin* as the reference genes). The quantitative polymerase chain reaction (qPCR) was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad; USA). The PCR mixture consisted of: 1 µL of template DNA (10 ng), 2 µL of EvaGreen (Biotium; USA), 0.2 µL of each forward and reverse *Rhf1* and *Actin* primer (0.5 µM) and added nuclease-free water to the final volume of 10 µL. The running qPCR condition were initiated by DNA denaturation at 95°C for 3 min, followed by 49 cycles of 95°C each for 5 s, 55°C for 10 s, 72°C for 20 s and 72°C for 10 min (modified from Jiang and Han, 2015). This work was performed in five replicates.

To determine the *Rhf1* and *Actin* amplicon, the gene products were amplified and cloned using a TOPO TA Cloning® Kit (Invitrogen; USA) and transformed to *E. coli* DH5α cells using the heat shock method by applying 42°C for 1 min (Sambrook and Russell, 2001). Subsequently, the positive clones were selected and extracted using a Rapid Mini Plasmid Kit® (Thermo Scientific; USA). The clones were confirmed using DNA sequencing analysis. The DNA standard curve was generated by plotting the threshold cycle (Ct) versus the logarithm of DNA concentrations of *Rhf1* and *Actin* using a series of 10-fold dilutions. The Ct values for each concentration were measured in five replicates and plotted against the logarithm of their template copy numbers. The copy number of each plasmid concentration was calculated based on the method from Lee et al. (2006) using the equation described by Whelan et al. (2003). The amplification efficiency (E) of the primers was calculated by using the equation 2 from Rasmussen (2001).

Quantification of Rhf1 transcript content

Reactions were performed in five replicates, with 50 ng of cDNA template. The qRT-PCR reactions were carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad; USA), following the amplification conditions described above using Evagreen (Biotium; USA) in a final volume of 20 µL. Standard curves (Ct versus log DNA copy number) were used to estimate *Rhf1* and *Actin* gene expression in mycelia for five generations as described above. Gene expression was expressed as the *Actin* and *Rhf1* cDNA copy number per 50 ng of total cDNA template.

Determination of adenosine and cordycepin

Standards of adenosine and cordycepin (both from Sigma-Aldrich; USA) were prepared at 1 mg/mL and then diluted to obtain the desired concentrations (0.39 µg/mL, 0.78 µg/mL, 1.36 µg/mL, 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL).

The crude extracts were prepared from mycelia by extracting with deionized water, incubating at 50°C for 3 hr and passing through a 0.45 µm membrane filter. The adenosine and cordycepin contents were analyzed using high-performance liquid chromatography, (HPLC; Waters Corporation; USA) by comparing to the standard compounds. This method was adapted from Xia et al. (2017). An aliquot of 1 µL was analyzed using a reversed-phase C18 column (4.6 mm × 250 mm, 5 µm particle size). The conditions used an isocratic 95:5 (0.2% of formic acid-to-methanol), flow rate of 0.2 mL/min and a column temperature 30°C. The adenosine and cordycepin were monitored and quantified at 260 nm. The metabolite production rate of each generation (expressed as µg/g dry weight/day) was determined by subtracting the amount of adenosine or cordycepin in mycelia after growing at day 21 with day 1, and divided by cultivation time (total 21 days) (Stanbury et al., 2016).

DNA methylation analysis

A 216 bp region of the *Rhf1* gene containing a *Hpa*II recognition site (CpG; 5′-CCGG-3′) was used to determine DNA methylation from G1–G5 using an EpiJet DNA methylation analysis kit (Thermo Scientific; USA). Fungal genomic DNA (200 ng) was prepared, followed by restriction cleavage using the isoschizomers *Hpa*II (sensitive to CpG methylation) or *Msp*I (insensitive to CpG methylation), according to Fulnecek and Kovarik (2014). A control with no enzyme was also prepared. Equal amounts of digested and undigested control and samples of genomic DNA were used with the master mix Evagreen® dye (Biotium; USA) according to the manufacturer's conditions. The qPCR reactions of all samples were performed in 20 µL of final volume consisting of 3 µL of template DNA, 2 µL of EvaGreen (Biotium; USA) and 0.2 µL of each forward and reverse primer of *Rhf1* as described above. To analyze the DNA methylation, changes in the threshold cycles of the digested and undigested DNA were used to calculate ΔCq as described in the manual for the EpiJet DNA methylation analysis kit (Thermo Scientific; USA).

Statistical analysis

All results were expressed as a mean ± SD. One-way analysis of variance (ANOVA) was used. Significant differences were determined based on Duncan's multiple range tests ($p < 0.05$). Data analysis used the SPSS version 19.0 software package (SPSS, Inc.; USA).

Results

Number of subcultures affecting mycelial growth rate and mycelium production of C. militaris

After subculturing, the mycelial growth and characteristics of mycelial morphology of the *C. militaris* were evaluated, as shown in Fig. 1 and 2. It was clear that the number of subcultures affected the fungal mycelial growth and biomass production of *C. militaris* but there were no changes in the fungal morphology during subculturing. The fungal colonies were circular in shape and had smooth edges; however, a pigmented appearance was slightly delayed compared to early subcultures after exposure to light for 7 d (Fig. 1A). Here, the G1 and G2 generations showed consistent growth based on measuring the mycelial colony diameters (7.67 ± 0.28 cm and 7.18 ± 0.10 cm, respectively, and $p < 0.05$). In the third generation, the mycelial growth was slower than in the previous generations, while diameter of the fungal colony was significantly reduced in the fourth generation (6.21 ± 0.09 cm). Then, the changes in cell growth were investigated using the mycelial growth rate and biomass production. The results showed that the difference in growth rate was noted in the first week for each generation. It started to decrease in G3 which was significantly less than previous generations, followed by G4 and G5 (Figs. 1A and B).

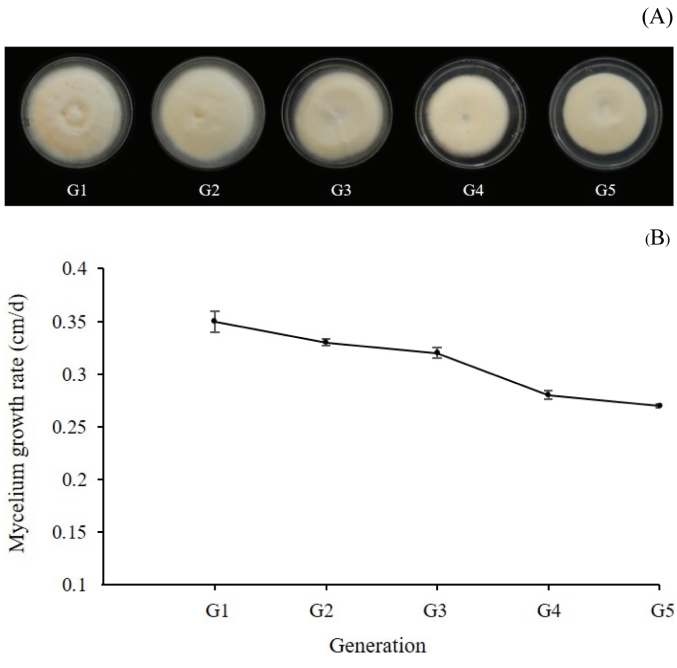

Fig. 1 Colony morphology and mycelial growth rate of *Cordyceps militaris* grown on agar medium at week 3 during subculturing for five generations: (A) morphology; (B) mycelium growth rate (mean ± SD of 10 replicates)

There was a significant 22% reduction in the growth rate from G1 to G5 (0.35 ± 0.01 cm/d to 0.27 ± 0.02 cm/d). Similarly, the fungal dry weight was decreased from G3 to G5 by about 42% (from 0.95 ± 0.04 g DW to 0.55 ± 0.01 g DW) as shown in Fig. 2.

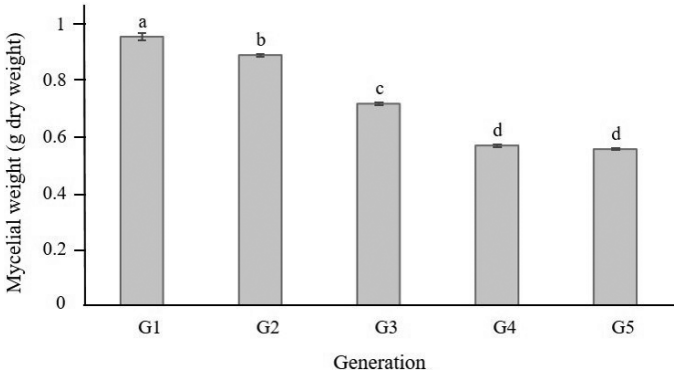

Fig. 2 Mycelial dry weight (mean ± SD of 10 replicates) of *Cordyceps militaris* biomass during subculturing for five generations, where error bars represent ±SD and different lowercase letters above bars indicate significant ($p < 0.05$) differences among mean values.

Changes of adenosine and cordycepin contents

To determine whether the reduction in mycelial growth during subculturing was associated with specific metabolites in the production of *C. militaris*, the adenosine and cordycepin contents in the mycelia and the culture media were quantified using HPLC analysis. The compounds were detected in the mycelia but not in the culture media, indicating that the adenosine and cordycepin contents decreased relatively from G1 to G5 (Figs. 3A and B), with significant reductions in adenosine (98.9%) and cordycepin (73.4%) production from G1 (1.97 ± 0.21 µg/g DW and 5.49 ± 0.11 µg/g DW) compared with G5 (0.03 ± 0.001 µg/g DW and 1.63 ± 0.02 µg/g DW). Consistently, the adenosine and cordycepin production rates rapidly decreased from G1 to G5, as shown in Table 1. Based on the G1–G5 results, the reduction in adenosine was more severe than for cordycepin, suggesting that they were linked directly to mycelial growth during subculturing.

Instability of Rhf1 gene during subculturing

Change in the *Rhf1* amplicon was monitored using quantitative PCR. The *Actin* and *Rhf1* genes were successfully amplified and sequenced for all generations. The *Actin* gene was consistent throughout G1–G5, indicating its suitability as a reference gene (supplementary S1) . There was a substantial reduction in the *Rhf1* gene amplicon from G1 to G5. The *Rhf1* gene started to significantly decrease during G2. The obtained amplicons for G3–G5 were significantly less than for G1 with 1×10^{12} copies/10 ng of fungal genomic DNA (Fig. 4).

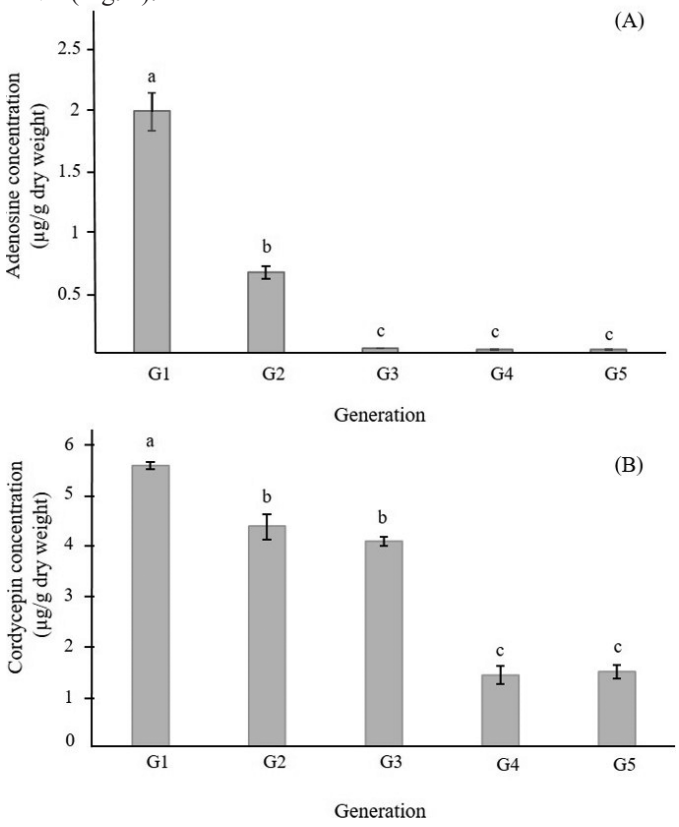

Fig. 3 Histograms showing adenosine (A) and cordycepin (B) contents (mean ± SD) in mycelia of *Cordyceps militaris* during subculturing, where error bars represent ± SD of triplicates and different lowercase letters above bars indicate significant difference ($p < 0.05$) among mean values.

Table 1 Biomass production and metabolite production rates of *Cordyceps militaris* during subculturing for five generations

Generation	Cell dry weight (g)	Adenosine production rate (µg/g DW/day)	Cordycepin production rate (µg/g DW/day)
G1	0.95±0.04[a]	0.0937±0.0104[a]	0.2613±0.0055[a]
G2	0.88±0.02[b]	0.0315±0.0039[b]	0.2047±0.0035[b]
G3	0.71±0.02[c]	0.0016±0.0001[c]	0.1911±0.0058[c]
G4	0.57±0.01[d]	0.0011±0.0002[d]	0.0662±0.0119[d]
G5	0.55±0.01[d]	0.0011±0.0001[d]	0.0694±0.0090[d]

DW = dry weight; Mean ± SD within the same column superscripted with different lowercase letters are statistically different ($p < 0.05$)

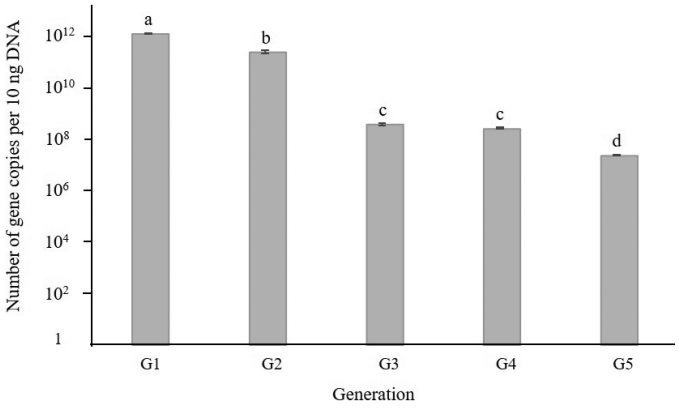

Fig. 4 Quantity of *Rhf1* gene amplicon (mean±SD) of *Cordyceps militaris* from G1–G5, Where error bars represent± SD of five replicates and different lowercase letters above bars indicate significant ($p < 0.05$) difference among mean values.

Next, to confirm whether the reduction in *Rhf1* copy number could affect gene stability during subculturing, quantitative gene expression of *Rhf1* was measured using real time RT-PCR. The reduction in gene expression was observed at G3 and clearly decreased at G5. Fig. 5 shows that the gene expression level of *Rhf1* significantly decreased from G1 (12.9×10^5 copy numbers) compared to G3 (2.1×10^5 copy numbers), which corresponded to the changes in the *Rhf1* gene copy number (DNA level), as shown in Fig. 4. This indicated that the obtained *Rhf1* gene expressions during G3–G5 were also less than for G1 (Fig. 5). In particular, G3 was critical in changes in *Rhf1* gene copy numbers and transcription. This investigation suggested that the number of amplicons of *Rhf1* might be used as the threshold to determine the quality of *C. militaris* before use, but it needs to be optimized.

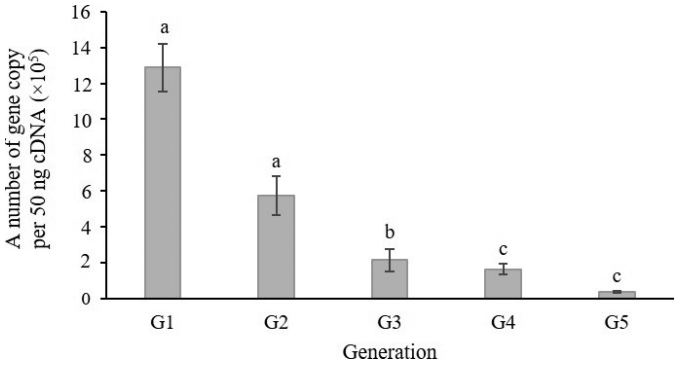

Fig. 5 Gene expression level (mean ± SD of five replicates) of *Rhf1* gene during subculturing for five generations, where error bars represent ± SD and different lowercase letters above bars indicate significant ($p < 0.05$) differences among mean values

DNA methylation of Rhf1 gene status during subculturing

A 216 bp region containing CpG at 125–341 bp was tested to examine the genetic variation in the *Rhf1* gene in association with methylation (supplementary S2) . The percentage of DNA methylation was determined for the CpG site at positions 206–209 bp of the *Rhf1* gene. Resistance to digestion by *Msp*I and susceptibility to *Hpa*II restriction enzymes indicated the completion of DNA methylation. For the qPCR assay, methylated and unmethylated DNA were digested by *Hpa*II or *Msp*I restriction enzymes. For the unmethylated DNA, the Cq values for *Hpa*II-digested DNA were 4.5 cycles higher than those for the *Msp*I-digested and control DNA (supplementary S3). The results revealed the percentage of DNA methylation was in two groups; in the first group, DNA methylation was less than 5% (G1–G2) whereas DNA methylation was completed in the range 50–68% (G3–G5) in the second group (Fig. 6). The percentage of DNA methylation rapidly increased after G3. The results demonstrated that DNA methylation for this generation may have caused the loss of *Rhf1* gene expression during subculturing. Thus, it seems that G3 is a transition point and the subsequent generations G4–G5 clearly showed complete DNA methylation (Fig. 6).

Discussion

Frequent degeneration of *C. militaris* during subculturing and preservation has constrained the development of the *C. militaris* industry. There have been few reports on the degeneration of this fungus in terms of genetic variations

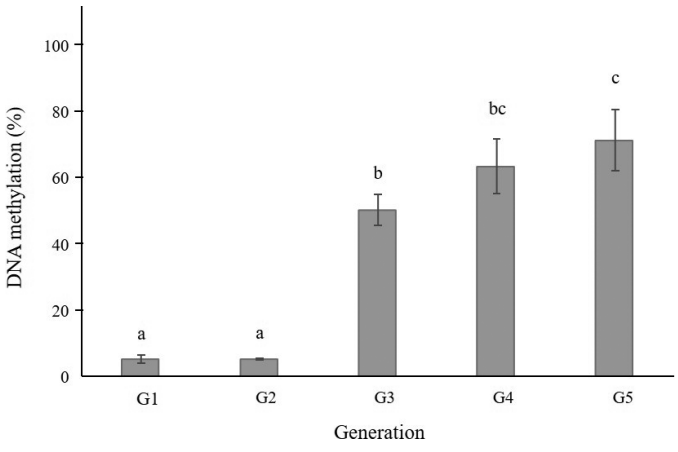

Fig. 6 DNA methylation (mean ± SD) at 125–341 bp of *Rhf1* gene from G1–G5, where error bars represent ± SD of five replicates and different lowercase letters above bars indicate significant ($p < 0.05$) differences among mean values

and environments (Lou et al., 2019). Similarly, degeneration of various strains of edible fungi, such as *Pleurotus eryngii* (Hua et al., 2017), *Lentinula edodes* (Guo et al., 2017) and *Volvariella volvacea* (Chen et al., 2019b), could be due to changes in the mating type, the number of subcultures, DNA methylation and infections from fungal and viral pathogens. However, the problem of fungal degeneration has not been solved and the underlying molecular mechanism of *C. militaris* is still little known.

In the current study, mycelial growth of *C. militaris* ATCC 34165 exhibited great differences in successive generations during subculturing (for five generations) using solid culture media, with the third generation being the transition point that was followed by generations with more progressive degeneration. Consistent with these findings, other evidence has shown that subculturing influences the growth and quality of fruiting body production in *C. militaris* (Yin et al., 2012; Sun et al., 2017) and degeneration of the mycelium in *V. volvacea* (Chen et al., 2019a). In the current study, the varying growth responses and levels of mycelial production of *C. militaris* may have been related to cellular stress and metabolites that contributed to cell maintenance.

The reductions in the levels of adenosine and cordycepin (Figs. 3A and B) indicated that cell growth and development were associated with adenosine production but not cordycepin production as was evident from cordycepin-producing mutants having normal growth (Xia et al., 2017). Alternatively, fungal growth degeneration may be linked to the cordycepin biosynthesis gene that mediates the dual biosynthesis of cordycepin and another adenosine analog, pentostatin. A change in the adenosine content may be critical for the dosage of cordycepin synthesis, which is located on the same gene cluster as cordycepin and both metabolites are synthesized when the cordycepin gene cluster is induced (Xia et al., 2017). The substantial reduction in the adenosine content as a cellular energy source in mycelia might be caused by mitochondrial DNA (mtDNA) which produce the majority of the adenosine triphosphate in cells and a second major function seems to be intimately linked to their generation of reactive oxygen species (ROS), which have been implicated in mtDNA mutations, aging, and cell death (Orrenius et al., 2007; Xiong et al., 2013; Li et al., 2014; Numata et al., 2019). However, these need to be clarified in detail by further studies. In the current study, the loss of adenosine was more severe than for cordycepin since it is required for mycelial growth and development as mentioned above. Thus, the degenerate mycelial growth in *C. militaris* may be critical in the change in adenosine production which demonstrated significant up-regulation of the genes in the adenosine biosynthetic pathway (Yin et al., 2017).

To date, number studies of the *C. militaris* degenerated strain have been reported about the gene expression in biological processes, and cellular components and functions, for example, primary metabolite (e.g. polysaccharides and ergosterol) and secondary metabolite production (e.g. cordycepin) but there few genes have been characterized (Dong et al., 2013; Yin et al., 2017; Xia et al., 2017). *Rhf1* is the gene that has been characterized as necessary for mycelial growth and fruiting body development (Jiang and Han, 2015). The current work attempted to address the impact of instability in the *Rhf1* gene during subculturing on the quality of *C. militaris* during maintenance or use. The number of *Rhf1* copies in each cell progressively decreased during continuous subculture (Figs. 4 and 5). These results indicated that *C. militaris* may deactivate the *Rhf1* gene by decreasing the number of filamentous proteins during the mycelial stage rather than fruiting body development (Jiang and Han, 2015; Liu et al., 2018a). Other results have suggested that *Rhf1* may be specifically required to form primordia (Jiang and Han, 2015) that are necessary for mycelial production during the G1–G5 generations.

Studies have shown that the changes of the number of gene copies in fungi depends on environmental responses (Salmon et al., 2004; Liu et al., 2018a) or the state of growth (Numata et al., 2019). Other work demonstrated that reduction in the *Rhf1* gene during subculturing may relate to cellular accumulation of ROS (Xiong et al., 2013; Liu et al., 2018a). Hence, reduction of the gene might be involved in the inactivation of DNA replication or DNA repair machinery in the cell cycle (Rowe et al., 2008; Shockley et al., 2013). In addition, changes in the number of mitochondrial DNA and transcription levels of genes such as *Cox1* and *Nadh4* appeared to be controlled by the copy number of mtDNA in *Grifola frondosa* during mycelial growth and fruiting body formation (Numata et al., 2019).

In the current study, the loss of the *Rhf1* gene may not have been directly linked to mycelial growth and metabolite production during subculturing; however, it seemed to be associated with the stress response by regulating the genes encoding antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Wang et al., 2005; Lin et al., 2010; Xiong et al., 2013; Liu et al., 2018a). An increase in antioxidant enzymes in *C. militaris* can promote or restore mycelial growth and fruiting body production in a degenerate strain (Xiong et al., 2013; Liu et al., 2018a). Additionally, the insertional mutant *C. militaris* strain g38 in the *Rhf1* gene has a low level

of gene expression of glutathione peroxidase compared to the degenerate strain; however, it did not restore fruiting body production (Liu et al., 2018a).

DNA methylation may be involved in the loss of *Rhf1* copy numbers due to gene expression during subculturing. The current results revealed that the third generation was a transition point for DNA methylation and degeneration followed in generations 4 and 5, as shown in Fig 6. This suggested that DNA methylation may be one factor that reduces gene expression (Fig. 5) during subculturing of *C. militaris*. As reported here, DNA methylation can cause the loss of gene expression during subculturing. Though genome instability is not fully accounted for, it appears to occur frequently in fungi. This might be explained together with chromosomal instabilities by: 1) the DNA methylation region of the *Rhf1* gene affects gene expression (Codón et al., 1997; Xin et al., 2019); 2) the dosage effect of cordycepin and analogs in mycelia might be caused by DNA methylation; and 3) the accumulation of ROS could damage DNA or affect gene stability (Li et al., 2014).

To date, there have been reports of successful genetic manipulation (Zheng et al., 2011b) and the development of a genome editing system using *CRISPR/Cas9* (Chen et al., 2018b) of *C. militaris* that will facilitate understanding the mechanism during subculturing or maintaining the quality of *C. militaris* cultures in large-scale production and commercialization.

The current study investigated the variations in mycelial growth, metabolite production and the *Rhf1* gene of *C. militaris* in successive subcultures. As subculturing continued, the growth rates and biomasses of mycelia gradually decreased from G1–G5. Consistent with the growth rate, differences in the quantity of the *Rhf1* gene were observed. Here, the amplicon of *Rhf1* showed its association with the degeneration using qPCR. Further the current study suggested that gene expression and DNA methylation of the *Rhf1* gene occurred during subculturing (after the fourth generation). Based on these findings, it was concluded that the quality of *C. militaris* depended on subculture degeneration. These results have provided insights into and offer a feasible rapid identification method for *C. militaris* strain degeneration.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Chen, A., Wang, Y., Shao, Y., Huang, B. 2017. A novel technique for rejuvenation of degenerated caterpillar medicinal mushroom, *Cordyceps militaris* (Ascomycetes), a valued traditional Chinese medicine. Int. J. Med. Mushrooms 19: 87–91. doi.org/10.1615/IntJMedMushrooms.v19.i1.90

Chen, B.X., Wei, T., Ye, Z.W., Yun, F., Kang, L.Z., Tang, H.B., Guo, L.Q., Lin, J.F. 2018a. Efficient *CRISPR-Cas9* gene disruption system in edible-medicinal mushroom *Cordyceps militaris*. Front. Microbiol. 9: 1157. doi.org/10.3389/fmicb.2018.01157

Chen, L., Liu, Y., Guo, Q., Zheng, Q., Zhang, W. 2018b. Metabolomic comparison between wild *Ophiocordyceps sinensis* and artificial cultured *Cordyceps militaris*. Biomed. Chromatogr. 32: e4279. doi.org/10.1002/bmc.4279

Chen, X., Zhang, Z., Liu, X., Cui, B., Miao, W., Cheng, W., Zhao, F. 2019a. Characteristics analysis reveals the progress of *Volvariella volvacea* mycelium subculture degeneration. Front. Microbiol. 10: 2045. doi.org/10.3389/fmicb.2019.02045

Chen, Y., Wu, Y., Liu, L., et al. 2019b. Study of the whole genome, methylome and transcriptome of *Cordyceps militaris*. Sci. Rep. 9: 898. doi.org/10.1038/s41598-018-38021-4

Codón, A.C., Lee, Y.S., Russo, V.E.A. 1997. Novel pattern of DNA methylation in *Neurospora crassa* transgenic for the foreign gene *hph*. Nucleic Acids Res. 25: 2409–2416. doi.org/10.1093/nar/25.12.2409

Das, S.K., Masuda, M., Sakurai, A., Sakakibara, M. 2010. Medicinal uses of the mushroom *Cordyceps militaris*: Current state and prospects. Fitoterapia 81: 961–968. doi.org/10.1016/j.fitote.2010.07.010

Dong, J.Z., Lei, C., Zheng, X.J., Ai, X.R., Wang, Y., Wang, Q. 2013. Light wavelengths regulate growth and active components of *Cordyceps militaris* fruit bodies. J. Food Biochem. 37: 578–584. doi.org/10.1111/jfbc.12009

Fulnecek, J., Kovarik, A. 2014. How to interpret methylation sensitive amplified polymorphism (MSAP) profiles? BMC Genet. 15: 2. doi.org/10.1186/1471-2156-15-2

Guo, M., Bian, Y., Wang, J., Wang, G., Ma, X., Xu, Z. 2017. Biological and molecular characteristics of a novel partitivirus infecting the edible fungus *Lentinula edodes*. Plant Dis. 101: 726–733. doi.org/10.1094/PDIS-07-16-0951-RE

Hua, S., Qi, B., Fu, Y.P., Li, Y. 2017. DNA methylation changes in *Pleurotus eryngii* Subsp. *tuoliensis* (Bailinggu) in response to low temperature stress. Int. J. Agric. Biol. 19: 328–334. doi.org/10.17957/IJAB/15.0286

Jiang, K., Han, R. 2015. *Rhf1* gene is involved in the fruiting body production of *Cordyceps militaris* fungus. J. Ind. Microbiol. Biotechnol. 42: 1183–1196. doi.org/10.1007/s10295-015-1637-2

Jiao, Z., Fen, W., Kuanbo, L., Qing, L., Ying, Y., Caihong, D. 2018. Heat and light stresses affect metabolite production in the fruit body of the medicinal mushroom *Cordyceps militaris*. Appl. Microbiol. Biotechnol. 102: 4523–4533. doi.org/10.1007/s00253-018-8899-3

Lee, C., Kim, J., Shin, S.G., Hwang, S. 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. 123: 273–280. doi.org/10.1016/j.jbiotec.2005.11.014

Li, L., Hu, X., Xia, Y., Xiao, G., Zheng, P., Wang, C. 2014. Linkage of oxidative stress and mitochondrial dysfunctions to spontaneous culture degeneration in *Aspergillus nidulans*. Mol. Cell. Proteomics. 13: 449–461. doi.org/10.1074/mcp.M113.028480

Lin, Q.Q., Qiu, X.H., Zhen, Z.L., et al. 2010. Characteristics of degenerate strain of *Cordyceps militaris*. Mycosystema 29: 670–677.

Liu, G.Q., Qiu, X.H., Cao, L., Han, R.C. 2018a. Scratching stimuli of mycelia influence fruiting body production and ROS-scavenging gene expression of *Cordyceps militaris*. Mycobiology 46: 382–387. doi.org/10.1080/12298093.2018.1544769

Liu, Q., Wang, F., Liu, K., Dong, C. 2018b. Influence of strain preservation methods on fruiting body growth and metabolite production by the medicinal mushroom *Cordyceps militaris* (*Ascomycetes*). Int. J. Med. Mushrooms. 20: 1003–1011. doi.org/10.1615/IntJMedMushrooms.2018028582

Lou, H., Lin, J., Guo, L., Wang, X., Tian, S., Liu, C., Zhao, Y., Zhao, R. 2019. Advances in research on *Cordyceps militaris* degeneration. Appl. Microbiol. Biotechnol. 103: 7835–7841. doi.org/10.1007/s00253-019-10074-z

Lu, Y., Xia, Y., Luo, F., Dong, C., Wang, C. 2016. Functional convergence and divergence of mating-type genes fulfilling in *Cordyceps militaris*. Fungal Genet. Biol. 88: 35–43. doi.org/10.1016/j.fgb.2016.01.013

Numata, F., Kawaguchi, N., Yamada, C., et al. 2019. Mitochondrial DNA dynamics during fruiting body formation in *Grifola frondosa*. Mycoscience 60: 147–150. doi.org/10.1016/j.myc.2019.01.003

Orrenius, S., Gogvadze, V., Zhivotovsky, B. 2007. Mitochondrial oxidative stress: Implications for cell death. Annu. Rev. Pharmacol. Toxicol. 47: 143–183. doi.org/10.1146/annurev.pharmtox.47.120505.105122

Rasmussen, R. 2001. Quantification on the lightcycler. In: Meuer S., Wittwer C., Nakagawara KI. (Eds.). Rapid Cycle Real-Time PCR. Springer. Berlin, Germany, pp. 21–34.

Rowe, L.A., Degtyareva, N., Doetsch, P.W. 2008. DNA damage-induced reactive oxygen species (ROS) stress response in *Saccharomyces cerevisiae*. Free Radic. Biol. Med. 45: 1167–1177. doi.org/10.1016/j.freeradbiomed.2008.07.018

Stanbury, F.P., Allan, W., Stephen, J.H. 2016. Principles of Fermentation Technology, 3rd ed. Butterworth-Heinemann. Oxford, UK.

Salmon, T.B., Evert, B.A., Song, B., Doetsch, P.W. 2004. Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. Nucleic Acids Res. 32: 3712–3723. doi.org/10.1093/nar/gkh696

Sambrook, J., Russell, D. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press. New York, NY, USA.

Shockley, A.H., Doo, D.W., Rodriguez, G.P., Crouse, G.F. 2013. Oxidative damage and mutagenesis in *Saccharomyces cerevisiae*: Genetic studies of pathways affecting replication fidelity of 8-oxoguanine. Genetics 195: 359–367. doi.org/10.1534/genetics.113.153874

Shrestha, B., Zhang, W., Zhang, Y. 2012. The medicinal fungus *Cordyceps militaris*: Research and development. Mycol. Progress. 11: 599–614. doi.org/10.1007/s11557-012-0825-y

Sun, S.J., Deng, C.H., Zhang, L.Y., Hu, K.H. 2017. Molecular analysis and biochemical characteristics of degenerated strains of *Cordyceps militaris*. Arch. Microbiol. 199: 939–944.

Sutton, L.M., Starzyk, M.J. 1972. Procedure and analysis of a useful method in determining mycelial dry weights from agar plates. Appl. Microbiol. 24: 1011–1012. doi.org/10.1128/am.24.6.1011-1012.1972

Wang, C. Butt, T.M., St Leger, R.J. 2005. Colony sectorization of *Metarhizium anisopliae* is a sign of ageing. Microbiology 151: 3233–3236. doi.org/10.1099/mic.0.28148-0

Wang, H., Cai, T., Wei, J., Feng, A., Lin, N., Bao, D. 2015a. Molecular markers to detect the formation of heterokaryon and homokaryon from asexual spores of the caterpillar medicinal mushroom, *Cordyceps militaris* (Ascomycetes). Int. J. Med. Mushrooms 17: 841–846. doi.org/10.1615/IntJMedMushrooms.v17.i9.40

Wang, Y.L., Wang, Z.X., Liu, C., Wang, S.B., Huang, B. 2015b. Genome-wide analysis of DNA methylation in the sexual stage of the insect pathogenic fungus *Cordyceps militaris*. Fungal Biol. 19: 1246–1254. doi.org/10.1016/j.funbio.2015.08.017

Wang, L., Zhang, W.M., Hu, B., Chen, Y.Q., Qu, L.H. 2008. Genetic variation of *Cordyceps militaris* and its allies based on phylogenetic analysis of rDNA ITS sequence data. Fungal Divers. 31: 147–155.

Whelan, J.A., Russell, N.B., Whelan, M.A. 2003. A method for the absolute quantification of cDNA using real-time PCR. J. Immunol. Methods. 278: 261–269. doi.org/10.1016/S0022-1759(03)00223-0

Xia, Y., Luo, F., Shang, Y., Chen, P., Lu, Y., Wang, C. 2017. Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. Cell Chem. Biol. 24: 1479–1489. doi.org/10.1016/j.chembiol.2017.09.001

Xin, X., Yin, J., Zhang, B., Li, Z., Zhao, S., Gui, Z. 2019. Genome-wide analysis of DNA methylation in subcultured *Cordyceps militaris*. Arch. Microbiol. 201: 369–375. doi.org/10.1007/s00203-019-01621-3

Xiong, C., Xia, Y., Zheng, P., Wang, C. 2013. Increasing oxidative stress tolerance and subculturing stability of *Cordyceps militaris* by overexpression of a glutathione peroxidase gene. Appl. Microbiol. Biotechnol. 97: 2009–2015. doi.org/10.1007/s00253-012-4286-7

Yang, T., Guo, M., Yang, H., Guo, S., Dong, C. 2016. The blue-light receptor *CmWC-1* mediates fruit body development and secondary metabolism in *Cordyceps militaris*. Appl. Microbiol. Biotechnol. 100: 743–755. doi.org/10.1007/s00253-015-7047-6

Yin, J., Xin, X., Weng, Y., Gui, Z. 2017. Transcriptome-wide analysis reveals the progress of *Cordyceps militaris* subculture degeneration. PLoS One. 12: e0186279. doi.org/10.1371/journal.pone.0186279

Yin, J., Xin, X.D., Weng, Y.J., Li, S.H., Jia, J.Q., Gui, Z.Z. 2018. Genotypic analysis of degenerative *Cordyceps militaris* cultured in the pupa of *Bombyx mori*. Entomol. Res. 48: 137–144. doi.org/10.1111/1748-5967.12246

Yin, Y., Yu, G., Chen, Y. et al., 2012. Genome-wide transcriptome and proteome analysis on different developmental stages of *Cordyceps militaris*. PLoS ONE. 7: e51853. doi.org/10.1371/journal.pone.0051853

Zemach, A., McDaniel, I.E., Silva, P., Zilberman, D. 2010. Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science 328: 916–919. doi.org/10.1126/science.1186366

Zheng, P., Xia, Y., Xiao, G., et al. 2011a. Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. Genome Biol. 12: R116. doi.org/10.1186/gb-2011-12-11-r116

Zheng, Z., Huang, C., Cao, L., Xie, C., Han, R. 2011b. *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in medicinal fungus *Cordyceps militaris*. Fungal Biol. 115: 265–274. doi.org/10.1016/j.funbio.2010.12.011

Zhou, X., Gong, Z., Su, Y., Lin, J., Tang, K. 2009. *Cordyceps* fungi: Natural products, pharmacological functions and developmental products. J. Pharm. Pharmacol. 61: 279–291. doi.org/10.1211/jpp.61.03.0002