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Genome-wide analysis of DNA methylation in the sexual stage of the insect pathogenic fungus *Cordyceps militaris*

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ABSTRACT

DNA methylation is a basic epigenetic mechanism found in eukaryotes, but its patterns and roles vary significantly among diverse taxa. In fungi, DNA methylation has various effects on diverse biological processes. However, its function in the sexual development of fungi remains unclear. *Cordyceps militaris*, readily performs sexual reproduction and thus provides a remarkably rich model for understanding epigenetic processes in sexual development. Here, we surveyed the methylome of *C. militaris* at single-base resolution to assess DNA methylation patterns during sexual development using genomic bisulfite sequencing (BS-Seq). The results showed that approximately 0.4 % of cytosines are methylated, similar to the DNA methylation level (0.39 %) during asexual development. Importantly, we found that DNA methylation in the fungi undergoes global reprogramming during fungal development. Moreover, RNA-Seq analysis indicated that the differentially methylated regions (DMRs) have no correlation with the genes that have roles during fungal sexual development in *C. militaris*. These results provide a comprehensive characterization of DNA methylation in the sexual development of *C. militaris*, which will contribute to future investigations of epigenetics in fungi.

Introduction

DNA methylation is a stable epigenetic modification found in most eukaryotes that plays crucial roles in many biological processes, including the regulation of gene expression, gene imprinting, and transposon silencing (Bird 2002; Zhang *et al.* 2006; Suzuki & Bird 2008). To date, interest in DNA methylation in fungi has been stimulated by confirmation of the existence of both methylated genes and active DNA methyltransferases, which attach methyl groups to DNA (Kouzminova & Selker 2001; Martienssen 2001; Lee & Freitag 2008; Dhillon *et al.* 2010; Zemach *et al.* 2010; Jurkowski &

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Jeltsch 2011). Methylome studies showed that DNA methylation of genomes in fungal species varies significantly, which suggests that DNA methylation may be involved in multiple important mechanisms in fungi (Rountree & Selker 1997; Zemach *et al.* 2010).

Recent studies of genome DNA methylation in fungi focused on the whole pattern of DNA methylation in the mycelium (asexual stage). Such reports showed that genomes of fungi display varying low levels of DNA methylation, ranging from imperceptible to just barely detectable for both CpG site methylation and non-CpG methylation (Antequera *et al.* 1984; Magill & Magill 1989; Zemach *et al.* 2010). Approximately 1.5 % of cytosines are methylated in the genome of *Neurospora*, while <0.1 % methylcytosines were found in *Schizosaccharomyces pombe* and *Anacystis nidulans* (Antequera *et al.* 1984; Selker & Stevens 1987; Foss *et al.* 1993). DNA methylation may be absent from *Aspergillus flavus*, though it is speculated that this organism may possess *de novo* DNA methylation that occurs transiently during the rare sexual stage (Liu *et al.* 2012). Variation in genome methylation patterns suggests that the role of DNA methylation is not strictly conserved among different fungal species. DNA methylation inhibits elongation, but not initiation, of transcription in *Neurospora crassa* and regulates phenotype-dependent transcriptional activity in *Candida albicans* (Rountree & Selker 1997; Mishra *et al.* 2011). In *Magnaporthe oryzae*, DNA methylation plays a role in fungal development and genome defence throughout development of the asexual life cycle (Jeon *et al.* 2015). However, there have been no surveys of genome-wide DNA methylation during the sexual development of fungi.

Cordyceps militaris, an entomopathogenic fungus in the phylum *Ascomycota*, is notable for easy artificial culturing of sexual fruiting bodies. This has enabled extensive opportunities for study of multiple mechanisms that operate at distinct stages of the life cycle of the fungus. The completion of genome sequencing provides a basis for methylome studies of *C. militaris* (Zheng *et al.* 2011). In-depth studies on the methylome of the sexual stage of *C. militaris* will not only identify the characteristics of genome DNA methylation but also help elucidate the role of genome DNA methylation in the sexual development of fungi.

The recently developed genomic bisulfite sequencing (BS-Seq) technology, which couples bisulfite conversion of unmethylated Cs to Ts to deep sequencing, has emerged as the gold standard for the study of genome-wide DNA methylation at single-nucleotide resolution (Sun *et al.* 2014). This technology has been applied in the production of DNA methylomes for more than 20 eukaryotic organisms including plants, invertebrates and vertebrates (Lister *et al.* 2009; Feng *et al.* 2010; Lyko *et al.* 2010; Xiang *et al.* 2010; Zemach *et al.* 2010; Bonasio *et al.* 2012). The high resolution of these studies identified patterns and functional effects of DNA methylation. Characteristics of DNA methylation of various fungi have also been described using BS-Seq, which provides important insights into the distribution and function of DNA methylation in fungi (Zemach *et al.* 2010; Su *et al.* 2011; Liu *et al.* 2012; Jeon *et al.* 2015).

In this study, we investigated the nature of DNA methylation in the nascent fruiting body (sexual development) of *C. militaris* by generating a DNA methylation map at single-base resolution across the genome using deep bisulfite sequencing. Subsequently, we explored the link between DNA methylation and gene expression in sexual development by profiling transcription in *C. militaris*. Together, this work provides the first insight into the DNA methylation landscape during the sexual development of *C. militaris*.

Materials and methods

Fungal strain and sample preparation

The fungal strain used in this study was *Cordyceps militaris* PM53 (isolated from lepidopteran insect pupae, Jiangsu, China), available from the Anhui Provincial Key Laboratory of Microbial Pest Control, Hefei, China. For the collection of different samples in asexual development, the strain was inoculated onto PPDA plates (20 % potato, 2 % dextrose, 1.5 % agar, and 1 % peptone, *w/v*) and incubated at 23 °C in the dark. For fruiting body production, the mycelia of this strain were inoculated into a 250-ml flask containing 50 ml liquid PPD medium (20 % potato, 2 % dextrose, and 1 % peptone, *w/v*). The flask was then incubated at 23 °C in a 150 rpm shaker for 7 d. Pupae were inoculated with fungal culture from the same flask, cultivated in the dark at 23 °C for 10 d, and then kept at 23 °C under a 17:7 h dark/light cycle.

Identification and real-time PCR analysis of genes encoding putative DNA methyltransferases

Genes encoding putative DNMTases in the fungal genome were identified through BLAST search using amino acid sequences of known DNMTases as queries. Total RNAs were extracted at different asexual developmental stages of *Cordyceps militaris*, i.e., 3-day-old (3M), 6-day-old (6M), 9-day-old (9M), and 15-day-old (15M), and from different sexual developmental stages, i.e., nascent (NF), stalk formation (MIF), mature fruiting bodies (MAF), and late stage fruiting bodies (LAF) (Fig S1). First-strand cDNA was synthesized from 1 μg of total RNA with a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. Specific primers for CMDim-2, CMDmtA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed for real-time PCR amplification (Table S1). cDNA templates derived from materials from different developmental stages were used for real-time PCR amplification with a SYBR Green Kit (Takara) and a 7500 Real-Time PCR System (Applied Biosystems). All reactions were run in triplicate. The threshold cycle (CT) was determined using the default threshold settings. The ΔΔCt method was used to calculate the relative expression levels of CMDim-2 and CMDmtA (Livak & Schmittgen 2001), using GAPDH as the internal control for each sample. All data are presented as the mean ± SE of three replicates.

BS-Seq library construction and sequencing

3M and NF were chosen for BS-Seq based on the results of the real-time PCR analysis of genes encoding putative DNA methyltransferases (Fig 1). DNA was fragmented by sonication with a Diagenome sonicator to a mean size of approximately 250 bp, followed by DNA-end repair, 3′-dA overhang and

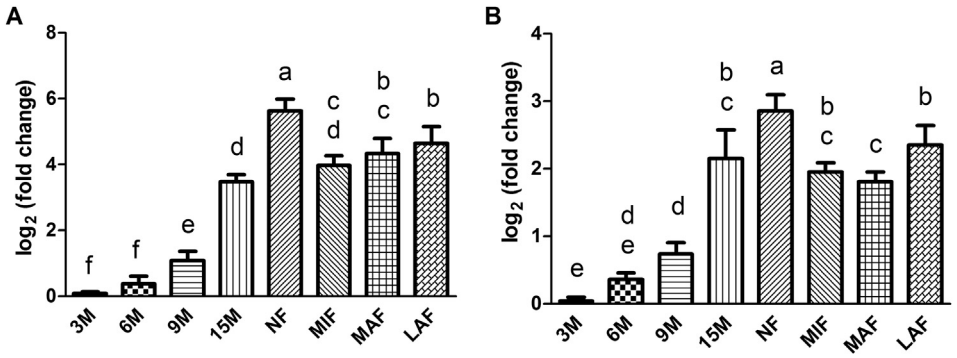

Fig 1 – Expression profile of DNA methyltransferase genes using real-time PCR. Expression patterns of CMDmtA (A) and CMDim-2 (B) genes in different developmental stages. Different letters above the bars denote samples that have significantly different levels of methylation ($P < 0.005$).

ligation of methylated sequencing adaptors according to the manufacturer's instructions (Illumina). The bisulfite conversion of *Cordyceps militaris* DNA was carried out using ZYMO EZ DNA Methylation-Gold kits (ZYMO). The bisulfite-converted DNA was amplified by 18 cycles of PCR, purified with a PCR purification kit (Qiagen), and a qualified library was constructed for sequencing after size selection. The resultant DNA was paired-end sequenced using an ultrahigh-throughput Illumina Genetic Analyzer (GA2) according to the manufacturer's instructions.

Read quality control and mapping

The raw paired-end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then, clean reads were separately aligned to the *Cordyceps militaris* CM01 genome (National Center for Biotechnology Information) with orientation mode using Tophat (http://tophat.cbcb.umd.edu/) software. Tophat is a program that aligns RNA-Seq reads to a genome in order to identify exon–exon splice junctions and gene expression, and this software was used to complete mapping and calculating gene expression levels using default parameters. All clean BS-Seq reads were mapped to the genome using the BSMAP aligner (Whole Genome Bisulfite Sequence MAPping Program), allowing up to two nucleotide mismatches, and uniquely mapped reads were used to determine the cytosine methylation levels (Xi & Li 2009).

Bioinformatic analysis of BS-Seq data

Identification of differentially methylated regions (DMRs) was conducted as previously reported (Zhang *et al.* 2013). Briefly, only cytosines with a depth of at least four in all libraries were considered and were used to identify whether those cytosines were methylated. The DNA methylation level was compared between NF and 3M using a sliding-window approach with a 200-bp window sliding at 50-bp intervals. Windows containing seven or more differentially methylated cytosines and P values < 0.01 (Fisher's exact test) between the two were reported as DMRs. Neighbouring DMRs were combined if the gap was $\leq$100 bp (Lister *et al.* 2009).

RNA-Seq

To better understand DNA methylation for the sexual development of *Cordyceps militaris*, 3M (as a control) and NF were chosen for RNA-Seq. Total RNAs were prepared for Illumina RNA-Seq according to previously reported methods (Wang *et al.* 2014). In brief, to maximize the coverage of target sequences, equal amounts of total RNA from the three replicates of NF and 3M were pooled for RNA-Seq library construction. Poly(A) mRNA from the total RNA was purified using oligo (dT) beads. Following purification, mRNA was fragmented into small pieces. The first cDNA strand was synthesized using random hexamer primers for reverse transcription with cleaved RNA fragments serving as templates. The second-strand cDNA was synthesized using RNase H and DNA polymerase I, and the sequencing library was constructed following the manufacturer's instructions (Illumina, San Diego, USA). The cDNA library was sequenced using an Illumina HiSeq 2000 with a single-end (single reads of 100 bases) sequencing strategy at the Beijing Genomics Institute (BGI). Clean reads, obtained by removing raw reads, were used for mapping to the *C. militaris* reference genome, and the expression level of each gene was normalized using the reads per kilobase per million reads (RPKM) method (Mortazavi *et al.* 2008). Rigorous algorithms were applied to identify differentially expressed genes (DEGs) based on previously described methods (Audic & Claverie 1997). Differentially expressed genes (DEGs) were identified using a false discovery rate (FDR) $\leq$0.001 and an absolute value of the $\log_2$ ratio $\geq$1 as the threshold.

BS-PCR and RNA-Seq validation

Five hypermethylated DMRs in NF were selected for experimental validation. Primers overlapping with the DMRs were used for amplification (Table S1). DNA (1 μg) was bisulfite converted as described (Espada *et al.* 2014). Two hundred nanograms of bisulfite-converted DNA were PCR-amplified, and the purified amplicons were cloned into a pMD18-T vector

(Takara) and then sequenced. An average of 15 clones was randomly chosen to sequence for each DMR; the results are summarized in Fig S2A.

cDNA was synthesized, and qPCR reactions were carried out as described above. The specific primers for genes that were used for qPCR to validate the RNA-Seq data are listed in Table S1. Three replicates were performed independently for each gene tested.

Nucleotide sequence accession

The raw Illumina sequencing data are deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE66919 and in SRA (http://www.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi) with the accession number SRP055724 at NCBI.

Results

Transcriptional profiling of two DMTase-encoding genes at different developmental stages

Through BLAST search using amino acid sequences of known DNMTases as queries, two genes encoding proteins containing the DNMTase domain were found (CM_04944 and CM_03836), which are closely related to DIM-2 and DmtA of *Uncinocarpus reesii*, respectively. These data indicate that *Cordyceps militaris* has a potential DNMTase homologous to DIM-2, which is capable of both *de novo* and maintenance DNA methylation, and a potential DNMTase homologous to DmtA responsible for repeat induced point-mutation (RIP) (Kouzminova & Selker 2001; Liu *et al.* 2012). Expression of DNA methyltransferase (CMDim-2 and CMDmtA) genes was evaluated at different developmental stages by quantitative real-time PCR. Both genes showed increased expression through asexual development with the production of spores and reached the maximum level at stage NF; therefore 3M (as a control) and NF of *C. militaris* were chosen for BS-Seq (Fig 1A and B).

Global mapping of DNA methylation in Cordyceps militaris

To investigate genome-wide DNA methylation during sexual development, whole-genome bisulfite sequencing was performed. In total, 30 535 314 and 24 315 200 raw reads were generated for NF and 3M samples, respectively (Table S2). After removing adaptor contaminants, unknown and low-quality reads, 27 664 250 and 22 695 904 clean reads were obtained (Table S2). Further analysis showed that sequence yields were 336 821 255 and 277 878 199 bases (Table S2). Each methylome was sequenced to >16-fold coverage per strand, and >89 % of the genomic cytosine positions were covered (Table S2). Initially, we obtained the following overall genome-wide methylation patterns: 1) For NF, 0.41 % including 0.39 % at CG, 0.38 % at CHG, and 0.43 % at CHH sites; 2) For 3M, 0.39 % including 0.37 % at CG, 0.39 % at CHG, and 0.40 % at CHH sites (Table 1). There were no significant differences in % mC of all genomic cytosines, but clear variations were found in the distribution of mCs between the two *C. militaris* stages. This result suggests that the fungal methylome undergoes global reprogramming during development, such that pre-existing mC sites are demethylated while C sites in different loci are methylated (Fig 2).

Table 1 – DNA methylation levels in different developmental stages of *C. militaris*.

Sample	C_rate	CG_rate	CHG_rate[a]	CHH_rate[a]
3M	0.39 %	0.37 %	0.39 %	0.40 %
NF	0.41 %	0.39 %	0.38 %	0.43 %

a H = A, T, or C.

DNA methylation patterns in gene regions during development in Cordyceps militaris *and analysis of differentially methylated regions*

To further explore the DNA methylation profile in and around genes during development in *C. militaris*, DNA methylation patterns were calculated from the reads. For CG, CHG, and CHH contexts, the methylation levels in genes were lower than in flanking regions (Fig 3). The DNA methylation level of the whole C context in the NF stage was higher than that in the 3M stage in the 0.5–2 kb region upstream of the TSS, while the converse was true between 0.5 kb upstream of the TSS and 0.5 kb downstream of the TTS; this pattern was similar to the DNA methylation levels for the CG and CHH contexts (Fig 3B and D). However, for CHG, the DNA methylation level in the NF was always lower than that in the 3M stage (Fig 3C). This change in the DNA methylation profile in and around genes suggests that DNA methylation may play regulatory roles in the transcription of genes.

To identify differentially methylated regions (DMRs), a search was carried out using a 200-bp sliding-window with 50 bp as the step-size. Windows with an adjusted *P* value <0.01 and a >1.5-fold change in the methylation level were retained. A total of 225 DMRs were identified, of which 141 were located in intergenic regions, while 84 DMRs were concentrated in 2-kb regions upstream of genes or gene bodies (Table 2, Table S3). There are 159 hypermethylated regions in NF and 66 hypermethylated regions in 3M, which suggests that DNA methylation undergoes changes during this fungal development (Table S3).

Global analysis of differentially expressed genes between 3M and NF samples

In the present study, we used FDR ≤0.001 and an absolute value of the $\log_2$ ratio ≥1 as the threshold to determine the significance of gene expression differences. Based on these criteria, a total of 3357 genes were differentially expressed between NF and 3M, including 2160 and 1197 genes upregulated in 3M and NF, respectively. Within those genes, we identified genes involved in the regulation of sexual development, such as Histidine biosynthesis His B (CCM_03120) and Cyclin-dependent kinase (CCM_05796); these results are consistent with previous studies (Zheng *et al.* 2011). To further understand the biological functions of DEGs, the KEGG database

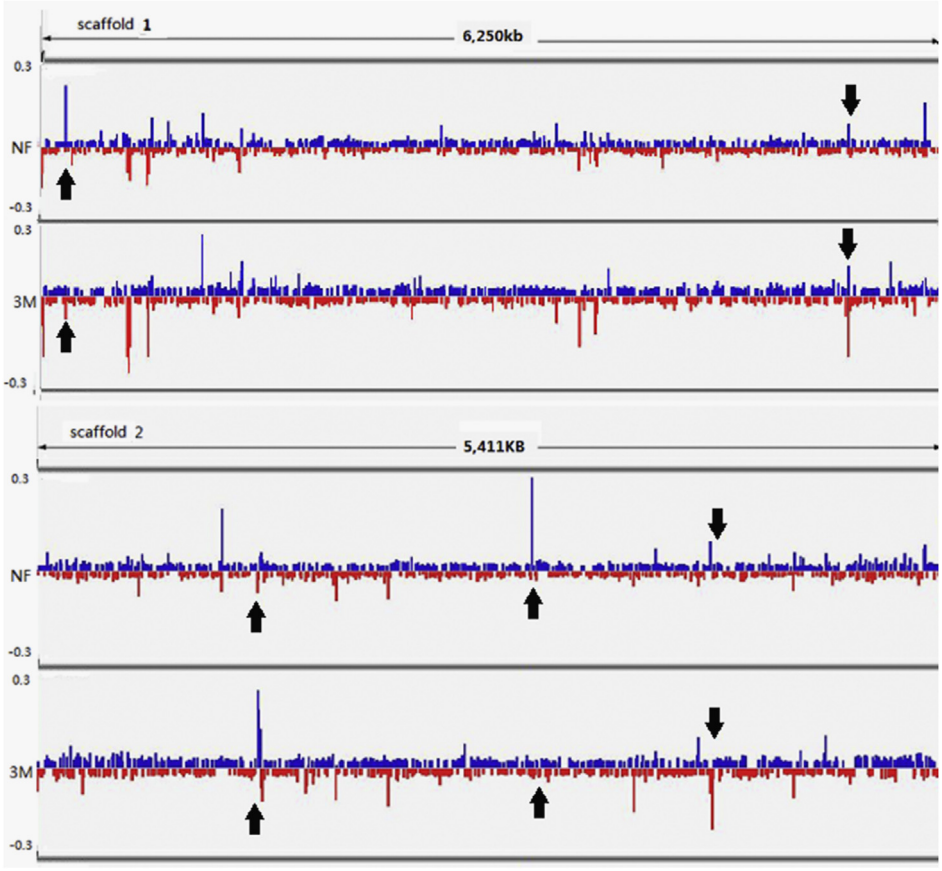

Fig 2 – Dynamics of DNA methylation in the genome of *C. militaris*. The density of methylcytosines (mCs) identified on each strand throughout scaffolds (scaffold 1 and scaffold 2) was calculated. Blue and red bars indicate methylation densities in Watson and Crick strands, respectively. Arrows indicate representative regions showing dynamic changes in DNA methylation during development. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was used to analyze pathways. A total of 31 different metabolic pathways were identified with at least 10 related DEGs ($P < 0.05$) in which metabolic pathways and biosynthesis of secondary metabolites showed significant enrichment (Table S4).

Effect of DNA methylation on transcript abundance of genes

To reveal the functional consequences of DNA methylation, we next evaluated the global impact of DMR location on transcriptional activity of the genome. DMR-associated genes were defined as genes with a DMR located in their gene bodies or within 2 kb upstream, and thus, 136 were identified. To assess more generally the function of the products of the 136 methylated genes, a gene ontology (GO) analysis was performed. All of these genes mapped to the GO terms in the three main categories (biological process, cellular component and molecular function) in the GO database (Fig S3A). KEGG pathway enrichment analysis was also carried out. Twelve pathways that showed the smallest Q values were selected in which protein processing in the endoplasmic reticulum and N-Glycan biosynthesis showed significant enrichment ($Q \leq 0.05$) (Fig S3B).

Regarding the relationship between DNA methylation and gene expression, hypermethylated DMR-associated genes were compared with down-regulated genes in NF and 3M, respectively. In NF, five down-regulated genes were found among 48 hypermethylated DMR-associated genes, and seven were found among 61 hypermethylated DMR-associated genes in 3M (Fig 4; Table 3). However, genes that play roles during sexual development were not found among these 12 down-regulated genes.

Validation of BS-Seq and RNA-Seq

To further confirm the results of the BS-Seq experiments, we performed traditional bisulfite-PCR and sequencing validation. A subset of five DMRs was selected for analysis, and a high percentage of mCs were validated (>92.7 %), which supports the validity of the BS-Seq data (Fig S2A).

The accuracy of the RNA-Seq results was assessed by qPCR for eight selected DMR-associated genes on the basis of their expression levels. The results (Fig S2B) confirm that the expression profiles of genes from the NF and 3M samples were similar to those determined in the analysis of the RNA-Seq transcriptome, validating our RNA-Seq results.

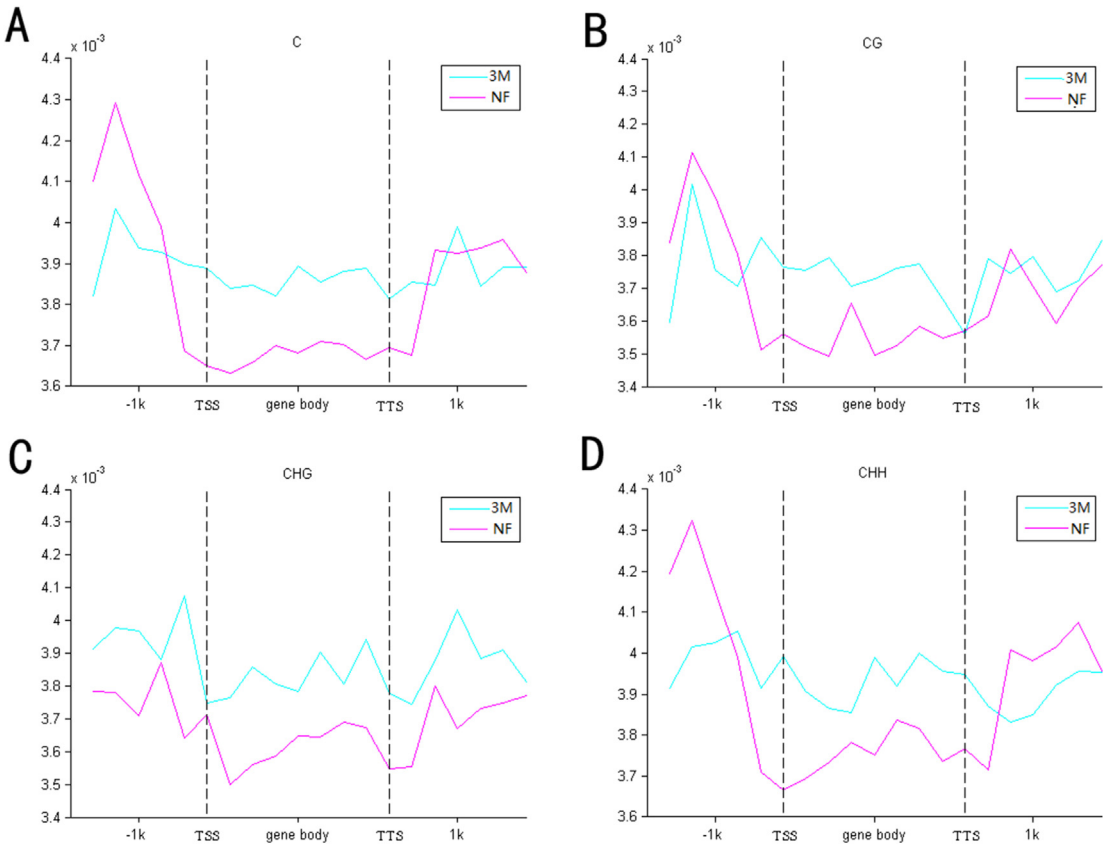

Fig 3 – Density of mCs in and around genes associated with DNA methylation during fungal development. DNA methylation profiles in gene regions were calculated using tags that were aligned with unique loci in the genome. The gene region was defined as the whole region from 2 kb upstream of the TSS, the gene body from the TSS to the TTS, and the 2-kb region downstream of the TTS. (A) Frequency of methylcytosine variation throughout the gene regions between 3M and NF. (B) Frequency of methylcytosine variation in the contexts of CG (B), CHG (C), and CHH (D).

Discussion

Previous studies have reported that DNA methylation in fungal species is phylogenetically widespread and ancient in origin but shows considerable variation that is reflected in both genome methylation patterns and diverse mechanisms of action (Zemach *et al.* 2010). Despite the potential importance of DNA methylation in fungi, the genome-wide patterns of methylation in fungal sexual stages remained poorly understood. In the work described herein, we have obtained the first genome-wide DNA methylation map at a single-base resolution with deep bisulfite sequencing during sexual development of fungi. To better understand DNA methylation in the sexual development of *Cordyceps militaris*, the 3M stage (asexual development) of this strain was also analyzed concurrently.

In contrast with the negligible genome DNA methylation levels in *Aspergillus flavus* (Liu *et al.* 2012), DNA methylation in *C. militaris* was unambiguously detected and accurately quantified at a low but significant level in both sexual and asexual developmental stages. When traditional bisulfite-PCR and sequencing to validate the methylation status were carried out in our work, most methylated sites were detected

Table 2 – The distribution of DMRs in 3M and NF.

	Total	Upstream[a]	Gene body	Upstream and Gene body	Intergenic regions[b]
Total	225	35	30	19	141
Hyper[c]	159	25	10	5	119
Hypo[d]	66	10	20	14	22

a Upstream 2 kb of gene body.
b Upstream 2 kb of gene body was deleted.
c Hypermethylation in NF.
d Hypomethylation in NF.

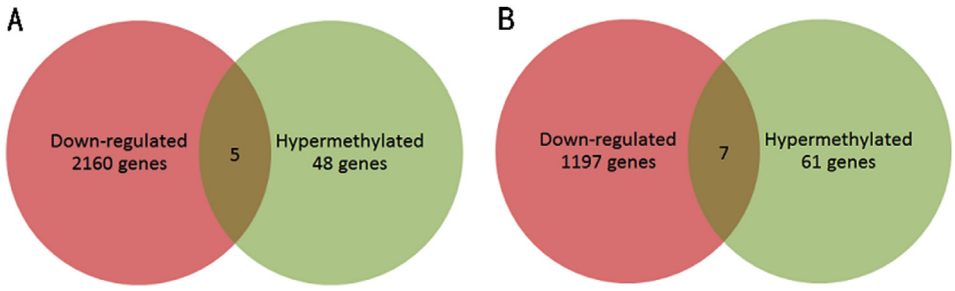

Fig 4 – Down-regulated genes and hypermethylated DMR-associated genes in *C. militaris*. (A) The number of down-regulated genes and hypermethylated DMR-associated genes in the NF stage. (B) The number of down-regulated genes and hypermethylated DMR-associated genes in the 3M stage.

in both the traditional bisulfite-PCR and sequencing and BS-Seq data, implying that methylation sites observed in this study are *bona fide* and are unlikely to be false positives resulting from an incomplete conversion reaction. As expected, we confirmed the presence of DNA methylation in *C. militaris*, at levels of 0.41 % and 0.39 % of cytosines methylated in sexual and asexual development stages, respectively.

Analysis of the DNA methylation patterns showed that *C. militaris* displayed substantial CpG site methylation and strong non-CpG methylation, corresponding to earlier reports in other fungi (Zemach *et al.* 2010; Su *et al.* 2011; Jeon *et al.* 2015). On further analysis of DNA methylation sites in *C. militaris*, we found that DNA methylation generally showed enrichment in regions with low G + C content, and cytosines located beside successive thymines tended to be unmethylated in highly methylated regions (Fig S2A). This distribution characteristic of methylcytosines was first reported in eukaryotic methylomes and suggests the possibility of sequence-specific methylation, that is, certain sequences may be potent or weak signals for *de novo* DNA methylation, and some sequences may be particularly inhibitory according to an earlier report (Tamaru & Selker 2003).

Comparative genomic analysis of DNA methylation in NF and 3M showed that although global genome methylation levels were not significantly different, considerable methylation variations existed in all three contexts (CG, CHG, and CHH). Intriguingly, the DNA methylation levels within genes are generally lower in NF than in 3M, suggesting that DNA methylation in genic regions changes during development, correlating well with patterns of DNA methylation in *Magnaporthe oryzae* as well as animals and plants (Gao *et al.* 2012; Zhong *et al.* 2013; Jeon *et al.* 2015). In-depth analysis of variations in the distribution of mCs between the two *C. militaris* stages showed that the overall change in the mC sites during development was not simply the result of a loss of methylation in pre-existing mC sites but rather a dynamic process by which losses of previous mC sites were accompanied by gains of new mC sites in different loci, indicating that DNA methylation undergoes global reprogramming during fungal development, which is similar to that observed in *M. oryzae* (Jeon *et al.* 2015). In many organisms, ten-eleven translocation (TET) proteins are 5-methylcytosine oxidases, which provide several chemically plausible pathways for the reversal of DNA methylation (Pastor *et al.* 2013). However, similar to *Ustilago maydis*, *C. militaris* lacks genes encoding TET proteins entirely, which suggests that some fungi use other mechanisms to complete the removal of mC marks (Wu & Zhang 2010; Iyer *et al.* 2014). Therefore, the mechanisms of both *de novo* and reverse DNA methylation in *C. militaris* are targets for future functional studies (Wu & Zhang 2014).

Table 3 – Down-regulated and hypermethylated DMR-associated genes in NF and 3M.

ID	DMR location	Annotation
NF		
CCM_01016	Gene upstream	Phosphoglycerate mutase,2,3-bisphosphoglycerate-independent
CCM_01017	Gene upstream	Hypothetical protein
CCM_05755	Gene upstream	CDK4/6, putative
CCM_05647	Gene body	Ubiquitin carboxyl-terminal hydrolase 2
CCM_06974	Gene body	Chitin synthase activator
3M		
CCM_04067	Gene upstream	Serine/threonine protein kinase, putative
CCM_00817	Gene body	Cellular retinaldehyde-binding protein
CCM_01893	Gene body	Isochorismatase family protein
CCM_05697	Gene body	Glutamine synthetase
CCM_06820	Gene body	YT521-B-like splicing factor, putative
CCM_06969	Gene body	RNA binding protein, putative
CCM_09538	Gene body	C6 finger domain protein, putative C6

DNA methylation in this fungus is a dynamic epigenetic entity, and its functions should be revealed further with analysis of DMRs. DMRs were identified, and more than half of them were located in intergenic regions, which suggests that intergenic DMRs in *C. militaris* may have roles that include the maintenance of genomic stability, chromatin condensation and regulation of non-coding RNAs (ncRNAs), as suggested in previous reports (Ahuja *et al.* 1997; Ballestar & Esteller 2002; Cheung *et al.* 2010). The enrichment of DMRs is localized in to genic regions in tomato and *Arabidopsis*, while DMRs localize to intergenic regions in *C. militaris* (Becker *et al.* 2011; Schmitz *et al.* 2011; Zhong *et al.* 2013). Moreover, in tomato, DMRs were distributed in regions 5′ upstream of genes, and in *Arabidopsis*, many transgenerational methylation variants were located inside genes, whereas DMRs in *C. militaris* were distributed both inside and in the 5′ region upstream of genes, which is similar to soybean (Song *et al.* 2013).

RNA-Seq was further used to elucidate whether DNA methylation has evolved in regulating transcriptional activity in *C. militaris* as reported in an earlier study on *Neurospora crassa* (Russell *et al.* 1987). Interestingly, genes of secondary metabolite biosynthetic pathways showed significant expression enrichment during sexual development, which corroborates previous studies on *C. militaris* cultured in different conditions and other fungi (Xiong *et al.* 2010; Yin *et al.* 2012; Lu *et al.* 2014). Further analysis showed that two groups, the polyketides and the non-ribosomal peptides, were enriched in sexual development, which is similar with *Cochliobolus heterostrophus*, *Gibberella zeae*, and *Sordaria macrospora* (Oide *et al.* 2007; Schindler & Nowrousian 2014). These results showed that secondary metabolites are essential for fungal sexual development. Regarding the integration of DNA methylation profiles and gene expression, we observed only 12 down-regulated or 15 up-regulated and hypermethylated DMR-associated genes among 109 DMR-associated genes, and we did not find an association between DNA methylation and gene expression (Figs 4 and S4; Tables 3 and S5). Therefore, a clear correspondence between DNA methylation and gene expression cannot be made. However, only less than 1 % of genes in the genome were shown thus far to have a major impact on fungal development. We argue that the absence of known genes in our data is not evidence of the absence of DNA methylation for particular function during fungal sexual development.

Taken together, the results of this study represent the first DNA methylomes described for an insect pathogenic fungus and provide an overview of methylation dynamics during fungal sexual development. The roles of DNA methylation have no correlation with the expression of genes that are involved in fungal sexual development in *C. militaris*. However, it is important to note that DNA methylation is only one level of multi-layered epigenetic regulation that also includes histone modifications and ncRNAs. Further work should investigate these epigenetic regulatory mechanisms during sexual development of fungi.

Conflict of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.08.017.

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