Analysis of Genomic DNA Methylation in Six Tissues of the Greater Horseshoe Bat (*Rhinolophus ferrumequinum*)

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A B S T R A C T

DNA methylation may play a vital role in tissue development and differentiation as it plays an important role in gene expression regulation. In previous studies, epigenetic variation in the muscles of bat populations were explored; however, the extent of the patterns and levels of genomic DNA methylation among tissues in chiropteran animals have yet to be investigated. In this study, the epigenetic variation of six tissues in normal state *Rhinolophus ferrumequinum* (i.e., no pregnancy, nursing, or hibernation) was explored using the F-MSAP technique, including brown adipose tissue (BAT), brain (B), heart (H), kidney (K), liver (L), and muscle (M) tissues. An average of 67.0% CCGG loci was methylated in each tissue. There were significant methylation differences among tissues in full-methylation ($p < 0.001$) and total methylation ($p = 0.001$). Moreover, various unique methylated loci (15–59) were also detected. Non-negligible level of hemi-methylation (31.4%) existed, indicating that CHG methylation may also play a potential role in tissue development and differentiation in *R. ferrumequinum*. BAT and B had different methylation profiles compared to the other four tissues, but the underlying mechanism for this requires further investigation. This work provides basic data for future studies on the intrinsic epigenetic mechanisms of vital biological processes, such as tissue development and differentiation, hibernating processes, and the environmental adaptability of Chiropteran animals.

INTRODUCTION

DNA methylation, one of epigenetic modifications, usually refers to the methylation of the fifth carbon of cytosine in CpG dinucleotides (i.e., 5-methylcytosine (^{5m}C)). In mammals, most methyl groups are erased in preimplantation embryos and re-established in implantation embryos (Cedar and Bergman, 2012). DNA methyltransferases (DNMTs) are key enzymes involved in establishing and maintaining DNA methylation. In detail, DNMT3a and DNMT3b establish new DNA methylation by adding a methyl group to an unmethylated cytosine in CpGs (i.e., *de novo* DNA methylation). DNMT1 recognizes and binds these hemi-methylated CpGs at the replicating forks and methylate the daughter strand during DNA replication (i.e., maintenance) (Lyko, 2018). ^{5m}C, especially the methylation at CpG islands (CGIs), often regulates gene expression by repressing transcription

processes (Jones, 2012). In one respect, DNA methylation prevents the binding between promoters and some transcription factors, which are sensitive to CpG methylation (Greenberg and Bourc'his, 2019). In another respect, DNA-methylated sequences are recognized and bound by methyl CpG binding domain proteins (MBDs) and transcription repressors, including histone deacetylases (HDACs) and several zinc finger proteins, resulting in gene silence (Jones and Takai, 2001). Therefore, due to the ability of regulating gene expression, DNA methylation plays an important role in vital biological processes of eukaryotes (Klose and Bird, 2006), such as development (Greenberg and Bourc'his, 2019), immunity (Morales-Nebreda *et al*., 2019), and senescence (Horvath and Raj, 2018).

Studies on DNA methylation in animals tend to focus on the pathogenesis and cure of various diseases (Michalak *et al*., 2019; Schulze *et al*., 2019). Recent works have extended to domestication, transgenerational inheritance, and the environmental adaptability of wild animals, such as differentially methylated loci that exist between the domestic dog and gray wolf (Banlaki *et*

al., 2017), and between Creole cattle and their Iberian ancestors (Sevane *et al.*, 2019). In male wild guinea pigs, various paternal diets induce transgenerational epigenetic modifications (Weyrich *et al.*, 2018). Differentially methylated regions (DMRs) of the genome exist between complete and low lateral plate morphs of three-spine sticklebacks, which possess various phenotypes that may be a result of the number of natural predators (Smith *et al.*, 2015). Notably, most previous studies have investigated one type of tissue at the same state. However, tissue-specific differentially methylated regions (TDMs) exist (Song *et al.*, 2005), meaning that the levels and patterns of DNA methylation vary in different tissues, which may be correlated with tissue-specific gene expression (Reizel *et al.*, 2018). Therefore, exploring the patterns and levels of DNA methylation in various tissues could facilitate the investigation of the relationship between tissue-specific methylation and gene expression, as well as the correlation among different functional states of the same tissue. Only a few studies have investigated the patterns and levels of genomic DNA methylation in various tissues in animals, including Zhikong scallop (*Chlamys farreri*) (Sun *et al.*, 2014), swine strain Laiwu (Yang *et al.*, 2011), and sika deer (*Cervus nippon*) (Yang *et al.*, 2018).

In most of these studies, the methylation-sensitive amplified polymorphism (MSAP), or fluorescence-labeled MSAP (F-MSAP) technique was utilized to detect the patterns and levels of DNA methylation. MSAP, the modification of amplification fragment length polymorphism (AFLP) technique (Vos *et al.*, 1995), uses two different DNA methylation-sensitive restriction isoschizomer enzymes *Hpa* II and *Msp* I to detect the methylation state of Cs of 5'-CCGG-3' sites. Because *Hpa* II cleaves the hemi-methylated external Cs and *Msp* I cleaves full methylated internal Cs in 5'-CCGG-3' sites, MSAP technique explores the information of CHG and CpG methylation. In addition, this technique provides massive methylated or non-methylated loci of several individuals concurrently without genomic information (Bossdorf *et al.*, 2008), indicating that it efficiently detects methylation differentiation in non-model species (Schrey *et al.*, 2013). Compared to traditional MSAP, F-MSAP is more sensitive and effective for obtaining epigenetic information using fluorescently labeled primers and capillary gel electrophoresis.

Many studies found that various survival environments could induce change in DNA methylation in both plants and animals (Hu and Barrett, 2017; Huang *et al.*, 2017; Thiebaut *et al.*, 2019). Bats are considered as the bioindicators because their sensitive to climate change and habitat deterioration (Jones *et al.*, 2009), and thus environmental changes may induce their epigenetic

variance. In previous studies, the epigenetic variation in muscles among bat populations were explored using MSAP (Liu *et al.*, 2012). However, the extent of DNA methylation variation among tissues in bats is still unknown. In this study, the F-MSAP technique was used to explore the methylation patterns and levels in six tissues of the greater horseshoe bat (*Rhinolophus ferrumequinum*) in a normal state (i.e., no pregnancy, nursing, or hibernation). This work will address and enhance our understanding of the intrinsic epigenetic mechanisms of tissue development and differentiation, hibernating processes, and environmental adaptability of chiropteran animals and provided basic data for future studies.

MATERIALS AND METHODS

Ethics statement and tissue sampling

Six adult bats (3♂ and 3♀) were collected in an artificial canal in Jiyuan City, Henan Province in China during September 2017. Six tissues from each individual, including brown adipose tissue (BAT), brain (B), heart (H), kidney (K), liver (L), and muscle (M), were collected and frozen in liquid nitrogen. Genomic DNA of each tissue was extracted by a genome DNA extraction kit (SK1205, Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.). Animal experiments were conducted in accordance with the National Animal Research Authority of Northeast Normal University, China (approval number: NENU-20080416).

F-MSAP assay

About 20 mg of each tissue were ground into powder using liquid nitrogen. Genomic DNA was extracted by a UNIQ-10 column animal genome DNA extraction kit (SK1205, Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.). The quality of DNA was verified by 1% agarose gel electrophoresis. All DNA samples were adjusted to 200 ng/μL and stored at -20°C. The F-MSAP technique using 5′-FAM labeled primers was employed to detect the methylation levels of each tissue sample. F-MSAP uses the isoschizomer enzymes, *Hpa* II and *Msp* I, to detect the methylation state of Cs in 5'-CCGG-3' sites. The procedures for F-MSAP, including DNA digestion, ligation, pre-amplified PCR and selective PCR, are described below.

Two copies of genomic DNA from each sample were digested with *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I at 37°C for 4 h. The digestion solution (20 μL) contained 5 U *Eco*R I, 5 U *Hpa* II (or *Msp* I), 2 μL 10 × T4 Buffer, 200 ng DNA template, and deionized water. Then, all DNA fragments were ligated to adapters overnight at 16°C. The sequences of adapters were in accordance with Xu *et al.* (2000). The

ligation solution (25 μL) contained digestion products with 5 pmol *Eco*R I adapter, 50 pmol *Hpa* II/*Msp* I adapter, 1.5 U T4 DNA ligase, 2.5 μL 10 × T4 buffer, and deionized water.

Pre-amplification PCR was a necessary for the preliminary selection and enrichment of ligated DNA fragments. It contained 2 μL digestion-ligation product, 2 μL 10 × PCR buffer, 1.5 μL dNTPs (2.5 mM each), 0.5 μL primer E+A (5'-GACTGCGTACCAATTCA-3') (10 μM), 0.5 μL primer HM+T (5'-GATGAGTCTAGAACGGT-3') (10 μM), 1 U Taq polymerase, and 18.3 μL deionized water. The PCR conditions were as follows: Predenaturation at 94°C for 5 min; 35 cycles at 94°C for 20 s; 56°C for 20 s; 72°C for 40 s; and a final extension at 72°C for 7 min.

Selective amplification PCR further amplified the selective primer pair-specific fragments from PCR products from the previous step. The reaction solution (25 μL) contained 1 μL 20 times diluted pre-amplification product, 2 μL 10 × PCR Buffer, 1.5 μL dNTPs (2.5 mM each), 1 μL primer E+AXX (10 μM), 1 μL primer HM+TXX (5'-FAM labeled) (10 μM), 1 U Taq polymerase, and 18.3 μL deionized water. The PCR conditions were as follows: predenaturation at 94°C for 5 min; 13 touch-down cycles at 94°C for 20 s; 65°C (reduced by 0.7°C in each cycle) for 20 s; 72°C for 40 s; 23 cycles at 94°C for 10 s; 56°C for 10 s; 72°C for 20 s; and a final extension at 72°C for 7 min. The selective amplification PCR products were analyzed by capillary electrophoresis. Genemarker v2.2.0 software was used to detect the fluorescent signals at different intensities and locations relative to the GS500 standard.

Statistical analyses

F-MSAP uses the isoschizomer enzymes, *Hpa* II, which cleaves the hemi-methylated external cytosine nucleotide when the external cytosine on one strand is methylated (referred to as hemi-methylation), and *Msp* I, which cleaves full methylated internal Cs (referred to as full-methylation), to detect the methylation state of Cs in 5'-CCGG-3' sites. Four patterns were used for comparing the amplification products from *Hpa* II and *Msp* I (bands were scored '+' as present and '-' as absent). Type I: +/+, non-methylated; Type II: +/-, hemi-methylation; Type III: -/+, full-methylation; Type IV: -/-, uninformative due to hypermethylation or the absence of a fragment (Liu *et al*., 2012). Methylation levels were calculated using the following formulas:

Hemi-methylation level = II/ (I+II+III)×100%,

Full-methylation level = III/ (I+II+III)×100%,

Total methylation level= (II+III)/(I+ II+III)×100%

The msap program in R software was used to obtain an epigenetic binary matrix (methylation-susceptible loci, including types II and III) from the F-MSAP raw data based on the error rate-based threshold (ERT) at 5% (Perez-Figueroa, 2013). The 'Mixed Scoring 2' approach of *MSAP_calc* was used to calculate the number of unique loci of both methylation patterns in each tissue (Schulz *et al*., 2013). ANOVA was used to estimate significant differences of methylation levels among different tissues ($p < 0.05$).

Nei's unbiased distance based on the epigenetic binary matrix was calculated to estimate the epigenetic differentiation among tissues. Additionally, multivariate analyses were used to further explore the potential relationship among tissues based on methylated loci. The Between-Group Eigen Analysis (Parisod and Christin, 2008) using ADE-4 software was employed to group individual PCA scores of each tissue (PCA in the covariance matrix was based on the epigenetic binary matrix) by maximizing the between-group variance (Thioulouse *et al*., 1997). Statistical significance was assessed by the Romesburg randomization test (10^4 permutations).

RESULTS

To explore the patterns and levels of global DNA methylation in *R. ferrumequinum*, genomic DNA methylation patterns of six tissues from six individuals were analyzed using the F-MSAP technique. Eight primer combinations (i.e., E-AAC/HM-TAC, E-AAC/HM-TCG, E-ACA/HM-TAG, E-ACA/HM-TGT, E-ACT/HM-TCG, E-ACT/HM-TTC, E-ACC/HM-TTC, and E-ACC/HM-TGT) were selected to explore epigenetic information. A total of 10,203 fragments were detected, including 2,706, 2,855, 1,118, 1,264, 1,138, and 1,122 fragments in BAT, B, H, K, L, and M, respectively.

Genomic DNA methylation patterns and levels in six tissues of R. ferrumequinum

Type II (hemi-methylation) and III (full-methylation) bands comprised 31.4% (3,203/10,203) and 35.6% (3,632/10,203) of the total fragments, respectively. Total methylated fragments accounted for 67.0% (6,835/10,203), and total methylation levels varied from 57.6% to 73.8% among tissues (Fig. 1). The levels of hemi-methylation were not significantly different among tissues, while significant differences in full-methylation patterns ($p< 0.001$) and total methylation ($p= 0.001$) were detected (Fig. 1). The levels of full-methylation patterns and total methylation in BAT and B were higher than the other four tissues. When focusing on each methylation pattern of each tissue, the full-methylation levels of BAT and B were significantly higher than hemi-methylation ($p= 0.005$ and $p < 0.001$, respectively). However, the opposite results were found for H ($p= 0.032$) and L ($p= 0.014$), indicating that

hemi-methylation was the major methylation pattern in these two tissues, rather than full-methylation. Significant differences were not detected between the levels of the two methylation patterns in K and M.
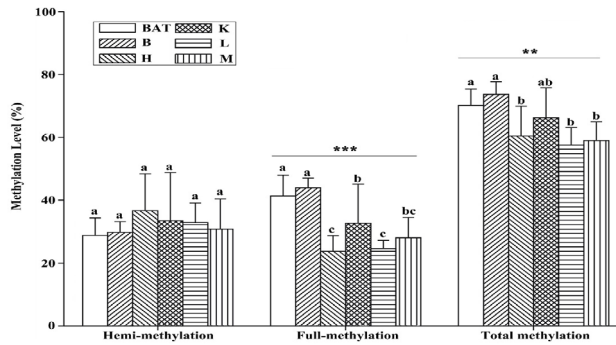

Fig. 1. Methylation levels in six tissues of *R. ferrumequinum*. Data are presented as the mean ± SE. Asterisks indicate significant differences: **, $p < 0.01$; *** $p < 0.001$.
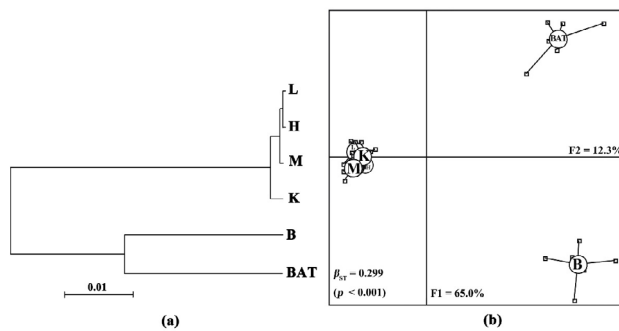

Fig. 2. Epigenetic differentiation among six *R. ferrumequinum* tissues. (a) Clustering tree based on Nei's unbiased epigenetic distance matrix. (b) Between-group eigen analysis using PCA values based on the epigenetic covariance matrix; the F1 and F2 axes explain 77.3% of the total variance ($p < 0.001$).

Epigenetic differentiation among tissues

Methylated loci and epigenetic distances were obtained to explore methylation differentiation among tissues. There were 44, 32, 59, 16, 15, and 42 unique methylated loci in BAT, B, H, K, L and M, respectively (Table I). BAT had the largest number of unique hemi-methylation loci, but the smallest number of unique full-methylation loci. L and M had the smallest number of unique hemi-methylation loci, while H had the largest number of unique full-methylation loci. Compared to BAT and B, the tissues of H, K, L, and M had the lowest epigenetic divergence (0.005–0.009) and clustered on one branch in the clustering tree based on Nei's unbiased distance matrix (Fig. 2a). Both B and BAT showed lower epigenetic divergence (0.025) and

were obviously different from other tissues (0.064–0.092). The results of the multivariate analyses, which further explored the potential relationship among tissues based on methylated loci, supported the above results. However, B and BAT were significantly different ($p < 0.001$) (Fig. 2b).

Table I. Number of unique methylation loci in six *R. ferrumequinum* tissues.

Tissue	Number of unique hemi-methylation loci	Number of unique full-methylation loci
BAT	43	1
B	28	4
H	14	45
K	9	7
L	6	9
M	6	36

DISCUSSION

DNA methylation regulates gene expression by binding MBDs and recruiting transcription repressors (i.e., HDACs) to repress transcription processes (Greenberg and Bourc'his, 2019), and thus, may play a vital role in tissue development and differentiation (Jones and Takai, 2001). Therefore, before further investigating the relationship between tissue-specific methylation and gene expression, the patterns and levels of DNA methylation in various tissues were explored first. In this study, the F-MSAP technique was used to explore the patterns and levels of methylation in six tissues of *R. ferrumequinum*.

It should be noted that the pattern and level of DNA methylation in the same tissue may vary seasonally. For example, Biggar and Storey (2014) observed a 1.7-fold increase in global DNA methylation of BAT tissue in hibernated 13-lined ground squirrels when compared to euthermic controls. Moreover, Weyrich *et al.* (2016) found that a temporary increase in ambient temperature induced methylation variation in the liver of male wild guinea pig (*Cavia aperea*), and these epigenetic modifications were transmitted to the F2 generation. Additionally, the age structure of free-living wild Bechstein's bats (*Myotis bechsteinii*) was established by DNA methylation of homologous genes (Wright *et al.*, 2018). The results of these previous studies indicate that different physiological states of the body or environment (e.g., temperature) could affect methylation status. Considering these findings, greater horseshoe bats in a normal state (i.e., adult; no pregnancy, nursing, or hibernation) were selected for observation in this study.

The genome-wide methylation levels in the six tissues of *R. ferrumequinum* were relatively high, ranging from 57.6% to 73.8% with an average of 67.0%. These values were slightly higher than that of swine (Yang *et al.*, 2011) and sika deer (Yang *et al.*, 2018). Because *Hpa* II cleaves the hemi-methylated external Cs and *Msp* I cleaves full methylated internal Cs in 5'-CCGG-3' sites, the hemi-methylation and full-methylation pattern would be represented as CHG methylation and CpG methylation in this study, respectively. Significant differences were detected in the levels of full-methylation pattern among tissues, while there were no significant differences detected in the levels of hemi-methylation patterns among tissues. However, the levels of CHG methylation in each tissue were non-negligible. The existence and roles of non-CpG methylation have been ignored for a long time. In fact, non-CpG methylation occurs in embryonic tissues and stem cells (Ramsahoye *et al.*, 2000) and may have higher frequencies in adult mammalian somatic cells (i.e., brain and skeletal muscle) (Pinney, 2014). Hao *et al*. (2016) found that there were similar CpG methylation proportions, but varying proportions in non-CpG methylation between control and heat-stressed pigs, indicating that non-CpG methylation was functionally involved in gene regulation (Pinney, 2014; Fuso, 2018; Fuso and Lucarelli, 2019). Notably, CHG methylation levels in H, K, L, and M tissues approached or exceeded corresponding CpG methylation levels, while BAT and B possessed a massive number of unique hemi-methylation loci. Thus, CHG methylation in each tissue may also play an important part in tissue development and differentiation of *R. ferrumequinum*.

The epigenetic differentiation among tissues, revealed by Nei's unbiased epigenetic distances and the multivariate analyses, indicated that H, K, L, and M were more similar than B or BAT. B and BAT had different methylation profiles, which remains unclear and is worth investigating in the future. The differences among H, K, L, and M were inconspicuous; however, the various methylation levels and diverse number of unique methylated loci indicate that there was considerable epigenetic differentiation among the four tissues, which may be related to gene expression during tissue development and differentiation (Yang *et al.*, 2018).

CONCLUSION

In summary, the patterns and levels of methylation in six tissues of *R. ferrumequinum* in a normal state were explored using the F-MSAP technique in this study. There were relatively high methylation levels and both hemi-methylation and full-methylation exhibited major methylation patterns. The tissues levels of full-methylation and total methylation were significantly different and possessed a diverse number of unique methylated loci. A massive hemi-methylation bands existed, indicating that CHG methylation may play a potential role in tissue development and differentiation in greater horseshoe bats. BAT and B differed from other four tissues in DNA in full-methylation and total methylation. This work provides basic data for future studies on the periodic variation of DNA methylation and intrinsic epigenetic mechanisms of vital biological processes in Chiropteran animals.

Statement of conflict of interest
The authors have declared no conflict of interest.

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