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Natural epigenetic variation in the female great roundleaf bat (*Hipposideros armiger*) populations

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Abstract Epigenetic modifications are considered to have an important role in evolution. DNA methylation is one of the best studied epigenetic mechanisms and methylation variability is crucial for promoting phenotypic diversification of organisms in response to environmental variation. A critical first step in the assessment of the potential role of epigenetic variation in evolution is the identification of DNA methylation polymorphisms and their relationship with genetic variations in natural populations. However, empirical data is scant in animals, and particularly so in wild mammals. Bats are considered as bioindicators because of their sensitivity to environmental perturbations and they may present an opportunity to explore epigenetic variance in wild mammalian populations. Our study is the first to explore these questions in the female great roundleaf bat (*Hipposideros armiger*) populations using the methylation-sensitive amplified polymorphism (MSAP) technique. We obtained 868 MSAP sites using 18 primer combinations and found (1) a low genomic methylation level (21.3 % on average), but extensive DNA methylation polymorphism (90.2 %) at 5′-CCGG-3′ sites; (2) epigenetic variation that is structured into distinct between- (29.8 %) and within- (71.2 %) population components, as does genetic variation; and (3) a significant correlation between epigenetic and genetic variations ($P < 0.05$). These results may also apply to other wild mammalian populations. The possible causes for the correlation between epigenetic and genetic variations are discussed.

Keywords Chiroptera · DNA methylation · Mammal · MSAP

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Introduction

Biodiversity faces challenges from habitat destruction, fragmentation, over exploitation, and global climate change (Jablonski 2004). Organisms have to modify their phenotype in response to such abiotic stresses (Agrawal 2001). Studies conducted in animals (Dolinoy et al. 2007; Kucharski et al. 2008) and plants (Bossdorf et al. 2010; Lira-Medeiros et al. 2010) have suggested that epigenetic processes play a crucial role in mediating environmentally induced phenotypic variations (Vogt et al. 2008; Angers et al. 2010; Gao et al. 2010). Those epigenetic patterns may be stably inherited by future generations (Richards 2006; Jablonka and Raz 2009; Skinner and Guerrero-Bosagna 2009), implying that epigenetic processes have potential evolutionary effects (Jablonka et al. 2005; Bossdorf et al. 2008; Richards 2008).

Epigenetics is the study of heritable changes in gene expression that result from covalent modifications of DNA and/or histone proteins, which do not entail a change in the DNA sequence per se (Richards 2006; Bird 2007). The epigenetic information is encrypted in genetic sequences (Bonasio et al. 2010; Migicovsky and Kovalchuk 2011). Before assessing their potential role in evolution, investigating epigenetic variations and their relationship with genetic variations that are observed in a real-world context are the important steps (Richards 2006; Bossdorf et al. 2008). Significant correlation suggests that epigenetic differentiation may be interpreted by genetic variation (Li et al. 2008; Ochogavía et al. 2009) or a downstream consequence of DNA sequence variation (Herrera and Bazaga 2010). In contrast, the lack of correlation between the epigenetic and genetic variances would suggest that natural variation in epigenetic modifications may be autonomous from the strict control of genetic variance (Richards 2006), as an additional system in evolution (Cervera et al. 2002; Vaughn et al. 2007).

DNA methylation is one of the best studied epigenetic mechanisms and methylation variability is crucial for promoting phenotypic variance of organisms under different environmental conditions (Gao et al. 2010; Massicotte et al. 2011). Natural genomic methylation variations have been investigated in plant populations (Li et al. 2008; Gao et al. 2010; Herrera and Bazaga 2010, 2011; Lira-Medeiros et al. 2010); however, studies on the origin, stability, variation, and inheritance of DNA methylation polymorphisms of wild animal populations in the evolution and ecological adaptation are scarce. There have only been three studies on fish (Blouin et al. 2010; Massicotte et al. 2011; Morán and Pérez-Figueroa 2011) and one on birds (Schrey et al. 2012). Wild animals constantly experience environmental variations; therefore, it is necessary to expand our understanding of their genomic DNA methylation patterns.

Bats are considered as bioindicators because they can reflect wider-scale impacts on the biota of interest because of their sensitivity to environmental variation, e.g., global climate change or habitat deterioration (Jones et al. 2009). Varied environmental conditions could induce epigenetic variance (Gao et al. 2010; Lira-Medeiros et al. 2010), so bats may present an opportunity to investigate epigenetic variation in wild mammalian populations. We selected female great roundleaf bat (*Hipposideros armiger*, Hodgson, 1835) populations, because this species is widely distributed in South China and has the wing morphology of short-distance migrants (Norberg and Rayner 1987), implying a low level of population mixing. Using the methylation-sensitive amplified polymorphism (MSAP) technique and five female populations, we explored: (1) the extent and pattern of genome-wide DNA methylation of the female *H. armiger* populations. In mammals, about 70 % of all CpG sites in somatic tissues are methylated (Ehrlich et al. 1982; Allis et al. 2007) and we should find high level of methylation in bats; (2) whether the DNA methylation variation varied within- and between-populations, as does genetic variation; and (3) the correlation between epigenetic and genetic variances in bats; a key aspect that has not been previously explored for any wild animal.

Materials and methods

Samples and DNA extraction

All female *H. armiger* samples were from the deposited specimens in the Museum of Natural History of Northeast Normal University, Jilin Province, China. Five populations comprising 25 females in total were chosen, including 6 from Sichuan Province (SCWY), 6 from Jiangxi Province (JXJGS), 5 from Guizhou Province (GZAL), 4 from Guangdong Province (GDSG), and 4 from Yunnan Province (YNYJ). According to Zhang et al. (1997), these populations belong to four zoological regions in China: JXJGS and GDSG belong to the East region, GZAL and SCWY belong to the West region, and YNYJ is from the edge of the Southwest and South Yunnan regions. Information on their caves of origin, including latitude, longitude, elevation, and climate data (mean annual temperature, MAT; mean annual rainfall, MAR; mean annual atmospheric pressure, MAAP; and mean annual wind velocity, MAWV; data from China Meteorological Data Sharing Service System, http://cdc.cma.gov.cn/shuju) are shown in Table 1. Genomic DNA was extracted from muscle tissues using a UNIQ-10 Column Animal Genome DNA Extraction Kit (SK1205, Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.).

MSAP technique

The MSAP method, based on the amplified fragment length polymorphism (AFLP) (Vos et al. 1995) technique, is well suited for non-model species with little genomic information and provides rapid epigenetic fingerprints for a large number of samples (Bossdorf et al. 2008). This technique uses the enzyme *Eco*RI (rare cutter) with either one of two methylation-sensitive isoschizomer restriction enzymes, *Hpa*II and *Msp*I (frequent cutters). The isoschizomer enzymes recognize the same restriction site (5′-CCGG-3′), but have different cytosine (Cs) methylation sensitivities. *Hpa*II is sensitive to full methylation (both strands methylated) of either Cs, but cleaves the hemimethylated external Cs (Mann and Smith 1977), whereas *Msp*I cleaves

Table 1 Location, latitude, longitude, elevation, and climate data of each bat colony

Colony	Location	Latitude (°N)	Longitude (°E)	Elevation (m)	MAT (°C)	MAR (m)	MAAP (kPa)	MAWV (m/s)
SCWY	Wanyuan, Sichuan, China	32.12	107.95	926	14.7	1.23	93.82	1.9
JXJGS	Jinggangshan, Jiangxi, China	26.60	114.20	1,112	18.4	1.52	100.72	2.2
GZAL	Anlong, Guizhou, China	25.28	105.54	1,061	14.9	1.01	81.06	2.1
GDSG	Shaoguan, Guangdong, China	24.77	113.56	147	20.4	1.58	100.66	1.5
YNYJ	Yuanjiang, Yunnan, China	23.55	102.27	1,444	18.7	0.86	86.85	3.3

MAT mean annual temperature, *MAR* mean annual rainfall, *MAAP* mean annual atmospheric pressure, *MAWV* mean annual wind velocity

sites with fully methylated internal Cs (Waalwijk and Flavell 1978; Ehrlich and Wang 1981) (the restriction enzyme database: http://rebase.neb.com/rebase/rebase.html). Both enzymes cleave fully non-methylated 5′-CCGG-3′ (Ehrlich and Wang 1981; McClelland et al. 1994).

The adapters and primers for *Eco*RI and *Hpa*II–*Msp*I digest fragments were designed according to Vos et al. (1995) and Xu et al. (2000), respectively. Two copies of each individual genome were digested by *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively. Eighteen selective amplification primer pairs were chosen for PCR (Eco + AGC − H/M + TAC/TAG/TCG/TTA/TGA/TGT, Eco + ACT − H/M + TAC/TCT/TGA/TGC, Eco + ACA − H/M + TAC/TAG/TCT/TGC, Eco + ACC − H/M +TCT/TGA/TGT, Eco + AAC − H/M + TTC). The PCR protocols were according to Vos et al. (1995). The products were electrophoresed for 3 h on 6 % denaturing polyacrylamide gels and visualized by silver staining (Bassam et al. 1991). To reduce the potential impact of size homoplasy, only unambiguous and intense bands, ranging in size from 150 to 500 bp, were scored (Caballero et al. 2008).

Statistical analysis

Methylation-sensitive amplified polymorphism bands were scored as present "+" and absent "−", hence there were three patterns for comparing amplification products from *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I (Table 2): (a) present in both (+/+), non-methylated; (b) present in only *Eco*RI/*Hpa*II (+/−, CHG methylation) or *Eco*RI/*Msp*I (−/+, CG

Table 2 *Hpa*II and *Msp*I sensitivities to 5′-CCGG-3′ methylation status from REBASE specifications

HpaII	MspI	Types	Notes
+	+	Non-methylated	
+	−	Methylated	CHG methylation
−	+	Methylated	CG methylation
−	−	Uninformative	Fragment absence or hypermethylation

+ and − represent the presence or absence of a fragment, respectively

methylation), methylated; (c) absent in both (−/−), uninformative because of the absence of a fragment or hypermethylation. MSAP fragments that differ in their presence/absence in the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns in at least one individual were considered as methylated bands; a site was considered as "methylation-sensitive polymorphism" (MSP) band if there was at least one individual in which the site was methylated and at least one individual for which the site was non-methylated or missing. The MSP binary matrix was scored as 1 (+/− or −/+), 0 (+/+), and missing (−/−). The other non-methylated loci, which were identical in their presence/absence in the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns, were considered as CCGG-genetic markers; a site was considered as "methylation-insensitive polymorphism" (MIP) band if the fragments were absent in both enzymes in at least one individual. The MIP binary matrix was scored as 1 (+/+) and 0 (−/−).

We calculated the gene flow among populations based on the MIPs matrix by the PopGen program (version 1.31) and used the Kruskal–Wallis H test to estimate the significance of the difference in the CG and CHG methylation patterns among populations. We calculated the Shannon diversity index based on the frequency of each band among the 25 individuals to estimate the within-population epigenetic diversity (H_{pop}). Significant differences in the Shannon index among populations were assessed by the Kruskal–Wallis H test, and the significance of the test was adjusted by the sequential Bonferroni correction (Rice 1989). The coefficient of epigenetic differentiation was computed as $G_{ST} = (H_{sp} - H_{pop})/H_{sp}$ (Bussell 1999), where H_{sp} is the within-species epigenetic diversity.

We used multivariate analyses to explore between-population epigenetic structure. Principal component analysis (PCA) on inter-profile covariance matrix based on MSP and MIP binary profiles were performed to provide a genome-wide variability point of view, summarized in a few synthetic variables. The between-group Eigen analysis [BPCA–PCA among groups based on PCA among individuals, (Parisod and Christin 2008)] was processed to group individual PCA profiles into populations maximizing

the between-group variance. Statistical significance was assessed by the Romesburg randomization test (10^4 permutations). Multivariate analyses were performed by ADE-4 software (Thioulouse et al. 1997).

Between-group Eigen analysis divides the variance into within- and between-population components and it is based on Euclidean distances; therefore, it can be considered as analogous to F statistics (called β_{ST}) (Parisod et al. 2005; Lira-Medeiros et al. 2010). The β_{ST} value equals the ratio of the inertia of the between PCA to the total inertia. However, β_{ST} are not equivalent to F statistics and β_{ST} values may be overestimated because BPCA maximizes the between-group variance (Parisod and Christin 2008).

To further explore the contribution of both epigenetic and genetic profiles to the female *H. armiger* population structure, we used the symmetrical co-inertia analysis, which maximizes shared structures among multiple data sets drawn from the same samples (Lira-Medeiros et al. 2010). This analysis can be safely used for multivariables that are related because it does not rely on linear regressions (Dolédec and Chessel 1994). Statistical significance was assessed by 10^4 Monte Carlo permutations in the ADE-4 software (Thioulouse et al. 1997).

We used Mantel's tests (Mantel 1967) to assess relationship of epigenetic (MSP) and genetic (MIP) profiles by two main indexes (10^4 permutations). First, according to Cervera et al. (2002), we compared Dice coefficients (Sneath and Sokal 1973), which were calculated independently from both MSP and MIP profiles in NTSYS 2.0 Software. Second, we compared pairwise β_{ST} (BPCA) values of both profiles (Parisod et al. 2005; Parisod and Christin 2008; Lira-Medeiros et al. 2010), which were calculated by the ADE-4 software (Thioulouse et al. 1997).

To exclude the effects of confounding environmental factors, we performed partial Mantel's tests while controlling environmental variables (geographical distance, climate variables) by PASSaGE v.2 (Rosenberg and Anderson 2011). This information was used to determine whether the residual variability in the epigenetic and genetic profiles was still significantly correlated after removing the effects of those environmental variables. The distance matrices of those environmental variables were calculated by a Euclidean method. The geographical distance was calculated from the latitude and longitude of each population, and was then logarithmically transformed (by $\log_e$). For climate variables, we implemented a PCA on a correlation matrix of the four climate variables (MAT, MAR, MAAP, and MAWV) and used the first two PC factor scores to obtain a matrix of Euclidean distances. All the Mantel's tests were randomly permuted 10^4 times.

Results

Variation in methylation profiles

To characterize variation in methylation profiles, we analyzed genomic DNA methylation patterns of 25 female *H. armiger* from 5 populations. We scored 868 MSAP sites using 18 primer combinations, with an average of 48.2 bands per primer pair. By comparing the presence/absence of restricted fragments in *Hpa*II and *Msp*I assays for each individual, 499 loci showed similar digestibility, but varied among populations, suggesting a non-methylated 5′-CCGG-3′ restriction site, i.e., 5′-CCGG-genetic variation, and showed low gene flow among those populations (Nm ranged from 1.03 to 2.23). The other 369 loci showed differential digestibility suggesting MSPs. Thirty-six loci were methylated in all 25 animals and 333 loci (90.2 %) were polymorphic among those individuals. Of these 333 loci, 46 polymorphic sites were found with both isoschizomers, while 287 were detected with only one of the isoschizomers, including 104 CHG methylated and 183 CG methylated sites.

The numbers of various fragments attributed to non-methylated (+/+), CHG methylated (+/−), CG methylated (−/+), and uninformative (−/−), respectively, were calculated for each population based on MSAP profiles. The total 5′-CCGG-methylation level ranged from 19.6 % in YNYJ to 23.5 % in GZAL, with a mean of 21.3 % at the species level (Fig. 1). The difference in the genome-wide methylation level (CG and CHG methylation) among all populations was significant (Kruskal–Wallis $\chi^2 = 13.33$, $df = 4$, $P = 0.010$); however, a non-significant difference was found in the level of CG (Kruskal–Wallis $\chi^2 = 0.566$, $df = 4$, $P = 0.967$) and CHG methylation patterns (Kruskal–Wallis $\chi^2 = 8.826$, $df = 4$, $P = 0.066$). Populations showed more difference in CHG than in CG methylation patterns, because isoschizomer *Msp*I is insensitive to
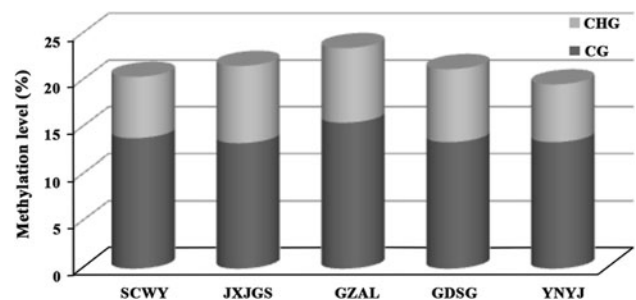

Fig. 1 MSAP-based cytosine methylation levels of two major types (CG and CHG) of the randomly sampled 5′-CCGG sites in five female *H. armiger* populations. Methylation level (%) was calculated by dividing MSAP bands representing methylated 5′-CCGG sites (differential presence/absence of restricted fragments in *Hpa*II and *Msp*I assays) against the total number of scored bands

methylation and shows similar CG pattern among populations (Blouin et al. 2010).

Epigenetic structure

We used frequency based and multivariate methods to assess the epigenetic structure of female *H. armiger* populations. The within-population epigenetic Shannon indices calculated for the five populations were 0.078 (SCWY), 0.071 (JXJGS), 0.084 (GZAL), 0.074 (GDSG), and 0.114 (YNYJ), respectively, and were significantly different (Kruskal–Wallis $\chi^2 = 50.982$, $df = 4$, $P < 0.001$). The YNYJ population showed a higher Shannon index than the others, implying higher variation. Based on the partition of Shannon diversity within and among populations, we calculated a G_{ST} of 0.327.

For the between-population analysis, the first axes of the PCA based on covariance matrix (MSP) summarized only 12.2 % of total inertia, and the first 19 PCA axes, which together represented 91.0 % of the information contained in the epigenetic data set, would be used for further analyses. The multivariate BPCA based on the above PCA scores resulted in a significant β_{ST} of 0.298 ($P < 0.001$), showing that epigenetic variance could be divided into distinct between- (29.8 %) and within- (71.2 %) population components. β_{ST} are considered as analogous, but not equivalent, to F statistics, and were used to represent the epigenetic structure (Parisod et al. 2005; Parisod and Christin 2008). In the new subspace (Fig. 2), the five populations showed obvious clustering in four quadrants by the first two axes (summarizing 62.2 % of the total inertia), while GZAL and GDSG populations (represented by 3 and 4, respectively) were intertwined, and YNYJ (represented by 5) showed more variation than the other populations.

Correlation between epigenetic and genetic profiles

Co-inertia analysis was used to evaluate the contribution of both genetic and epigenetic profiles to the *H. armiger* population structure. The first two axes explained 41.6 % of the total co-variation between the epigenetic and genetic profiles, and this association was significantly different from the value expected for random association ($P < 0.001$). Epigenetic and genetic profiles displayed similar distributions (Fig. 3). Epigenetic and genetic profiles were significantly correlated based on Dice coefficients ($r = 0.913$, $P < 0.001$) and pairwise β_{ST} ($r = 0.882$, $P = 0.009$). To remove the effects of environmental variables, we performed partial Mantel's tests while controlling geographic distance and climate variables, and the results still showed significant correlation (Dice coefficients: $r = 0.967$, $P = 0.034$; β_{ST}: $r = 0.943$, $P = 0.027$). These
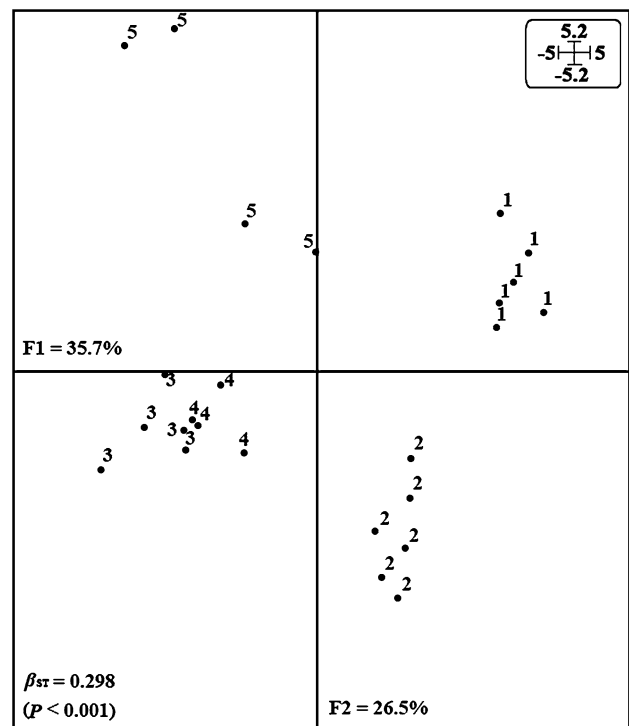

Fig. 2 Between-group Eigen analysis of the five female *H. armiger* populations using PCA values based on the epigenetic covariance matrix (MSP). *F1* and *F2* values show the contribution of the two principal components summarizing the total variance of each data set. The first two axes summarize 62.2 % of the total variance. The numbers from *1* to *5* represent the five populations: SCWY, JXJGS, GZAL, GDSG, and YNYJ. β_{ST} were calculated by BPCA for epigenetic profiles and tested with 10^4 Romesburg randomization permutations

results suggested a significant correlation between epigenetic variance and nucleotide sequence variation.

Discussion

The MSAP technique only detects methylation at 5′-CCGG-3′ sites and cannot discriminate between methylation and fragment absence when both cytosines are hypermethylated; therefore, the level of genomic DNA methylation may be underestimated. Bearing in mind this inherent limitation of the technique, our study has explored the level and pattern of genome-wide 5′-CCGG-methylation in five female *H. armiger* populations. From our results, those populations have a low genomic methylation level (21.3 % on average), but extensive DNA methylation polymorphism (90.2 %) at 5′-CCGG-3′ sites. The difference in the genome-wide methylation level among populations was significant. Significant relative β_{ST} values suggested that epigenetic variation was structured into distinct between- and within-population components in the
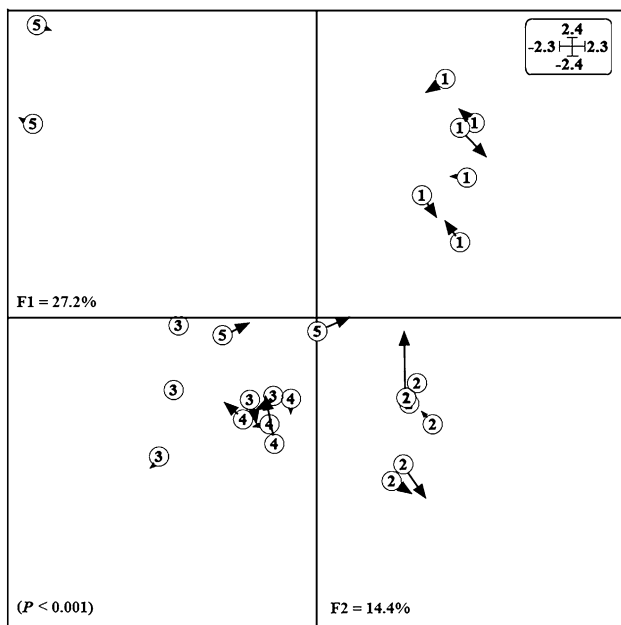
Fig. 3 Co-inertia analysis of female *H. armiger* populations using PCA scores based on genetic (MIP) and epigenetic (MSP) covariance matrices. The co-inertia analysis maximized the covariance of PCAs. *F1* and *F2* values show the contribution of the two principal components summarizing the total variance of each data set. *Circles* correspond to the projection of genetic profiles (MIP) and *arrowheads* correspond to the projection of epigenetic profiles (MSP). The numbers from *1* to *5* represent the five populations: SCWY, JXJGS, GZAL, GDSG, and YNYJ. The significance test of this association was done with 10^4 permutations (Monte Carlo test)

female *H. armiger* populations, similar to plants (Keyte et al. 2006; Li et al. 2008), in a manner that could be considered analogous to genetic structure (Herrera and Bazaga 2010).

The BPCA plot based on methylation profile also showed that female *H. armiger* populations were obviously separated to four major groups, as if the environmental factors covarying across geographical locations exert a relatively greater impact on DNA methylation (Fig. 2). However, GZAL and GDSG populations, which came from different zoological regions, were intertwined. Additionally, individuals in the YNYJ population showed more variation than the other populations. YNYJ is located at the edge of the Southwest and South Yunnan regions, implying migration in this population. Moreover, epigenetic and genetic profiles displayed similar distributions in the co-inertia subspace (Fig. 3) and had a significant association ($P < 0.001$). The above findings raise an interesting question: is the differentiation of genome-wide DNA methylation among populations dependent on nucleotide sequence variation in bats?

To address this question, we estimated the correlation between epigenetic and genetic profiles. It is a matter of debate amongst ecologists as to whether epigenetic

variance is independent (Cervera et al. 2002; Vaughn et al. 2007) and/or dependent from genetic variation (Li et al. 2008; Ochogavía et al. 2009; Herrera and Bazaga 2010) in natural populations. In our study, Dice coefficients and/or pairwise β_{ST} (based on inter-individual distance matrices of MSP and MIP with control of environmental variables) were used to address this question by partial Mantel's tests, respectively. Although we used genetic characterization of the populations by multilocus approaches based on the whole set of non-methylated MSAP fragments (Ashikawa 2001; Cervera et al. 2002), we found a clear and significant correlation between epigenetic and genetic variations in the female *H. armiger* populations. Similar result has been reported in humans (Zhang et al. 2010).

The significant correlation suggested that methylation-based epigenetic variance might be associated with the control of genetic instability. Bjornsson et al. (2008) found that DNA methylation varies over time with familial clustering in humans, which implies that epigenetic variance may be under genetic control. In addition, genetic variation in the form of a transposon, e.g., absence, would directly affect the epigenetic state of the retroelement, which could induce varied phenotype in the genetically identical offsprings (Morgan et al. 1999; Michaud et al. 1994). Those researches in mammals have found that the influence of some epigenetic effectors is sequence dependent or allele specific (Meaney and Ferguson-Smith 2010). Therefore, the significant correlation between epigenetic and genetic variances in the female *H. armiger* populations in our study is probably caused by methylation variation that depends on DNA sequence difference through interactions involving multiple genes (Liu et al. 2010).

However, neutral drift could also account for the significant correlation between epigenetic and genetic variations. If epigenetic differences are determined entirely by genetic control, strong divergent selection would be required to cause the epigenetic variations in a situation of high gene flow between populations. That means high gene flow might minimize the population differences caused by the drift. But in this study, we detected limited gene flow among female *H. armiger* populations studied (Nm ranged from 1.03 to 2.23), which implies that stochastic processes of drift might result in epigenetic population patterns that develop in parallel to genetic differences, causing a correlation without a functional link between the two (Richards et al. 2010).

Investigating DNA methylation polymorphisms in natural populations and evaluating the relevance of their epigenetic and genetic profiles are critical steps for assessing the potential role of epigenetic variation in wild mammalian microevolution. Using MSAP, we found extensive epigenetic polymorphism, distinct epigenetic structures, and a significant correlation between epigenetic

and genetic variations in the female great roundleaf bat (*H. armiger*) populations, which may also apply to other wild mammalian populations. The latter observation suggests that the epigenetic variation is under genetic control and/or the correlation was caused by neutral drift.

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