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Morphology variation among eleven local populations of the endemic *Myotis davidii* in China

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Abstract: The David's myotis (*Myotis davidii*) is a endemic species of China. In our research, we investigated the relationships between the genetic, environmental, geographic variations and the external variations. Mantel test indicated influencing the external variable by genetic distance, geographic distance and environmental Euclidean distance. When we used stepwise linear regression to analysis the influencing extent of each environmental factor on the phenotypic variation, the results indicated that the temperature was the most important factor influencing phenotypic variation among ten environmental variables. We also reconstituted the phylogenetic tree based on the mitochondrial DNA HIV sequence, external or combined data of eleven local *Myotis davidii* populations, respectively, in order to infer the biogeography distribution patterns of external and genetic variables. ML tree, BI tree and phenotypic minimum spanning tree were all divergent into three lineages (region IF, IIA and IIC in lineage A; region IVB, IVD and IVE in lineage B; region IID in lineage C). And the external and genetic variation patterns paralleled with unique Chinese topographic and physiogeographic features in general. However, individuals from southeast mid-low elevation mountain areas (IID) showed a difference compared to other regions and might be a transition area connecting the eastern plain and western plateau. Our results highlight the importance of adaptive divergence for geographical patterns. And the external variables along the genetic divergence, environmental and geographic gradient, evolutionary branching can occur much more easily, which increased availability of phylogeographical and ecological data should facilitate further understanding of population divergence drivers at broad scales.[*Life Sci J* 2021;18(3):54-63]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 11. doi:10.7537/marslsj180321.11.

1. Introduction

The populations of the same species can exhibit marked variation in morphology, genetic, physiology and particularly body size (Mayr 1963, Yablokov 1966; Endler 1977, Jetz *et al*. 2009). In general, these divergences are affected by many factors, such as geographical distribution, environmental heterogeneity, spatial separation, as well as the intra-population evolution, (Mayr 1956, Mayr 1963, Ellison *et al*. 2004, Jetz *et al*. 2009, You *et al*. 2010). Geographic variation of vertebrate characters usually represented the local adaptation, and exhibited parallel eco-geographic variation and the eco-geographic trends (Bergmann 1848, Endler 1977, James 1983, Mittelbach *et al*. 1999) across a large biogeography region. Natural selection maintained the clines of phenotype character variations, the genetic and non-genetic components of phenotypic variation must co-vary (Thorpe 1976, James 1983).

In China, topography was in three levels with the highest in the west and the lowest in the east (Zhang *et al*. 2008). The climate varieties with the centre of Tibetan Plateau which induces the special vegetation distribution pattern in China (Tong *et al*. 1999). Thus, geographic variation in genetic, morphology and cranial responded to the environmental gradients and population evolution history (Ellison *et al*. 2004, Goodman *et al*. 2009). The phylogeographic contained the potential information about geographical components within species (Bermingham and Moritz 1998, Avise 2000, Arntzen *et al*. 2007). However, morphological variation correlated with environmental and geographic variation has been mostly excluded from molecular analyses. Even though contain relatively small numbers of characters in comparison to the molecular data, morphological data may contribute more support and stability to the combined analysis (Gatesy 2002, Jetz *et al*. 2009), which allow a closer approach to the true phylogeny (Querioz 1993, Nylander *et al*. 2004). The evolutionary history and the pattern of external variation is of special interest, due to the hierarchical nature of development and the possible constraints that morphological integration could pose for ontogenetic and evolutionary changes (Blomberg *et al*. 2003, Ivanovic *et al*. 2008, Ivanovic *et al*. 2012). The phylogenetic framework is essential

in assessing evolutionary trends (Ivanovic *et al.* 2012). In addition, morphological integration may be responsible for coordinated changes in diverse parts of morphology on phylogenetic time scales (Zelditch and Fink, 1995). Testing for phylogenetic signal, defined as the degree to which phenotypic similarities are congruent with phylogeny, can help to discern between these two possibilities (Blomberg *et al.* 2003, Cardini and Elton 2008).

Myotis davidii is an endemic species of China, living in several province of China (Smith et al. 2009), and geographically distributing primarily in Oriental Realm and Palaearctic (Zhang 1997). The molecular phylogeography research of *M. davidii* populations indicated the demographic history and population structure were associated with climatic oscillations, topography and eco-environmental variation of China (You *et al.* 2010). Thus, we predict that phenotypic plasticity vs. genetic control, geographical distance and environmental heterogeneity should explain much of the phenotypic variability among populations of *M. davidii*. Here, we use the external data and mtDNA HVI sequences to (1) find out whether morphologic and cranial variation of *M. davidii* population are associated with the genetic divergences and environmental conditions (such as the geomorphologic and climate categories, latitude, longitude, elevation, precipitation, humidity, temperature, barometric pressure and wind velocity across a large geographic region), and to assess the important factors

influencing phenotypic variation, (2) reconstitute the phylogenetic relationships based on the genetic, morph-metric and the combined data, respectively, and to examine the relationships among each population.

2. Material and Methods
2.1 Climate and geomorphology

In our study, latitude, longitude, and elevation of each site were determined using GPS (global positioning system, ± 50 m) (Table 1). Climatic variables, such as annual precipitation, annual humidity, annual temperature, annual barometric pressure and annual wind velocity, were obtained for each site from China Meteorological Administration (http://www.cma.gov.cn/).

* IF represents the Yan-mountain and Liaoxi mid-low elevation mountains, IIA represents the Zhe-Min mid-low elevation mountains, IIC represents the low elevation mountains and plain in the region of middle Yangtze River, IID represents the Gui-Xiang-Gan mid-low elevation mountains, IVB represents the E-Qian-Dian middle elevation mountains, IVD represents the mid-high elevation mountains of southwestern Sichuan and mid-Dian, IVE represents the middle-high elevation mountains of southwestern Dian.

We also created distance matrices based on the Euclidian distance of the environmental variables for all unique pair wise combinations of populations (Jetz *et al.* 2009).

Table 1. The geomorphologic regions, climate categories, and sample sizes.

Province	Location marker	Geomorphologic regionalization*	Climate region	N
Anhui	CH			7
Jiangsu	YX		Northern subtropical zone humid monsoon climate	10
Anhui	WH	ⅡA		7
Zhejiang	HZ			17
Jiangxi	JGS	ⅡC		11
Guangxi	GL	ⅡD		7
Guangdong	GZ		Central subtropical zone humid monsoon climate	14
Guizhou	ZY	ⅣB		13
Chongqing	WL			7
Yunnan	KM	ⅣD		6
Yunnan	SM	ⅣE	Southern subtropical zone humid monsoon climate	13

Abbreviations for each location (Fig 1) are assigned to three major topographic types: eastern low elevation mountain and plain areas (I), southeast mid-low elevation mountain areas (II) and southwestern mid-high elevation mountain areas (IV) (Table 1, Zhang 1997).
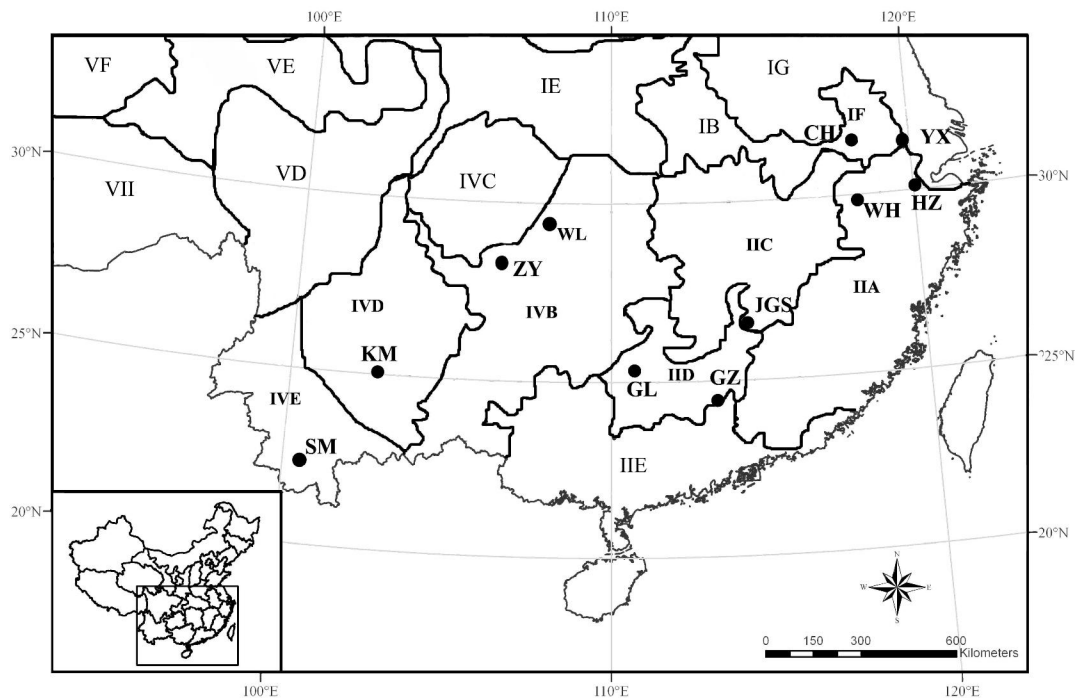
Figure 1. Sampling localities and the geomorphologic regions (see Table 1 for detail) of the Chinese endemic species, *M. davidii*

2.2 Sampling and Morphological data

Protocols for capturing and handling live bats followed guidelines established by the Declaration of Helsinki (52nd WMA General Assembly, Edinburgh, Scotland, October 2000) and Wildlife Conservation of the People's Republic of China (No. 24). Samplings were approved by Wildlife Conservation Association of China.

We combined the populations within 70 km in the same geomorphologic regionalization. Jishou from Hunan, Kaixian from Chongqing and Lijiang fromYunnan were excluded from the analysis due to lack external measurement variables. Finally, 112 individuals and 11 populations were analysis in our study (Fig.1 and Table 1). Measurements (in mm, unless stated otherwise) were: length of head and body (HB), length of forearm (FA), length of metacarpal of 3rd digit (M-III), length of 1st phalanx of 3rd metacarpal (P1-III), length of 2nd phalanx of 3rd metacarpal (P2-III), length of metacarpal of 4th digit (M-IV), length of metacarpal of 5th digit (M-V), length of wing (WL), wingspan (WS), width of wing (WW), length of tibia (TB), length of hind feet (including claws, LHF), length of calcar (CCL),length of ear (E), width of ear (EW), and body mass (M, in g). Cranial measurements included skull length (SL), condylobasal length (CBL), braincase height (BCH), braincase width (BCW), zygomatic width (ZW), interorbital width (IOW), rostral width (M3M3WU), crown breadth of molars (M3-M3U), upper toothrow length (CM3U), distance between auditory bullae (DBA), lower toothrow length (CM3L), mandibula length (ML) and mandibula height (MH).

2.3 Molecular analysis

We obtained nucleotide data from the 112 individuals for the mtDNA HVI region. A repeated sequence of 81 bp within the mtDNA HVI region was excluded from the analysis by using the software TRF404.win. Therefore, 340 bp was used in genetic analysis. Haplotype sequences were deposited in Genbank (Accession nos. GU013475-GU013508, GU013516-GU013527). Total genomic DNA was isolated from those tissues using the QIAGEN DNAeasy Tissue Kit. The mtDNA control region was amplified by polymerase chain reaction (PCR) using the primers P-F (Wilkinson & Chapman 1991). DNA sequences were edited using BioEdit version 7.0.5.3 (Hall 1999) and aligned using Clustal W version 1.81 (Thompson *et al*. 1994).

Multivariate and geographical variation analysis

Kolmogorov-Smirnov test was used to test whether external and cranial measurements were normally distributed. Analysis of Variance (ANOVA) was performed to assess patterns of sexual dimorphism. Principal Component Analysis

(PCA), with morphological and cranial data, was used to further differentiate among *M. davidii* colonies. In order to find out which is the important environmental factors (such as topographical, climatic, latitude, longitude, elevation, precipitation, humidity, temperature, barometric pressure and wind velocity) influencing the morphological and cranial variations, a stepwise linear regression analyses, with stepwise entry and removal variable selection, were used to evaluate the response to the morphological and cranial variation (Altman 1991). We used a probability of F≤0.05 to enter and F≥0.10 to remove a variable. We also used mantel test to assess the statistical significance of the correlation between genetic and among external Euclidean distance, genetic distance (Fst), geographical distance and environmental Euclidean distance.

All analyses above used SPSS version 14.0 (SPSS Inc. 2005), except that the genetic distance (Fst) was calculated by Arlequin 3.0. All the significant level was 0.05.

Phylogenetic analysis

Phylogenetic analyses of unique haplotypes in maximum likelihood (ML) algorithms performed in PAUP 4.0b10. We used standard substitution models calculated by PAUP 4.0b10. Then, a GTR+ G + I model was used. ML analyses used heuristic searches with starting trees obtained by NJ followed by TBR branch-swapping. ML nonparametric bootstrap analyses used 100 heuristic searches with starting trees obtained with NJ based on *p* distances followed by TBR and nearest-neighbor interchange branch-swapping, saving all optimal trees. Stochastic models developed morphologic data inclusion any kind of characters used today to infer phylogeny in such analyses, and the computational efficiency of the Bayesian Markov chain Monte Carlo (MCMC) approach allows each data partition to be treated using more realistic evolutionary models (Nylander *et al*. 2004).

The BI tree was based on combined data to reconstruct the phylogenetic tree. The approach "character congruence", combines all morphological and molecular characters into a single data matrix from which a tree is constructed (Kluge 1989). The effectiveness of different characters is then evaluated according to their congruence with the resulting tree (Larson 1998). The combined analysis is most effective when all characters are independent within and between data sets (Querioz 1993). Analytical methods facilitating the use of molecular and morphological characters as complementary sources of phylogenetic information are explored (Larson 1998). A Mk-GTR+I model was used to construct a Bayesian tree with combined data (Ronquist & Huelsenbeck 2003). Bayesian methods for the

analyses were combined with mtDNA HVI sequences and 29 external variables. Each variable was divided into ten levels according to the variation range. Then, each variable of 112 individual was marked with level of 0 to 9 based on the measurement, and a 0 to 9 matrix was obtained for further Bayesian analysis. Parameter estimated was for each sequence and external variable simultaneously. A Markov chain Monte Carlo (MCMC) was employed for 1 000 000 generations and sampled every 100 generations with combined data (Nylander *et al*. 2004). Four chains were run, and 5000 initial trees were discarded. Bayesian posterior probabilities were estimated based on the 50% majority-rule consensus of the trees.

We calculated Euclidean distances on component morphospace as overall indices of morphological similarity. Minimum spanning trees were calculated on Euclidean distances. Euclidean distances tree was computed with SPSS version 14.0.

We used mtDNA HVI sequences (GenBank Accession no. U95332) and external variables of *M. myotis* as outgroup to root the trees.

3. Results
3.1 Morphological analysis

Kolmogorov-Smirnov tests showed no significant differences in variances for all variables, which indicated all parameters, were in normal distribution. ANOVA analysis indicated that there were no obvious differences between males and females of the 29 external parameters (P>0.05). Therefore, data for both sexes were combined in analyses.

PCA variables indicated that all the variables had high loadings on the PC1, except for P2-III, HW and DBA (Table 2). The positive and relatively uniform metrics variables associated with the first component axis of PCA represented variation in general size and explained the greatest part (52.43%) of variation across colonies. Loadings on components 2 and 3 explained 28.15% and 9.72% of the total variance, respectively, represented variations in shape and explained the variations in magnitude and sign.

3.2 Multivariate and impact factors analysis

When used the stepwise linear regression analyses to analyze the relationship between environmental variables (such as the geomorphologic and climate categories, latitude, longitude, elevation, precipitation, humidity, temperature, barometric pressure and wind velocity) and the external factor score variables, the results showed that PC1 was significant associated with annual temperature (B = -0.029, β = -0.776, t = -3.476, P = 0.008*), PC2 was significant associated with latitude (B =-0.430, β = -

0.886, t = -2.230, P = 0.001**) and longitude (B = -0.583, β = -0.592, t = -2.660, P = 0.002*), PC3 was no associated with environmental factors. Body size appeared increasing from northeast to southwest, and the shape variations in magnitude and sign were fluctuant along with the latitude and longitude.

　　　Mantel test showed that the Euclidean distance of PC1 and PC2 scores distance for the metric variables were significant associated with geographic distance (PC1: R^2=0.524, P=0.001**; PC2: R^2=0.610, P=0.001**), the environmental Euclidean distance (PC1: R^2=0.440, P=0.001**. PC2: R^2=0.522, P=0.001**) and genetic distance (PC1: R^2=0.665, P=0.001**. PC2: R^2=0.771, P=0.001**).

Table 2. Loading variables of principal component analyses of metric variables of Chinese endemic species, *M. davidii*. Metric variables explained by the percentage (% VAR).

Variable	Principal Component			Variable	Principal Component		
	PC1	PC2	PC 3		PC 1	PC 2	PC 3
HB	0.542	-0.558	-0.178	SL	0.952	-0.026	-0.125
FA	0.831	0.015	-0.188	CBL	0.944	-0.019	-0.133
M-Ⅲ	0.555	0.663	-0.075	BCH	0.448	0.145	-0.064
P1-Ⅲ	0.633	-0.448	-0.107	M	0.573	-0.447	-0.055
P2-Ⅲ	0.197	0.522	-0.134	BCW	0.838	0.022	-0.059
M-Ⅳ	0.560	0.607	-0.326	ZW	0.847	0.089	-0.009
M-Ⅴ	0.562	0.688	-0.177	IOW	0.721	0.177	-0.366
WL	0.566	-0.057	0.473	M3M3WU	0.635	-0.085	-0.107
WS	0.894	-0.059	-0.046	M3-M3U	0.700	-0.238	-0.029
WW	0.604	0.485	0.314	CM3U	0.807	-0.209	0.143
TB	0.710	-0.416	-0.112	DBA	0.193	0.774	-0.175
LHF	0.501	-0.392	0.342	CM3L	0.885	-0.093	-0.015
CCL	0.601	0.125	-0.133	ML	0.937	-0.115	-0.107
E	0.466	0.066	0.489	MH	0.821	0.227	-0.134
HW	0.068	0.221	0.762	% of Var	52.429	28.147	9.719

3.3 Phylogenetic analysis

　　　When reconstituted the phylogeny trees by the genetic data, the combined data and the external data, separately. Three lineages of phylogenetic trees based on the combined data and the external data were striking concordance with the single genetic phylogenetic trees (Fig. 2). And all the phylogenetic trees supported the recognition of a monophyletic *M. davidii* group (Fig. 2). A minimum spanning tree with external data revealed strong phylogeographic structuring into three longitudinal groups (Fig. 2C), which is lineage A, lineage B and lineage C.

　　　A monophyletic clade was strongly supported by BI tree (Fig. 2B). In the morphological and sequences' Bayesian analysis, the average standard deviation of split frequencies was less than 0.01 after 1 000 000 generations, indicating that they were converging to a similar stationary distribution. We set this point as the burn-in sample size to calculate the posterior distribution of the topologies. The consensus tree was congruent with the highest likelihood score tree. The BI tree formed three lineages (lineage A, lineage B and lineage C) (Fig. 2B). Individuals from lineage A presented the regions IF, IIA and IIC. Lineage B was composed of the IVB, IVD and IVE regions. Individuals from lineage C presented the region IID (Fig. 2B).

　　　We calculated Euclidean distances on component morphospace as overall indices of morphological similarity. The minimum spanning tree of the eternal variables formed three lineages (Fig. 2C). The result was consistent with the ML and BI trees.
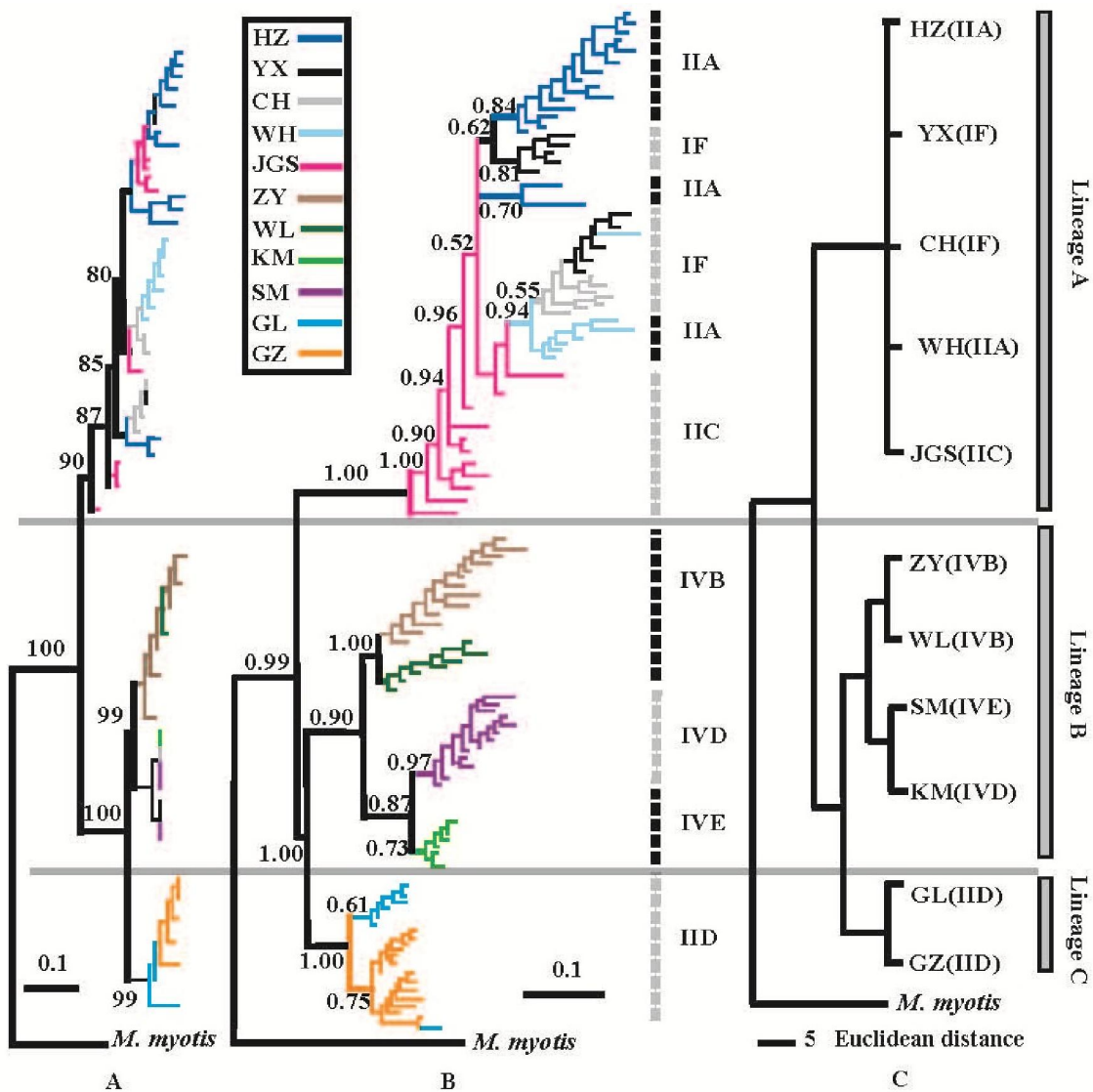
Figure 2. Phylogenetic tree of Chinese endemic species,*M. davidii.* (A) Maximum-likelihood phylogenetic tree with DNA data. (B) Bayesian inference (BI) tree with DNA and eternal data. (C) Minimum spanning tree with external data. The bootstrap values above 50% are shown. Different populations were in different line color (the location markers see Table 1 and Fig. 1).

4. Discussions
4.1 Phylogenies reconstitution

The divergence of morphology and crane in the rate and direction of ontogenetic shape changed were corresponding to phylogeny relationships amongst *M. davidii* populations. Geographic distances, strong selection, and local adaptation were more likely to evolve because each lineage represents a gene pool (You *et al*. 2010, Slatkin 1987). Like horse shoe bat, *Rhinolophus affinis* (Hisheh *et al*. 2000), the most notable outcome of the present study

was that the external morphologic differences of *M.davidii* carried a significant phylogenetic signal.

The mtDNA HIV of *M.davidii* showed the haplotypes exclusively within region I-II, IV and IID to the western and eastern China (You *et al*. 2010). Our analysis also indicated the *M. davidii* populations were in a significant phenotype differences among lineages (Fig. 2C). Some association was observed between mtDNA phylogenetic groups and the different ecological morphs exhibited in the populations. In this study, all the phylogenies analysis indicated the same result,

which presented in Fig. 2 imply three geographic populations (lineage A, lineage B and lineage C) consistent to the Chinese geomorphologic regions. Individuals in lineage A had a broad genetic homology. However, when the phylogenies analysis used the combined data, the individuals from JGS formed a separately lineage which was consistent to the geomorphologic region IIC (Fig. 2B, Table 1). The individuals from HZ, YX, WH were classified into geomorphologic region IF and IIA. This phenomenon might be due to region IF and IIA were all in the northern subtropical zone humid monsoon climate region. And the external and genetic variables were associated with environmental or even the microhabitat climate affected in different topographies during a long evolutionary time. In lineage B, the individuals from ZY, WL, SM and KM were all in geomorphologic region IV. Individuals from GL and GZ in region IID were divergent from geomorphologic region IIA and IIC and formed a sister groups with colonies from geomorphologic region IV. Therefore, individual phenotype from region IID was much more similar with region IV, which might due to the similar geomorphology and environmental characteristic. In the evolutionary perspective, the region GL, GZ and JGS populations were between west and east populations, and formed the gradient groups associated with the environmental variations. In addition, the genetically differentiated from three lineages were clearly indicated that ecological morphs represent natural assemblages resulting from monophyletic divergence. Genetically and ecological morphological variation of *M. davidii* were corresponded to geomorphologic and physigeographic feature variations, but does not completely coincide with climate categories. Though in the same climate category, different topography with different elevation, temperature, plant species and coverage (Ashcroft 2006, Joly & Brossard 2007, Lu *et al.* 2006, Huber 2005, Caumul & Polly 2005) contribute to variations in microhabitat, and constrained *M. davidii* external variation within the context of topography of China. And a combination of aerodynamic and sensory constraints might also limit size in aerial insectivores (Kunz & Fenton 2005). The dependence of external variations and the genetic differentiation on a gradient of environmental variables is in agreement with the theoretical model (Doebeli & Dieckmann 2003), showing that processes of evolutionary diversification may lead to sharp geographical differentiation along environmental gradients. The environmental gradients existed for an extended period of time which reflected in the genetic or even the morphology.

4.2 Impact factors for the external variations

In China, studies on the geographic distribution pattern of vertebrates, such as bats (Xu *et al.* 2010), birds (Song *et al.* 2009) and amphibians (Zhang *et al.* 2008), have described evolutionary histories following the environmental or climatic oscillations in the context of complex regional scenarios (Flanders *et al.* 2009). However, macrogeographic distribution was likely to encounter greater environmental heterogeneity. And numerous pieces of evidence support the view that drastic shifts in environmental character during evolution caused changes in morphology (Hisheh *et al.* 2000, Ivanovi *et al.* 2008, Ivanovi *et al.* 2009).

In this research, when a genetic and non-genetic factors considered together, the external variable of *M. davidii* were all tend to associated with the geographic variable, environment variables or even the genetic variable, based on the result of mantel test analysis. The former phylogeographic study based on the mtDNA indicated a less gene flow among *M. davidii* populations (You *et al.* 2010). Geographical distance via its effect on gene flow was important in their plastic in size and shape, which were also responded to environmental variations.

Among the non-genetic factors, temperature was an important factor in phenotype plasticity, which heavily influencing not only the size, but also the shape (Ivanovi *et al.* 2009). Therefore, we inferred the body size and shape of *M. davidii* were influenced by the outer or inner factors. In this research, according to the results of metric factor analysis, PCA1 represented variation in size and PCA2 represented the variation in shape, which were all associated with the environmental variables. And the results of the stepwise linear regression analyses further showed that the inferred above was right. Thus, we got the conclusion that the body size variation was associated with the temperature among the ten environmental factors. However, the shape variation of *M. davidii* populations appeared common associated with latitude and longitude under unique topographic and eco-environmental characters of China. Therefore, in spanned a wide range of environments variation, an organism trait was tightly associated with local condition which was expected as a consequence of local adaptation or phenotypic plasticity (Mayr 1956, Mayr 1963, Meiri *et al.* 2007). A positive relationship between variation in organism traits and geographical range size would arise due to the greater environmental variation encountered over a larger geographical extent (Mayr 1956, Mayr 1963). Therefore, the effect of environmental variation and genetic control might be induced phenotype deviation and the extent to which the environment is likely to induce character

changes directly depends upon the phenotype plasticity of *M. davidii*. Different features can be affected by the environment to varying extents. And the size is more likely to be affected than the shape in *M. davidii* based on the result of the contribution scores of the factor analysis. Morphological difference was closely associated to the genetic and ecological characters where they located. This phenomenon also appeared in many animal species (Arntzen *et al*. 2007, Furtula *et al*. 2008).

Our research highlighted the importance of adaptive divergence for geographical patterns. And the external variables along the genetic divergence, environmental and geographic gradient, evolutionary branching can occur much more easily, which increased availability of phylogeographical and ecological data should facilitate further understanding of population divergence drivers at broad scales.

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