Molecular genetics and quantitative traits divergence among populations of *Eothenomys miletus* from Hengduan Mountain region

Running title: Genomics and phenotypic adaptation of voles

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ABSTRACT:

An important objective of evolutionary biology has always been to grasp the evolutionary and genetic processes that contribute to speciation. The present work provides the first detailed account of the genetic and physiological adaptation to changing environmental temperatures as well as the reasons causing intraspecific divergence in the Eothenomys miletus from the Hengduan mountain (HM) region, one of the biodiversity hotspots. 161 *E. miletus* individuals from five populations in the HM region had their genomes simplified sequenced, and one additional individual from each community had their genomes resequenced. We then characterized the genetic diversity and population structure of each population and compared the phenotypic divergence in traits using neutral molecular markers. We detected significant phenotypic and genetic alterations in *E. miletu*s from the HM region that were related to naturally occurring diverse habitats by combining morphometrics and genomic techniques. The *E. miletus* existed asymmetric gene flow patterns, indicating that five *E. miletus* populations exhibit a isolation-by-island model, and this was supported by the correlation between F_{ST} and geographic distance. Finally, P_{ST} estimated by phenotypic measures of most wild traits were higher than differentiation at neutral molecular markers, indicating directional natural selection favouring different phenotypes in different populations must have been involved to achieve this much differentiation. Our findings give information on the demographic history of *E. miletus*, new

insights into their evolution and adaptability, and literature for studies of a similar nature on other wild small mammals from the HM region.

KEYWORDS: *Eothenomys miletus*, F_{ST} , genetic diversity, population genomic, P_{ST}

INTRODUCTION

Early flora and fauna cradleland and refuges are hotspots for biodiversity. Some biodiversity hotspots serve as "evolutionary forewords" that spur fast divergence of tropical plant groupings and the junction of long-distance species distribution, having a significant impact on the establishment and evolution of the world's flora and fauna.

Due to the Hengduan Mountains (HM) region's high northwest and low southeast latitudes, as well as its significant height fluctuations, and its climate, which is characterized by a modest yearly temperature difference and a huge daily temperature difference, a wider range of animal species can survive there (Ren et al., 2020a). As a result, the HM region is listed as one of the 25 worldwide biodiversity hotspots (Li et al., 2014; Qu et al., 2014). Located in the Tibetan Plateau, the HM region is a section of the Qinghai-Tibetan Plateau (QTP). It covers 364,000 square kilometers and is made up of the western Yunnan, northwestern Yunnan, western Sichuan, southeast Tibet, southeast Qinghai, and southwest (Qu et al., 2014). The dramatic topography of the HM region, caused by tectonic uplift during the late Pliocene, resulted in dramatic ecological stratification. As a result, the current HM region is made up of a series of parallel mountains, with elevations ranging from 1000 meters on valley floors to over 5000 meters on mountain peaks (Hwang, 2003). These resemble "sky islands," with deep valleys and "ocesans" of alternate vegetation surrounding them (He and

Jiang, 2014). Populations have consequently gotten separated from one another and evolved independently. These "sky islands" mimic an archipelago of islands and mountain ranges by isolating creatures into separate subregions and mountain chains (Zhang, 2012; Li et al., 2014).

The refugia theory is one well-known explanation for the high biodiversity (Zhang, 2002; He et al., 2016). Throughout Quaternary ice-age cycles, the HM region in mainland China is regarded as one of the most notable glacial refugia (Qiu et al., 2011; Li et al., 2021). In times of unfavorable climatic circumstances, the complex and diverse ecosystem in the HM region allows for species to move up and down the mountains in search of suitable habitats, reducing the risk of extinction. According to intraspecific phylogeographic studies among species, large valleys functioned as physical barriers for smaller terrestrial animals, and the HM region featured a number of refugia where populations were able to escape the Pleistocene glaciations (He and Jiang, 2014). Another model hypothesizes that the intricate "sky island" split the ecosystems in the highlands, isolating populations of certain species, which led to allopatric speciation (He and Jiang, 2014; He et al., 2016). However, the reasons for this particular diversity are not well understood.

Animals display a variety of phenotypic alterations as a result of selection forces acting on heritable features as a result of geographical and temporal heterogeneity (Leinonen et al., 2008). Animals may go through these phenotypic changes to better fit their environment at the physiological, behavioral, and especially morphological levels. Although phenotypic plasticity has been extensively studied and its significance in adaptation and evolution has been well-discussed, the basic driving mechanisms are still unknown (Kelly et al., 2012;

89 Sommer, 2020).

90 Comparative analyses of quantitative genetic and neutral marker differentiation have
91 given researchers a way to assess the relative contributions of stochastic genetic drift and
92 natural selection to the explanation of among-population divergence (Leinonen et al., 2008).
93 In several species, the comparison of quantitative trait across populations (Q_{ST}) and
94 differentiation at neutral molecular markers (F_{ST}), commonly referred to as the Q_{ST}-F_{ST}
95 comparison, revealed that natural selection played a significant role in the cause of
96 differentiation in quantitative traits. In several cases, putative F_{ST} and Q_{ST} differentiation in
97 various populations is compared in order to evaluate their evolutionary signatures and
98 discover potential features implicated in local adaptation.

99 However, raising animals from various populations in a common environment is
100 typically required for estimating the additive genetic variances needed for Q_{ST} (Leinonen et
101 al., 2008; Brommer et al., 2011). As a result, for some wild species, particularly endangered
102 species, the breeding test for estimating the Q_{ST} becomes impractical. Currently, most species
103 substitute quantitative trait analysis (Q_{ST}) with phenotypic divergence in a trait (P_{ST}), and P_{ST}
104 counts are based on phenotypic assessments of a wild trait of several individuals across
105 numerous populations (Brommer et al., 2011). P_{ST}-F_{ST} comparisons, on the other hand, rely
106 on the unrealistic presumption that nonadditive genetic effects and environmental effects may
107 be reduced and that phenotypic variation equals additive genetic variance (Wójcik et al.,
108 2006).

109 *Eothenomys* of subfamily Arvicolinae, which belong to the family Cricetidae in Rodentia,
110 is wildly distributed throughout the Holarctic realm and parts of the Oriental realm (Luo et al.,

2004). Long-standing controversy surrounds the precise phylogenetic position of *Eothenomys*. Recently, according to research on the species of *Eothenomys* utilizing molecular and morphological evidence revealed that *Eothenomys* has three subgenera, which includes *Eothenomys*, *Anteliomys*, and *Ermites*. *Eothenomys* consists of *Eothenomys colurnus*, *Eothenomys melanogaster*, *Eothenomys eleusis*, *Eothenomys miletus*, *Eothenomys cachinus*, *Eothenomys fidelis,* and *Eothenomys shimianensis. Anteliomys* consists of *Eothenomys chinensis*, *Eothenomys custos*, *Eothenomys olitor*, *Eothenomys proditor*, and *Eothenomys wardi. Ermites* is the newly distinguished subgenus, which includes five species, *Eothenomys hintoni*, *Eothenomys tarquinius*, *Eothenomys jinyangensis*, *Eothenomys meiguensis*, and *Eothenomys luojishanensis*, respectively (Liu et al., 2012; Zeng et al., 2013).

E. miletus is a naturally occurring species in Hengduan mountain region (Zhu et al., 2010； Ren et al., 2020b), and is listed in International Union for Conservation of Nature (IUCN). *E. miletus* is one of the representative species for studying the evolution of biodiversity in HM region (Zhu et al., 2008). Despite the numerous of population studies that looked at their distribution, phenotypic morphology (Zhu et al., 2008, 2010, 2011, 2014, 2017; Ren et al., 2020b), and microsatellites (Zhu et al., 2013), our understanding of evolution and adaptation within *E. miletus* populations is limited due to the lack of genomic studies. The primary objective of this paper, we use simple genome sequencing and resequencing to explore the genetic variations and genetic structure among five *E. miletus* populations from HM region, as well as compare the quantitation of the P_{ST} based on the collected the morphological data with F_{ST} estimated using sequencing to assess the relative roles of natural selection. Finally, we provide literature for the similar studies on other wild small mammals

from HM region.

MATERIALS AND METHODS

Subjects and experimental design

From November 2018 to January 2019, the voles (*Eothenomys miletus*) used in this study were caught in five sites with gradually varying altitudes: Deqin (DQ, n=33); Xianggelila (XGLL, n=33); Lijiang (LJ, n=34); Jianchuan (JC, n=33); and Ailaoshan (ALS, n=33). Figure 1A and Table 1 contain comprehensive sampling data. The study's latitude, elevation, and annual mean temperatures came from regional weather services. The livers of animals that were caught in the wild were immediately dissected and frozen in liquid nitrogen after they were weighted and anesthetized. Samples were transported to the Yunnan Normal University lab in dry ice and maintained there in a refrigerator at a temperature of 80℃ until they were analyzed. Using the phenol/chloroform method, the total genomic DNA of the animals was extracted from tissue samples. With the Covaris system, 1-3 g of DNA from each person were cut up into fragments of 200-500 bp (Gene Company, Ltd., Hong Kong, China). The Institutional Animal Care and Use Committee granted its approval to all experimental methods.

Morphometrics

We created small mammal skull specimens using the Tenebrio molitor larval method. The analysis of the fractured skull specimens was not carried out. At Yunnan Normal University's animal specimens room, 112 complete skull specimens were kept (Kunming, China). Vernier calipers were used to measure external and cranial morphometrics to the nearest 0.01 cm. For each specimen, twenty-one cranial and external characteristics were

mentioned. Nine external measurements, including body length (BL), tail length (T1L), torso length (T2L), chest width (CW), chest depth (CD), ear length (EL), ear width (EW), fore limb length (FLL), and hind limb length (HLL), were taken from specimen tags referring to Gao (2017). Twelve cranial measurements were made after Yang (2005) and Xia's measurements (2006). The measurements of the cranium included cranial length (CL), cranial basal length (CBL), cranial height (CH), pillow nose length (PNL), zygomatic breadth (ZB), neurocranium width (NW), covering cap length (CCL), interorbital breadth (IB), eye socket length (ESL), auditory vesicle length (AVL), upper tooth row length (UTRL), and lower tooth row length (LTRL). In order to maximize the sample size, combining males and females for morphological analysis works well because their sexual dimorphism does not differ from groups (Zhang et al, 2019).

Sample sequencing, read mapping and quality control.

161 voles were utilized in this investigation to produce 464-494 mid-depth specific site-specific amplification fragments (SLAF) of 464-494 insertion lengths using the Rsal enzyme from Beijing Baimai Technology Co., Ltd. (Sun et al., 2013). In SLAF labeling, the target fragment is identified for processing after PCR amplification, purification, mixing, and enzyme digestion (Kozich et al., 2013). A is added at the 3' end to connect the connectors of the double-labeled sequences. Using the Illumina HiSeq 2500 platform, we processed and sequenced the fragments that we had identified. The raw readings were initially filtered using the following criteria: reads that had more than 10% of unidentified nucleotides (N) and more than 50% of low-quality bases (phred quality 5) were both excluded. Then, using the "MEM" approach of Burrows-Wheeler Aligner (BWA 0.7.12-r1039) (Li and Durbin, 2009), and the

clean reads were mapped to the reference genome of Prairie voles (*Microtus ochrogaster*) (https://www.ncbi.nlm.nih.gov/genome/10848) (Fan et al., 2019).

A 42-degree depth of coverage was repeated with one vole at each point. The BGI platform was then used to process and sequence DNA fragments. Following that, raw reads were first filtered employing Beijing Genomics Institution Co. LTD's SOAPnuke 1.5.6 software. Clean reads were then mapped to the Microtus ochrogaster reference genome using the "MEM" algorithm of BWA 0.7.12-r1039 software with the option "-t 8 -k 19 -M -R" (Fan et al., 2019). The SortSam.jar methodology of Picard 1.117 and the RealignerTargetCreator and IndelRealigner tools of GATK 3.3.0 were used, respectively, to sort and correct the final BAM files used in the subsequent analysis (McKenna et al., 2010).

SNP Calling and Filtering

In order to estimate the sequencing quality value Q, the reads considered to be of low quality were those with joint and 50% bases with a Q10 value. Q = -10*log10e. From the straightforward genome sequencing of 161 voles, we obtained the SNPs using the innovative technology SLAF-seq (Beijing Baimai Biotechnology Co. LTD). Using the call function in Bcftools 1.10, we called variants after using SAMtools 1.2 to gather summary data from BAM files and calculate the likelihood of potential genotypes (Li et al. 2009). Segments of the reference genome were separated and examined simultaneously. Segments of the reference genome were separated and subjected to parallel analysis. The raw SNPs were then filtered using a customized script using the following criteria to obtain high-quality variants: Completeness > 0.5 and minimal allele frequency > 0.05 are the criteria.

Clean paired-end reads from individuals were aligned to the resequenced assembled vole

reference genome using BWA 0.7.12-r1039. Then, SNPs were identified using GATK 3.3.0, and the clean SNPs were aligned using GATA 3.3.0 hard filter with the following filter parameters: QD 2.0, FS > 60.0, MQ 40.0, ReadPosRankSum -8.0, and MQRankSum -12.5. Only SNPs with high second-order credibility were retained for further analysis after the SNPs were filtered by minimum allele frequency (MAF) = 0.06 and maximum missing rate = 25%.

Population structure

Population structure analysis was done using the ADMIXTURE 1.3.0 (Alexander et al., 2009), which calculates individual ancestry and admixture ratios based on K ancestral populations. We examined the number of genetic clusters (K) ranging from one to 10 while running ADMIXTURE five times to gauge convergence (Alexande et al., 2009). Additionally, we performed a cross-validation test with frappe to determine the best match K value (Tang et al., 2005). Using EIGENSOFT 3.0 software, principal component analysis (PCA) was carried out (Patterson et al., 2006). The neighbor-joining (NJ) approach in MAGA 7.0.26 was employed to reconstruct phylogenetic trees of 161 individuals (Koichiro et al., 2011; Ren et al., 2022).

Genetic Diversity

The expected heterozygosity (He) and observed heterozygosity (Ho) were calculated using PLINK 1.9 (Purcell et al., 2007) to test the genetic diversity indices of five populations based on high-consistency SNPs, and the, observed allele number, expected allele number, Nei diversity index, and polymorphy information content (PIC) were calculated using a customized Perl script. We used SPSS 26.0) to calculate Pearson's correlation coefficient (r^2)

between each pair of variables in order to further quantify the impact of environmental variables, such as altitude, temperature, and latitude, on genetic diversity at five geographic populations (Qu et al., 2014).

Demographic history reconstruction and gene flow

The maximum likelihood method and a Bayesian statistical model were employed in Perl to estimate pairwise relative migration rates and direction based on the retroactive theory (Beijing Baimai Biotechnology Co. LTD) (Sundqvist et al., 2016; Schiffels and Durbin, 2014). The Bayesian statistical model of MIGRATE-N software was used to estimate pairwise relative migration rates and directionality between populations based on the ancestor tracing theory. Additionally, five populations' gene flow was examined using the TREEMIX software. The miss rate is 0.8 at its highest. R becomes 0.6 after the chain-unbalanced SNP is instantly removed. Additionally, the pairwise sequentially Markovian coalescent (PSMC) model, which has been extensively used in other mammals, was used to estimate changes in effective population size based on heterozygous sites across the genome. In this study, the generation time and the mutation rate were separately set at 0.5 years and 2.96×10^{-9} (Teng et al., 2017). The remaining high-credibility SNPs from genome resequencing were used for PSMC analysis after SNPs with a minimum allele frequency of 0.06 and a maximum missing rate of 25% were filtered.

Neutral genetic differentiation and phenotypic differentiation

SNPRelate package in R 3.6.3 was used to calculate pairwise F_{ST} (Zheng et al., 2012), and Prism 9 was used to build a heat map of the pairwise F_{ST} value. Based on Pearson's product-moment correlation, the Mantel test of matrix correspondence (Mantel, 1967) was

243 applied to test correlations between geographic distance, environment distance, altitude
244 distance, temperature distance, precipitation distance, pairwise F_{ST}, and $F_{ST}/(1-F_{ST})$ in order
245 to test the effects of geographic distance and environmental differences on genetic
246 differentiation. This was done using the Ecodist package in R 3.6.3 (Rousset, 1997). On
247 topographic maps of the study area, point-to-point geographic distances were calculated
248 (Browne & Ferree, 2007). Moreover, we gathered environmental data from WorldClim 2.0
249 for sampling locations using 19 common bioclimatic variables (Fick & Hijmans 2017).
250 ArcMap 10.2 was used to convert the data. The 19 standard bioclimatic variables that
251 correlate to temperature were utilized as temperature data, while the 19 typical bioclimatic
252 variables that relate to precipitation were used as precipitation data.

253 To calculate the distance in environment, temperature, and precipitation, we employed
254 the Pearson distance measurement method. General linear regression analysis in R 3.6.3 was
255 used to investigate the relationship between geographic distance and environmental distance.
256 The pairwise F_{ST} or $F_{ST}/(1-F_{ST})$ was employed as the response, the geographic distance as the
257 predictor, and the environmental distance as the condition factor to assess isolation by
258 distance (IBD). The geographic distance was utilized as the condition element to investigate
259 isolation by environment (IBE), isolation by temperature (IBT), and isolation by precipitation
260 (IBP). Moreover, the distance in altitude between paired sampling sites was calculated.
261 Finally, we utilized Canoco 5 to perform redundancy analysis (RDA).

262 Using the SPSS 26.0 program, the body mass and twenty-one exterior and cranial
263 character data were evaluated. One-way analysis of variance (ANOVA) and LSD post-hoc
264 tests were used to assess group differences in attributes; $P < 0.05$ was deemed statistically

265 significant, while $P < 0.01$ was deemed extremely significant. Prism 9 was used to perform
266 Highcharts and Boxplot. Using the online Heatmapper, a cluster analysis plot and correlation
267 matrix map between physical characteristics and environmental factors were created.

268 Divergence at phenotypic traits will be greater than that seen for neutral loci under
269 divergent selection. Common garden and reciprocal transplant studies are not viable for the
270 species since the voles employed in this study are wild populations. P_{ST} measures the
271 percentage of among-population phenotypic variance in quantitative characteristics and is
272 equivalent to Q_{ST} (Spitze, 1993), which quantifies the proportion of among-population
273 genetic variance in quantitative traits:

274 $$P_{ST} = \frac{c\sigma^2{}_B}{c\sigma^2{}_B + 2h^2\sigma^2{}_W}$$ (Raeymaekers et al., 2007)

275 where σ^2_B is the variance between populations, σ^2_W is the variance within populations, and h^2
276 the heritability. The scalar c expresses the proportion of the total variance that is presumed to
277 be because of additive genetic effects across populations, assuming that environmental
278 variance among samples is randomly distributed or absent and that heritability (h^2) within
279 samples is 0.5. The consequences of departure from these assumptions are considered below
280 in the Discussion sections. Phenotypic variance components were estimated following Sokal
281 & Rohlf 1995. The pairwise P_{ST} values for individual attributes were compared with the
282 pairwise F_{ST} (P_{ST}/F_{ST} value) to assess the degree of phenotypic divergence with neutral
283 genetic divergence. The two-way clustering heat map of the P_{ST}/F_{ST} value between paired
284 populations was built using the online Heatmapper. We tested correlations between
285 geographic distances, population pairwise altitudinal differences, pairwise F_{ST}, and pairwise
286 P_{ST} using the Mantel test of matrix correspondence (Mantel, 1967) as implemented in the

Ecodist package in R 3.6.3. To determine if neutral genetic differentiation accounts for the divergence in quantitative characteristics, a correlation test between pairwise F_{ST} and pairwise P_{ST} was first carried out for each trait. In order to find out whether divergence in quantitative traits was connected to geographic distance and altitudinal differences, a correlation test between pairwise altitudinal differences, geographic distance, and pairwise P_{ST} was run for each variable. Geographic distances between two points were calculated using topographic maps of the study area.

RESULT

SNP Calling

Five *E. miletus* populations from the Hengduan mountain regions were sampled by us, totaling 161 individuals (Figure 1A, and Table 1). 363.16 million pair-end reads with an average of 92.23% Q30 and 42.09% GC were produced after quality control (Supplementary table 1). 161 individuals had a total of 847,420 SLAF labels, including 470,440 polymorphism labels, which were gathered (Supplementary table 2). After quality control, we successfully identified 2,221,486 SNPs from 161 voles (Supplementary table 3). Additionally, we obtained 0.645 Tb of clean data with average Q20 and Q30 values of 97.72% and 92.85%, respectively, by sequencing at a depth coverage of 38.36, and 108,005,364 SNPs were gathered by comparing with the first 40 chromosomes of the reference genome (Supplementary figure 1 and table 5).

Population Structure

Five populations of voles could be distinguished using mixture analyses based on the

same SNPs and assuming various numbers of ancestry components (K) (Figure 1B). Population structure was evident, with K = 4 providing the strongest statistical evidence. First, at K = 1, the five populations of mice united to form one ancestor. The ALS group displayed unique ancestries from other populations when K = 2. Additionally, with K = 3, the JC population was further distinguished from the other populations. This is consistent with the PCA results, which distinguished the JC population from the ALS population using the first and second main components (PC1 and PC2). Moreover, with K = 4, a portion of the XGLL individuals and the JC population formed one ancestra, and the remainder XGLL individuals and the DQ population formed one ancestra, in accordance with PCA, which further divided the LJ population and the DQ population (Figure 1C and Supplementary Figure 2). The five groups spread over these locations showed varying degrees of mixed ancestry as K climbed from 5 to 10. The line chart in Figure 1B displays cross-validation errors for various K values, with K = 4 having the lowest cross-validation error rate.

Following that, we used phylogenetic reconstruction to categorize the individuals (Figure 1D). The clustering of populations, which showed four clusters, revealed that the ALS and JC populations each formed one cluster, while a portion of the XGLL population with DQ individuals formed one cluster and the remaining XGLL population with LJ individuals formed another. This is in line with what our structure analysis and PCA revealed.

Genetic Diversity

Table 2 contains a summary of the various population genetic diversity indicators, such as the nucleotide polymorphism ($\theta\pi$), Tajima D, observed allele number, expected allele number, observed heterozygous (Ho), expected heterozygous (He), Nei diversity index,

Shanon wiener index, as well as polymorphysm information content (PIC). The genetic diversity of the five *E. miletus* populations from the Hengduan mountain regions was highest in the ALS population, followed by JC population, and least in the XGLL population.

The impact of environmental factors on genetic diversity was then further investigated using general linear analysis and multiple linear regression analysis, as shown in Table 1. Some intriguing links have been found. With the exception of Tajima, D, and observed heterozygotes, there was no link between altitude and genetic diversity indices ($P > 0.05$), however there were substantial relationships between ambient temperature, latitude, and indexes ($P < 0.05$).

Demographic history and gene flow

To estimate the pairwise relative migration rates and direction between pairwise populations, we employed the Migrate-N. (Figure 2A). Although average migration rates across all groups were more than one migrant per generation, there were asymmetric gene flow (*Nm*) patterns. According to the F_{ST} technique, there were 0 to 62.52 migrants on average per generation between all populations. There were asymmetric patterns of gene flow between the DQ and XGLL populations and the XGLL and LJ populations, with the Nm between the DQ and XGLL populations having the highest mean of 62.92. The next Nm was from the XGLL population to the LJ population. Furthermore, there were no Nm between the JC and ALS populations as well as the LJ and JC populations. Additionally, the maximum likelihood tree of *Nm* between five populations was constructed using Treemix (Supplementary Figure 3); the outcome closely matched the finding from our Migrate-N result.

353 Changes of the effective population size (Ne) over time were evaluated with the PSMC
354 model for each five populations (Figure 3B), and showed a similar pattern. There were
355 variety phases of Ne, and the variations in Ne aligned well with the changes in historical
356 world temperature. First, Ne began to increase during Quaternary glaciation (2000~3000Kya,
357 Ehlers and Gibbard, 2008) until Marine Isotope Stage 12 (500Ka ± 5Ka BP. (Howard, 1997).
358 Second, there were two population bottleneck effect which happened about 500 Ka and 30Ka
359 years ago, the two period of low temperature in history (Howard, 1997).
360 Third, the second increasing time of the Ne during Marine Isotope Stage 5 (MIS5,
361 130Ka-80Ka BP. Lisiecki and Raymo, 2005), the last major interglacial stage in history, and
362 reach a higher level during Marine Isotope Stage 3 (MIS3, 60Ka-25Ka BP. Siddall et al.,
363 2008), a special period in the last glacial period which has the extremely unstable climatic
364 conditions and many climatic abrupt events, while the Ne begin to decrease during the colder
365 substage MIS3c (39.3Ka-26.5Ka BP. Wulf et al. 2018) until the end of the last glacial period
366 (11.5Ka BP. Blunier, 2001). After the periods of fluctuation, the Ne decreased.
367 ***Neural genetic differentiation and phenotypic differentiation***
368 The pairwise fixation (F_{ST}) ranged from 0.019 to 0.188 (average, 0.124) in this study.
369 Moreover, the heat map of the pairwise F_{ST} showed that JC population and ALS population
370 have high genetic differentiation with the other three populations, and there were medium
371 score genetic differentiation between the remainder populations (Figure 3A). In addition,
372 there was the largest values generally pairwise F_{ST} between ALS population and JC
373 population as well as the lowest pairwise F_{ST} between DQ population and XGLL population.
374 Mantel tests for groups revealed a strong relationship between pairwise F_{ST} and $F_{ST}/(1-F_{ST})$

375 as well as temperature distance (IBT) (mantel r_{FST}= 0.741; P_{FST} < 0.05; mantel $r_{FST/(1-FST)}$=
376 0.766; $P_{FST/(1-FST)}$ < 0.01, Figure 3D, E), while the other distances, including geographic
377 distances (IBD) (mantel r_{FST}= 0.618; P_{FST} > 0.05; mantel $r_{FST/(1-FST)}$= 0.627; $P_{FST/(1-FST)}$ > 0.05,
378 Figure 3B, C), altitude distance (IBA) (mantel r_{FST}= 0.182; P_{FST}> 0.05; mantel $r_{FST/(1-FST)}$=
379 0.166; $P_{FST/(1-FST)}$ > 0.05, Figure 3F, G), climate distance (IBC) (mantel r_{Fst}= -0.528; P_{FST}>
380 0.05; mantel $r_{Fst/(1-FST)}$= -0.520; $P_{FST/(1-FST)}$ > 0.05, Figure 3H, I), precipitation distance (IBP)
381 (mantel r_{FST}= -0.443; P_{FST} > 0.05; mantel $r_{FST/(1-FST)}$= -0.442; $P_{FST/(1FST)}$ > 0.05, Figure 3J, K),
382 had no significant correlation with pairwise F_{ST} and $F_{ST}/(1-F_{ST})$. Moreover, RDA analysis
383 showed that there was a highest contribution of temperature distance on genetic diversity
384 (Figure 3L).

385 There were extremely significant differences in body mass as well as twenty external
386 and cranial characters, expect AVL, between five populations (P < 0.01) (Figure 4 A, B). The
387 body mass and size of LJ population, JC population and ALS population were greater than
388 DQ population and XGLL population. Moreover, The results of single cluster analysis
389 revealed that revealed the grouping of populations, which showed two clusters, DQ
390 population and XGLL population formed one cluster, and JC population, LJ population and
391 ALS population formed one clusters (Figure 4C). Finally, there were significant correlations
392 between most phenotypic traits and environment factors, which had positive correlation with
393 annual environment temperature, and had negative relationship with altitude and latitude (P <
394 0.05) (Figure 4 D).

395 We further calculated the pairwise P_{ST} of all phenotypic traits between five populations,
396 and compared with the pairwise F_{ST}. First the results of violin diagram show that the

probability of P_{ST} more than F_{ST} is large (Figure 5 A). Moreover, the results of independent sample t test showed that P_{ST} of all tested traits was significantly greater than F_{ST} ($P < 0.01$). From the two-way clustering heat map of P_{ST}/F_{ST} value, several interesting findings have emerged. First, most of pairwise P_{ST} of phenotypic traits were higher than the pairwise F_{ST} (Figure 5 B, Supplement table 6). Moreover, the P_{ST}/F_{ST} value differed significantly, and there was the highest P_{ST}/F_{ST} value between DQ population and XGLL population than the other pairwise population, followed by the ratio of between XGLL population and LJ population.

Mantel tests showed no relationship between pairwise P_{ST} and F_{ST} for most traits (Table 3), but the pairwise P_{ST} for BM, EL, CL, CBL and AVL in *E. miletus* were significantly correlated with population pairwise F_{ST}. Mantel tests showed a significant correlation between pairwise P_{ST} for BM, BL, T_1L, CW, FLL, HLL, IB and UTRL in *E. miletus* and population altitudinal differences, however, there were no significant correlation between pairwise P_{ST} for traits except for the ZB in *E. miletus* and population geographic distance (Table 3).

DISCUSSION

Phenotypic changes at the morphological, physiological and behavioral levels to adapt the diverse environment in HM region were found in *E. miletus* (Zhu et al., 2014; Zhang et al., 2019; Ren et al., 2020b). Genetic variations were also found in five *E. miletus* populations from HM region in this study, and although sharing a similar demographic history, the populations had a clear genetic structure. According to the result of population structure,

there were four clusters in genetic level, which grouped together a part of XGLL individuals and JC population, and the remainder of XGLL individuals and DQ population, and JC population as well as ALS population respectively formed a single cluster. This is different from the statistic of phenotypic variations, which clustered together the DQ population and XGLL population, and grouped together the LJ population, JC population and ALS population (Zhang et al., 2019; Ren et al., 2020a).

High genetic variation can serve as the basis for adaptability to environmental change through natural selection, which is essential to the long-term survival of populations (Ellegren et al., 2016; Bijak et al., 2018), as seen in this study with *E. miletus*. Geographical differences result in populations displaying varying degrees of genetic diversity (Ellegren et al., 2016). The study is selected populations ascend in altitude order. LJ population, JC population and ALS population belong to a relative low altitude with range from 2000m to 3000m, as well as XGLL population and DQ population belong to a relative high altitude which over 3000m. The annual average temperature of the environment is counter with the altitude. Our data show that the relative low altitude populations had higher genetic diversity than the relative high altitude populations, but there were no correlation between genetic diversity indexes and altitude. The reason may attribute to the altitude selected in the present study, as the altitude of five population over 2000m reached a high altitude level. Nevertheless, most of genetic diversity indexes had significant correlation with annual average temperature and latitude in this study, indicating that annual average temperature and latitude may play important roles in the genetic diversity of *E. miletus*, while, whether the other factors, such as food, gut microbiota and so on, can play a role in genetic diversity

441 remains to be explored.

442 It is interesting to note that there were asymmetric gene flow patterns in five *E. miletus*
443 populations. First, there was relative high gene flow between DQ population and XGLL
444 population as well as between XGLL population and LJ population, and these better proves
445 the population structure of *E. miletus* in this study, which clustered together respectively. In
446 addition, JC population and ALS population had low gene flow with the other populations,
447 and there was even no gene flow between LJ population as well as ALS population and JC
448 population. This result is consist with that the JC population and ALS population form a
449 cluster respectively. These data may indicate that five *E. miletus* populations exhibit a
450 isolation-by-island model. This contrasts with the isolation-by-distance concept that is
451 present in red-backed vole in southern Virginia (Reese et al., 2001) and southern Appalachia
452 (Browne and Ferree, 2007). The isolation-by-island model predicts that there is no
453 relationship between the distance separating populations and the amount gene flow, in
454 contrast to the isolation-by-distance mode, which asserts that populations separated by shorter
455 distances will experience higher rates of gene flow than populations separated by longer
456 distances (Browne and Ferree, 2007). Isolation-by-island concept typically manifests in
457 animals whose habitat is cut off by an extreme environment, and in those species, the
458 distributions of the sub-populations are typically entirely discontinuous in that environment
459 (Qu et al., 2004). These findings show that barriers to gene flow among *E. miletus*
460 populations existed as a result of the extreme topography of the HM region caused by the
461 geological uplift that occurred during the late Pliocene.

462 It seems conceivable that relatively stable habitats appropriate in the HM region, known

as refugia, emerge after the fast uplift of the HM region towards the end of the Pliocene for *E. miletus* population to survive extreme climate in Quaternary glaciation (Qu et al., 2014; He et al., 2016; Zhou et al., 2006). Moreover, most probably *E. miletus* populations were pushed up and down the hillsides in response to the varying extent of glaciers during the Pleistocene, causing populations interflow increase. Thus, there was a increase in Ne during the begging of Quaternary glaciation. While, climate fluctuations strongly affected the Ne of the species after the formation of geographical isolation in HM region, as the effective population size historically decreased during cold periods, especially during the last ice age.

There was medium or high score genetic differentiation between five *E. miletus* populations, and Mantel test between pairwise F_{ST} and geographic also support the isolation-by-island model, which showed that there was no correlation between pairwise F_{ST} and geographic distance in the present study (Browne and Ferree, 2007). Phenotypic changes at the morphological levels to adapt the diverse environment in HM region were also found in *E. miletus* in this study. This is consist with the previous studies (Zhang et al., 2019; Ren et al., 2020a). Moreover, morphological changes had negative correlation with altitude and latitude, and positive correlation with annual environment temperature, indicating that morphological traits of *E. miletus* dose not obey the Bergmann's rule (Bergmann, 1847; Ashton et al., 2000).

No data were available to estimate the genetic variances of traits in this study due to the fact that the animals in this study are wild, but we can determine the effect on P_{ST} under different h^2 conditions. We further calculated the P_{ST} value using four different heritability estimates (0.25, 0.5, 0.75, and 1), based on the assumption that there is no environmental

485 variance. The graphs in Figure 6 showed the value that the P_{ST}-F_{ST} ratio would take for
486 different values of h^2. The majority of P_{ST} values were greater than pairwise the F_{ST} value,
487 even though the pairwise F_{ST} value was at its minimum when the h^2 was assumed to be one.
488 However, it is well understood that the h^2 can not be one, and must be less than one. With our
489 original assumptions, we concluded that most traits are the consequence of natural selection.
490 Except for a few exceptions, the only conditions under which P_{ST} would be much lower than
491 F_{ST} are if environmental variance is close to zero, and the critical value of c when the h^2 is
492 one is shown in Supplement table 7. These conditions are unlikely to be compatible in nature
493 because nonheritable variance should be environmentally pliable (Wójcik et al., 2006).
494
495 **CONCLUSION**
496 In this study, we investigated the widely dispersed *E. miletus* in the HM region and used
497 population genomic techniques to provide insights on its differentiation, adaptation, and
498 history. In conclusion, our data show that *E. miletus* from the HM region exhibits phenotypic
499 and genetic alterations related to naturally occurring diverse environments. It's interesting to
500 note that there are two phenotypic clusters and various phenotypic and genetic change
501 patterns. Furthermore, phenotypic and genetic changes are linked to environmental factors,
502 such as latitude, altitude, and average annual temperature, and phenotypic traits are more
503 influenced by environmental factors; however, it is still unknown whether other
504 environmental factors may also have an impact on phenotypic and genetic changes.
505 Additionally, the significant biological stratification brought on by the tectonic uplift of the
506 HM region during the late Pliocene results in spectacular topography, which has an impact on

the asymmetric gene flow patterns found in *E. miletus*. Five *E. miletus* populations demonstrate an isolation-by-island model, which is supported by gene flow and a link between FST and geographic distance. Last but not least, PST estimates for the majority of wild traits are higher than differentiation at neutral molecular markers, indicating that directional natural selection favoring various phenotypes in various populations was likely involved in achieving thus much divergence. Our findings provide as a foundation for studies on other HM region wild small animals.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare no competing financial interests.

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732 Table 1. The information of sample site.

Region	Sample number	Site	Altitude(m)	Annual average temperature(℃)	Precipitation(mm)	Vegetation types
DQ	29	99°03′15″E, 28°35′14″N	3459	4.7	633.7	Alpine meadow
XGLL	33	99°83′16″E, 27°90′13″N	3321	5.5	984.2	Subalpine meadow
LJ	33	100°23'30″E, 26°87'53″N	2478	12.6	975.0	Subalpine meadow and shrub
JC	33	99°75′03″E, 26°44′35″N	2219	13.9	987.3	Lobular shrub
ALS	33	100°42′49″E, 24°90′30″N	2183	19.7	597.0	Savanna Shrub and Grass

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738 Table 2. The valve of nucleotide polymorphism ($\theta\pi$), Tajima. D, expected allele number, observed heterozygous, expected heterozygous, Nei
739 diversity index, and polymorphysm information content (PIC), and the correlations analysis between environment factors, including altitude,
740 annual average temperature, and latitude, with genetic diversity indexes of five *E. miletus* populations from Hengduan mountain.

Population	**DQ**	**XGLL**	**LJ**	**JC**	**ALS**	**Altitude (km)**		**Annual average temperature(℃)**		**Latitude**	
						r^2	***P* value**	r^2	***P* value**	r^2	***P* value**
Nucleotide polymorphism ($\theta\pi$)	2.75E-05	2.82E-05	2.74E-05	2.79E-05	2.94E-05	0.152	*>0.05*	0.389	*>0.05*	0.538	*>0.05*
Tajima. D	1.076	1.075	1.061	1.092	1.28	0.278	*>0.05*	0.577	*>0.05*	0.695	*>0.05*
Expected allele number	1.566	1.559	1.571	1.576	1.6	0.579	*>0.05*	0.847	*<0.05*	0.882	*<0.05*
Observed heterozygous	0.223	0.213	0.229	0.223	0.239	0.48	*>0.05*	0.708	*>0.05*	0.665	*>0.05*
Expected heterozygous	0.338	0.335	0.34	0.343	0.354	0.576	*>0.05*	0.842	*<0.05*	0.883	*<0.05*
Nei diversity index	0.345	0.341	0.347	0.349	0.36	0.566	*>0.05*	0.832	*<0.05*	0.86	*<0.05*

Polymorphysm information content (PIC)	0.273	0.271	0.274	0.276	0.284	0.533	*>0.05*	0.813	*<0.05*	0.864	*<0.05*

741 DQ: DeQin, XGLL: XiangGeLiLa, LJ: LiJiang, JC: JianChuan, ALS: AiLaoShan

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753 Table 3. Mantel test between pairwise F_{ST} and environment distance as well as P_{ST}.

Traits	Pairwise F_{ST}		Geographic distance		Temperature distance		Altitude distance		Climate distance		Precipitation distance	
	r	***P value***	***r***	***P value***	***r***	***P value***	***r***	***P value***	***r***	***P value***	***r***	***P value***
BM	0.541	*P > 0.05*	0.546	*P > 0.05*	0.342	*P > 0.05*	0.777	*P < 0.05*	-0.014	*P > 0.05*	0.074	*P > 0.05*
BL	-0.148	*P > 0.05*	0.246	*P > 0.05*	0.102	*P > 0.05*	0.862	*P < 0.05*	0.248	*P > 0.05*	0.216	*P > 0.05*
T1L	0.243	*P > 0.05*	0.287	*P > 0.05*	0.219	*P > 0.05*	0.812	*P < 0.05*	0.171	*P > 0.05*	0.248	*P > 0.05*
T2L	0.455	*P > 0.05*	0.170	*P > 0.05*	0.475	*P > 0.05*	0.133	*P > 0.05*	0.274	*P > 0.05*	0.430	*P > 0.05*
CW	0.354	*P > 0.05*	0.613	*P > 0.05*	0.372	*P > 0.05*	0.929	*P < 0.05*	0.079	*P > 0.05*	0.151	*P > 0.05*
CD	-0.211	*P > 0.05*	0.444	*P > 0.05*	0.433	*P > 0.05*	0.305	*P > 0.05*	0.506	*P > 0.05*	0.405	*P > 0.05*
EL	0.742	*P > 0.05*	0.477	*P > 0.05*	0.654	*P < 0.05*	0.248	*P > 0.05*	-0.098	*P > 0.05*	0.060	*P > 0.05*
EW	-0.025	*P > 0.05*	0.390	*P > 0.05*	0.473	*P > 0.05*	-0.034	*P > 0.05*	0.370	*P > 0.05*	0.290	*P > 0.05*
FLL	-0.125	*P > 0.05*	0.321	*P > 0.05*	-0.030	*P > 0.05*	0.810	*P < 0.05*	0.382	*P > 0.05*	0.406	*P > 0.05*
HLL	0.168	*P > 0.05*	-0.108	*P > 0.05*	-0.118	*P > 0.05*	-0.086	*P > 0.05*	-0.643	*P < 0.05*	-0.737	*P < 0.05*

CL	0.886	*P < 0.01*	0.687	*P < 0.05*	0.588	*P > 0.05*	0.296	*P > 0.05*	-0.754	*P < 0.05*	-0.704	*P < 0.05*
CBL	0.797	*P > 0.05*	0.524	*P > 0.05*	0.443	*P > 0.05*	0.493	*P > 0.05*	-0.254	*P > 0.05*	-0.108	*P > 0.05*
CH	0.209	*P > 0.05*	0.010	*P > 0.05*	-0.012	*P > 0.05*	0.447	*P > 0.05*	-0.028	*P > 0.05*	0.011	*P > 0.05*
PNL	0.362	*P > 0.05*	-0.034	*P > 0.05*	0.042	*P > 0.05*	0.214	*P > 0.05*	-0.040	*P > 0.05*	-0.008	*P > 0.05*
ZB	-0.171	*P > 0.05*	-0.459	*P > 0.05*	-0.665	*P > 0.05*	-0.055	*P > 0.05*	-0.055	*P > 0.05*	0.004	*P > 0.05*
NW	-0.015	*P > 0.05*	-0.162	*P > 0.05*	0.130	*P > 0.05*	-0.441	*P > 0.05*	-0.514	*P > 0.05*	-0.550	*P > 0.05*
CCL	0.412	*P > 0.05*	0.317	*P > 0.05*	0.265	*P > 0.05*	0.297	*P > 0.05*	-0.612	*P > 0.05*	-0.679	*P > 0.05*
IB	-0.29	*P > 0.05*	0.179	*P > 0.05*	0.025	*P > 0.05*	0.861	*P < 0.05*	0.348	*P > 0.05*	0.285	*P > 0.05*
ESL	-0.462	*P > 0.05*	-0.270	*P > 0.05*	-0.405	*P > 0.05*	-0.147	*P > 0.05*	0.475	*P > 0.05*	0.395	*P > 0.05*
AVL	0.715	*P > 0.05*	0.472	*P > 0.05*	0.778	*P < 0.05*	-0.147	*P > 0.05*	-0.468	*P > 0.05*	-0.449	*P > 0.05*
UTRL	0.414	*P > 0.05*	0.253	*P > 0.05*	0.130	*P > 0.05*	0.715	*P < 0.05*	0.063	*P > 0.05*	0.182	*P > 0.05*
LTRL	0.147	*P > 0.05*	0.011	*P > 0.05*	-0.025	*P > 0.05*	-0.202	*P > 0.05*	0.142	*P > 0.05*	0.186	*P > 0.05*

754 BM: Body mass, BL: body length, T_1L: tail length, T_2L: torso length, CW: chest width, CD: chest depth, EL: ear length, EW: ear width, FLL:

fore limb length, HLL: hind limb length, CL: cranial length, CBL: cranial basal length, CH: cranial height, PNL: pillow nose length, ZB: zygomatic breadth, NW: neurocranium width, CCL: covering cap length, IB: interorbital breadth, ESL: eye socket length, AVL: auditory vesicle length, UTRL: upper tooth row length and LTRL: lower tooth row length. Data were analyzed by Mantel test.

765 **Figure Legends**

766 **Figure 1** Population structure. (A) Sampling information of *E. miletus* used in this
767 study. (B) Genetic structure of the 161 individuals from five populations. Groupings
768 of samples from 1–10 ancestral clusters are shown. Groupings of samples from one to
769 ten ancestral clusters are shown. (C) Scatter plot of principal components 1 versus 2
770 (PC1 versus PC2 showed in left) and principal components 1 versus 3 (PC1 versus
771 PC3 showed in right) for the five populations. (D) Neighboring-joining phylogenetic
772 tree of five populations. DQ: DeQin, XGLL: XiangGeLiLa, LJ: LiJiang, JC:
773 JianChuan, ALS: AiLaoShan.

774 **Figure 2** Demographic history and gene flow of *E. miletus*. (A) Diagram of relative
775 magnitude and direction of gene flow. Arrowheads show the estimated direction of
776 gene flow. (B) Demographic history inferred by PSMC. The major stage, the
777 Quaternary glaciation (3000~10 Ka BP), includes twice increase (2000Kya and 90kya)
778 and twice decrease (Marine Isotope Stage 12 (500Ka ± 5Ka BP) and Marine Isotope
779 Stage 3 (60Ka-25Ka BP)). DQ: DeQin, XGLL: XiangGeLiLa, LJ: LiJiang, JC:
780 JianChuan, ALS: AiLaoShan.

781 **Figure 3** Genetic differentiation and linear regression lines showing the correlations
782 among genetic, geographic, and environmental distances. (A) The heat map of
783 pairwise F_{ST} between *E. miletus* populations, Groups: DQ: DeQin population, XGLL:
784 XiangGeLiLa population, LJ: LiJiang population, JC: JianChuan population, ALS:
785 AiLaoShan population. Mantel test between pairwise F_{ST} and $F_{ST}/(1-F_{ST})$ as well as
786 geographic distance (IBD: B, C), temperature distance (IBT: D, E), altitude distance

(IBA: F, G), climate distance (IBC: H, I), and precipitation distance (IBP: G, K). Data were analyzed by Mantel test. $P < 0.05$. (L) RDA ordination ts of genetic diversity in *E. miletus*.

Figure 4 Group differences in body mass (A) and twenty-one phenotypic traits (B) of five *E. miletus* populations from HM region. Data were analyzed by one-way ANOVA followed by the LSD post-hoc test. Significant differences were indicated by different alphabetic letters. One-way clustering heat map based on the body and skull traits in *E. miletus* (C). The correlation matrix between altitude, annual average temperature and latitude with twenty-two phenotypic traits (D). DQ: DeQin, XGLL: XiangGeLiLa, LJ: LiJing, JC: JianChuan, ALS: AiLaoShan; BM: Body mass, BL: body length, T_1L: tail length, T_2L: torso length, CW: chest width, CD: chest depth, EL: ear length, EW: ear width, FLL: fore limb length, HLL: hind limb length, CL: cranial length, CBL: cranial basal length, CH: cranial height, PNL: pillow nose length, ZB: zygomatic breadth, NW: neurocranium width, CCL: covering cap length, IB: interorbital breadth, ESL: eye socket length, AVL: auditory vesicle length, UTRL: upper tooth row length and LTRL: lower tooth row length.

Figure 5 Two-way clustering heat map of the value of pairwise P_{ST} vs F_{ST} value between five *E. miletus* populations from Hengduan mountain regions. DQ: DeQin, XGLL: XiangGeLiLa, LJ: LiJiang, JC: JianChuan, ALS: AiLaoShan; BM: Body mass, BL: body length, T_1L: tail length, T_2L: torso length, CW: chest width, CD: chest depth, EL: ear length, EW: ear width, FLL: fore limb length, HLL: hind limb length, CL: cranial length, CBL: cranial basal length, CH: cranial height, PNL: pillow nose length,

ZB: zygomatic breadth, NW: neurocranium width, CCL: covering cap length, IB: interorbital breadth, ESL: eye socket length, AVL: auditory vesicle length, UTRL: upper tooth row length and LTRL: lower tooth row length.

Figure 6 The heat map of comparison value between P_{ST} estimated by phenotypic measures using four different heritability estimates (0.25 (A), 0.5 (B), 0.75 (C) and 1 (D)), based on the assumptions that there is no environmental variance, and pairwise F_{ST} calculated using differentiation at neutral molecular markers. DQ: DeQin, XGLL: XiangGeLiLa, LJ: LiJiang, JC: JianChuan, ALS: AiLaoShan; BM: Body mass, BL: body length, T_1L: tail length, T_2L: torso length, CW: chest width, CD: chest depth, EL: ear length, EW: ear width, FLL: fore limb length, HLL: hind limb length, CL: cranial length, CBL: cranial basal length, CH: cranial height, PNL: pillow nose length, ZB: zygomatic breadth, NW: neurocranium width, CCL: covering cap length, IB: interorbital breadth, ESL: eye socket length, AVL: auditory vesicle length, UTRL: upper tooth row length and LTRL: lower tooth row length.