

Geographical variation in echolocation vocalizations of the Himalayan leaf-nosed bat: contribution of morphological variation and cultural drift

Aiqing Lin, Tinglei Jiang, Jagmeet S. Kanwal, Guanjun Lu, Jinhong Luo, Xuewen Wei, Bo Luo and Jiang Feng

A. Lin, T. Jiang, G. Lu, J. Luo, X. Wei, B. Luo and J. Feng (fengj@nenu.edu.cn), Jilin Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal Univ., 2555 Jingyue Street, Changchun 130117, China, and: Key Laboratory of Vegetation Ecology of Education Ministry, Inst. of Grassland Science, Northeast Normal Univ., 5268 Renmin Street, Changchun 130024, China. – J. S. Kanwal, Dept of Neurology, Georgetown Univ. Medical Center, Washington DC, USA.

Ecologists and evolutionary biologists have a long-standing interest in the patterns and causes of geographical variation in animals' acoustic signals. Nonetheless, the processes driving acoustic divergence are still poorly understood. Here, we studied the geographical variation in echolocation vocalizations (commonly referred to as echolocation 'pulses' given their short duration and relatively stereotypic nature, and to contrast them from the communicative vocalizations or 'calls') of a widespread bat species *Hipposideros armiger* in south China, and assessed whether the acoustic divergence was driven by either ecological selection, or cultural or genetic drift. Our results revealed that the peak frequency of echolocation pulses varied significantly across populations sampled, with the maximum variation of about 6 kHz. The peak frequency clustered into three groups: eastern and western China, Hainan and southern Yunnan. The population differences in echolocation pulses were not significantly related to the variation in climatic (mean annual temperature, mean annual relative humidity, and mean annual precipitable water) or genetic (genetic distance) factors, but significantly related to morphological (forearm length) variation which was correlated with mean annual temperature. Moreover, the acoustic differences were significantly correlated with geographical and latitudinal distance after controlling for 'morphological distance'. Thus, neither direct ecological selection nor genetic drift contributed to the acoustic divergence observed in *H. armiger*. Instead, we propose that the action of both indirect ecological selection (i.e. selection on body size) as well as cultural drift promote, in part, divergence in echolocation vocalizations of individuals within geographically distributed populations.

Animals' acoustic signals often vary geographically and are considered as good models to improve our understanding of ecological processes, such as population divergence, niche differentiation and speciation, and of the role of adaptation, selection and drift. Acoustic divergence may arise from the action of different forces of evolution, among which ecological selection is the most common (Podos and Warren 2007, Wilkins et al. 2013). Population divergence in acoustic signals can arise when signals undergo selection for optimal transmission in the local acoustic environment (Morton 1975, Wiley and Richards 1982). In this case, the evolution of acoustic signals is influenced directly by environmental factors such as temperature, vegetation types and ambient noise (Slabbekoorn and Smith 2002, Nemeth et al. 2013, Luo et al. 2014). Ecological selection can also contribute to acoustic divergence indirectly. Geographical variation in acoustics may emerge as an epiphenomenon of ecological selection on other traits, rather than through direct selection of the sounds themselves (Andrew 1962, Ballentine and Pfennig 2006). Compared with direct ecological selection,

the role of indirect ecological selection in acoustic divergence is reported relatively less frequently. Moreover, the evolution of acoustic signals may be a neutral process. Acoustic features could evolve as a result of the accumulation of copying 'errors' in vocal learning; random drift in cultural evolution would then lead to vocal differences among diverging populations (Slater 1986). Genetic drift represents another potential driver of acoustic divergence. Random changes at genetic loci that underlie vocal ontogeny and production and the structure and function of the brain nuclei involved in vocal learning would be expected to impact vocal geographical evolution (Podos and Warren 2007). Under the drift scenarios, a positive correlation can be expected between acoustic and geographical (cultural drift) (Yoshino et al. 2008, Sun et al. 2013) or genetic distance (genetic drift) (Irwin et al. 2008, Campbell et al. 2010).

Echolocation vocalizations in bats are primarily used for orientation, navigation and prey capture, and sometimes play an additional communicative role (Jones and Siemers 2011). This type of vocalization can prove useful for empiri-

cal tests of the role of selection and drift in the processes of acoustic divergence. We already know that the transmission of echolocation signals is influenced by ambient temperature, humidity, type of vegetation, and other environmental factors (Guillén and Ibanez 2000, Jiang et al. 2010b, Snell-Rood 2012, Sun et al. 2013). Thus, bats may evolve to produce relatively lower frequency echolocation pulses in habitats where sound absorption rates during aerial transmission are high (Snell-Rood 2012). The predominant frequency present within echolocation pulses, however, is essentially determined by size and shape of the vocal apparatus (Neuweiler 2000); a large larynx for example produces relatively low frequency sounds.

Given the physics of acoustics, the mass of sound-producing structures are often positively correlated with body size, and exhibit a negative relationship between body size and the predominant frequency within the dominant vocalization, which has been commonly observed among comparisons of sex, population and species in bats (Francis and Habersetzer 1998, Jones 1999, Guillén and Ibanez 2000, Jiang et al. 2010b). Therefore, natural selection acting on body size may indirectly lead to changes in characteristic frequency of echolocation pulses that improves adaptation to different environments. In addition, cultural drift has been suggested to contribute partly to the geographical variation in echolocation pulses in several bat species, such as *Rhinolophus cornutus* (Yoshino et al. 2008), *R. monoceros* (Chen et al. 2009), and *R. ferrumequinum* (Sun et al. 2013). As predicted by the ‘cultural drift hypothesis’, population differences in echolocation pulses are significantly correlated with geographical distance in these species. However, the results of studies available on geographical variation of vocalizations emitted by bats do not support the ‘genetic drift hypothesis’ (Yoshino et al. 2008, Chen et al. 2009, Puechmaile et al. 2011, Jiang et al. 2013, Sun et al. 2013).

The Himalayan leaf-nosed bat, *Hipposideros armiger* (Chiroptera: Hipposideridae) typically roosts in caves and is widely distributed in south China, mainland southeast Asia and the south Himalayas (Bates et al. 2008). This species emits echolocation pulses that are characterized by a constant frequency (CF) component followed by a downward frequency modulation (FM) component, with the most energy being normally present in the CF component of the second harmonic of the pulses (Fig. 1). Recent molecular evidence suggests that the divergence in major genetic lineages of *H. armiger* can be dated to ca 0.24 Ma and multiple refugia may have existed for this species in China (Lin et al. 2014). Obvious population genetic structure was found based on maternally inherited genetic markers (mitochondrial DNA, mtDNA), but weak structure based on biparentally inherited markers (microsatellites, nSSR). The different levels of genetic structure may be largely due to a male biased dispersal mechanism. In particular, populations from eastern China (EC), western China (WC), and southern Yunnan (SY) were genetically divergent among each other and the population in Hainan (HN) had a closed genetic relationship with the EC populations based on mtDNA data, but with the WC populations based on nSSR. Furthermore, substantial gene flow was found across geographical regions despite the presence of many barriers and high environmental heterogeneity in south China. These factors make the

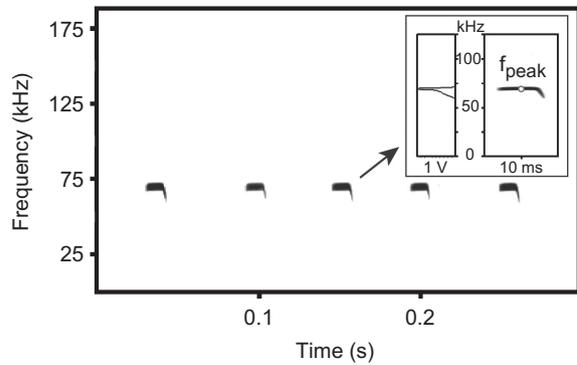


Figure 1. Spectrogram of an echolocation call sequence of *Hipposideros armiger*. The upper right plot shows power spectra (left section), plotting amplitude versus frequency, and spectrogram (right section) of a selected echolocation pulse illustrating the peak frequency (f_{peak}) of the pulse.

Himalayan leaf-nosed bat an ideal organism for investigating the role of ecological selection, cultural and genetic drift on the evolution of acoustic traits.

In this study, we recorded echolocation pulses emitted in the resting state of *H. armiger* in south China, and documented the presence of systematic geographical variation in their echolocation pulses. We then tested whether direct or indirect ecological selection, or cultural or genetic drift contributes to acoustic divergence of echolocation pulses emitted by *H. armiger*. Per the direct selection hypothesis, we predicted that population differences in echolocation pulses would be related to variation in environmental factors, such as temperature and humidity. Per the indirect selection hypothesis, we predicted that echolocation pulses would covary with body size and that population differences in body size would be associated with changes in environmental factors. Per the cultural drift hypothesis, we predicted that acoustic distance between populations would be correlated with geographical distance. Per the genetic drift hypothesis, we predicted that acoustic distance would be correlated with genetic distance. We used our acoustic, morphological, climatic, geographical and genetic data to test the relevance of all of these hypotheses on observed variation in the acoustic characteristics of vocalizations used for echolocation in geographically dispersed populations of *H. armiger*.

Material and methods

Sound recording, morphological measurement and molecular sampling

In 2008 and 2011, we collected 286 adults of *Hipposideros armiger* from 17 localities in south China, including individuals from Chongyi (CY), Fanchang (FC), Jिंगgangshan (JGS), and Shaoguan (SG) in EC, Anlong (AL), Beichuan (BC), Baise (BS), Emeishan (EMS), Guilin (GL), Hanzhong (HZ), Jiangkou (JK), Kaixian (KX), and Xishui (XS) in WC, HN, and Hekou (HK), Menglun (ML), and Simao (SM) in SY (Fig. 2, Supplementary material Appendix 1 Table A1). Bats were taken into a temporary laboratory (9 m long \times 9 m wide \times 5 m high) near the roost. Resting echolocation

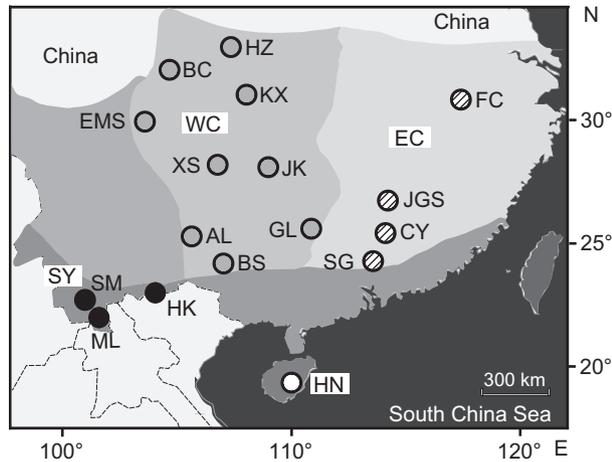


Figure 2. Localities for echolocation call recording for *Hipposideros armiger* in China. Gray levels delineate different zoological regions of south China. EC, eastern China; WC, western China; SY, southern Yunnan; HN, Hainan. Definitions of site abbreviations and coordinates are provided in the Supplementary material Appendix 1 Table A1.

pulses were recorded for each individual, with the microphone located at a distance of ~2 m from the bat. Pulses were recorded using an ultrasound recording system, with a sample rate of 375 kHz at 16 bits/sample. We measured the lengths of forearm (FA) of these bats with electronic vernier calipers to the nearest 0.01 mm. The mean of three repeated measurements for each individual was used for the analysis. Moreover, a 3-mm wing membrane biopsy punch was taken from each of 181 individuals for genetic analysis (Supplementary material Appendix 1 Table A1). All bats were released after completion of data and sample collection. Latitude, longitude and elevation for each locality were recorded using GPS. Linear geographical distances between sampling localities were calculated based on the coordinates recorded.

Climatic data collection

We used climatic variables to describe environmental features relevant to sound propagation of the sampling localities. Temperature and humidity have a direct influence on sound transmission, and annual rainfall has also been suggested to have an effect on call frequency of bats (Jiang et al. 2010b). We therefore selected mean annual temperature (MAT), mean annual relative humidity (MARH), and mean annual precipitable water (MAPW) to test for correlations between bat acoustic feature and climate conditions at different sampling localities. Climatic data for each locality across the years 1971–2010 were obtained via the R-package RNCEP (Kemp et al. 2012).

Sound analysis

The peak frequency (f_{peak}) of the second harmonic is relatively constant in a resting CF bat that has been widely used to describe the acoustic feature of echolocation pulse for CF bats (Yoshino et al. 2008, Chen et al. 2009, Jiang et al. 2010a, b, Sun et al. 2013), although daily

variations have been reported (Huffman and Henson 1993, Hiryu et al. 2006). We selected high quality echolocation pulses from call sequences of each individual for acoustic parameter measurement and used the software Avisoft SasLab Pro to determine the f_{peak} of the second harmonic of each pulse. The f_{peak} was measured from power spectrum (FFT) using a sample rate of 250 kHz and a Hamming window, giving a frequency resolution of 61 Hz. A mean value was calculated for each individual from 15 randomly chosen pulses.

Analysis of genetic differentiation

We amplified partial mitochondrial control region (358 bp) and complete cytochrome b (1140 bp) sequences and seven nuclear microsatellite loci (nSSR: BAM09, CHANG242, TE2, P6D12, PT5B2, P541 and PE4) from 181 individuals. Among these samples, 120 individuals from 11 populations have been reported in Lin et al. (2014). The sequence amplification and microsatellite genotyping for the remaining samples collected from BC, CY, FC, HK, HZ and JK were performed following the protocols reported by Lin et al (2014). All sequences were aligned using CLUSTAL_X (Thompson et al. 1997) and revised manually (Genbank accession numbers for new sequences: KF739413–KF739425). For nSSRs, Hardy–Weinberg equilibrium (HWE) status, and extent of linkage disequilibrium were calculated using GENEPOP 4.0 (Rousset 2008). Null allele frequencies for each locus were estimated using MICRO-CHECKER (Van Oosterhout et al. 2004). We quantified genetic differentiation between populations using ARLEQUIN ver. 3.5 (Excoffier et al. 2005) by calculating pairwise $F_{ST}/(1 - F_{ST})$ separately for concatenated mtDNA sequences and nSSR.

Geographical variation and correlation analysis

To assess an effect of geographical locality and sex on echolocation pulses, we tested for differences in f_{peak} among localities and between sexes using a generalized linear model (GLM) in which both variables were treated as factors. The GLM was performed using the R statistical software. A multidimensional scaling (MDS) analysis was carried out in SPSS 16.0 using the matrix of acoustic distances between populations to represent the patterns of acoustic differentiation in a two-dimensional space. Once distinct groups were found, post hoc multiple comparison Tukey tests were used to test the differences among groups.

To test the correlations between acoustic differences and morphological, climatic, geographical and genetic variation, we first calculated pairwise differences of population mean values for FA and each climatic and geographical variable, respectively. We also calculated combined climatic Euclidean distances with MAT, MARH and MAPW to represent the overall climatic variation between sampling localities. We then used pairwise Mantel tests to detect the relationships between acoustic distance matrix and morphological, climatic, geographical and genetic distance matrices, respectively. Partial Mantel tests were used to test for associations between acoustic distance matrix and important morphological, climatic and geographical matrices after removing the effects of climatic, morphological, or genetic matrices, respectively.

All Mantel tests and partial Mantel tests were implemented in PASSaGE ver. 2 (Rosenberg and Anderson 2011). We corrected critical α values for multiple Mantel and partial Mantel tests using Rom's modified Bonferroni adjustment (Rom 2012). Additionally, simple linear regression models were used to test the relationship between f_{peak} and important factors as well as between these factors.

Results

Geographical variation of echolocation pulses

Across all populations, the mean f_{peak} of echolocation pulses varied markedly, ranging from 66.80 to 72.51 kHz (Fig. 3A). The GLM revealed that significant variation in f_{peak} was explained by geographical locality ($F_{16,247} = 32.62$, $p < 0.001$), but not by sex (females: 69.23 ± 1.10 kHz; males: 69.06 ± 1.15 kHz; $F_{1,247} = 2.41$, $p = 0.12$). The MDS revealed three distinct acoustic groups ($r^2 = 1.0$), i.e. EC and WC, HN and SY (Fig. 3B). Post hoc multiple comparison tests confirmed the significant differentiation of the f_{peak} of echolocation pulses between these three groups (all $p < 0.001$) and the insignificant differentiation between EC and WC ($p = 0.08$).

Genetic differentiation

Both concatenated mtDNA sequences and nSSR data exhibited significant global genetic differentiation (mtDNA: $F_{ST} = 0.799$; nSSR: $F_{ST} = 0.086$; both $p < 0.001$). Pairwise population F_{ST} values based on mtDNA ranged from -0.048 to 0.937 (Supplementary material Appendix 1 Table A2), and 91.17% of them were significant ($p < 0.05$). Pairwise population F_{ST} values based on nSSR ranged from -0.016 to 0.258 , and 74.26% of them were significant ($p < 0.05$).

Correlates of acoustic divergence

Both Mantel and partial Mantel tests showed a significantly positive correlation between acoustic and morphological differences across overall populations (Table 1). Mantel tests

Table 1. Correlations between acoustic distance of echolocation pulses (EP) of *Hipposideros armiger* and pairwise distances of morphological, environmental, geographical and genetic factors across populations. WC, western China; FA, forearm; MAT, mean annual temperature; MARH, mean annual relative humidity; MAPW, mean annual precipitable water; mtDNA, mitochondrial DNA sequences; nSSR, nuclear microsatellite markers. Overall climatic Euclidean distances (climatic) between sampling localities were calculated with MAT, MARH and MAPW. The significant p-values corrected by Rom's modified Bonferroni adjustment are shown in bold.

Variables	Variables	Total range		Within WC	
		r	p	r	p
<u>Mantel tests</u>					
EP	FA	0.59	<0.001	-0.01	0.974
	MAT	0.43	0.016	-0.04	0.810
	MARH	0.00	0.980	0.06	0.769
	MAPW	0.36	0.027	0.00	0.987
	Climatic	0.36	0.037	0.03	0.852
	Longitudinal	0.13	0.380	-0.07	0.702
	Latitudinal	0.39	0.005	-0.01	0.941
	Elevation	0.11	0.436	0.18	0.307
	Geographical	0.41	0.002	0.00	0.991
	mtDNA	0.20	0.203	0.09	0.660
FA	nSSR	0.37	0.030	-0.12	0.505
	MAT	0.39	0.020	-0.23	0.230
	mtDNA	0.08	0.582	-0.11	0.653
mtDNA	nSSR	0.14	0.388	-0.19	0.341
	Geographical	0.35	0.004	0.35	0.047
	nSSR	0.64	<0.001	0.71	<0.001
<u>Partial Mantel tests</u>					
EP	FA (MAT)	0.51	<0.001	-0.02	0.936
	FA (latitudinal)	0.55	<0.001	-0.01	0.962
	FA (geographical)	0.61	<0.001	-0.01	0.970
	FA (mtDNA)	0.58	<0.001	0.00	0.984
	FA (nSSR)	0.58	<0.001	-0.03	0.874
	MAT (FA)	0.27	0.061	-0.04	0.797
	MAT (latitudinal)	0.21	0.181	-0.05	0.791
	Latitudinal (FA)	0.32	0.013	-0.01	0.933
	Latitudinal (MAT)	0.09	0.530	0.03	0.879
	Geographical (FA)	0.43	<0.001	0.00	0.983
FA	MAT (mtDNA)	0.38	0.024	-0.18	0.302
	MAT (nSSR)	0.37	0.021	-0.10	0.508
mtDNA	nSSR (EP)	0.30	0.006	0.35	0.046
	nSSR	0.58	<0.001	0.72	<0.001

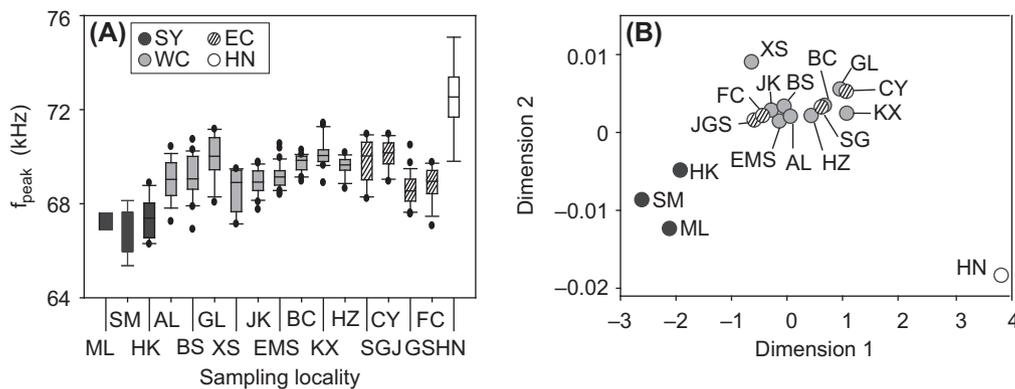


Figure 3. Box plot of peak frequency (f_{peak}) of echolocation pulses (A), and multidimensional scaling plot (B) of acoustic distance (population pairwise differences) for echolocation pulses of *Hipposideros armiger* sampled in eastern China (EC), western China (WC), southern Yunnan (SY) and Hainan (HN). Site abbreviations are defined in Supplementary material Appendix 1 Table A1.

also showed a significant correlation between acoustic and geographical distances (Table 1, Fig. 4A), and the correlation was still highly significant after controlling for morphological distance. Moreover, a significant correlation was found between acoustic and MAT and latitudinal distances, respectively; however, the correlation was not significant when controlling morphological or MAT distance in partial Mantel tests (Table 1, Supplementary material Appendix 1 Table A3). There was an insignificant relationship between acoustic and genetic distance based on either nSSR (Table 1, Fig. 4B) or mtDNA (Table 1, Fig. 4C). Morphological distance was significantly correlated with MAT distance, but insignificantly correlated with genetic distance (Table 1). Both mtDNA and nSSR based genetic distances were strongly correlated with geographical distance across all populations. Linear regression models showed a significantly negative correlation between f_{peak} and FA ($r = -0.78$, $p < 0.001$, Fig. 4D), but an insignificantly positive correlation between f_{peak} and MAT ($r = 0.23$, $p = 0.373$, Fig. 4E), a negative but insignificant correlation between FA and MAT ($r = -0.28$, $p = 0.277$, Fig. 4F), and a weak correlation between f_{peak} and latitude ($r = 0.03$, $p = 0.921$). We also focused on the relationships between echolocation pulses and the morphological, climatic, geographical and genetic factors within WC regions, but no significant relationship was found for these comparisons revealed in Mantel and partial Mantel tests (Table 1, Supplementary material Appendix 1 Table A3).

Discussion

In the present study, we found that echolocation pulses of *Hipposideros armiger* varied significantly across populations

in south China, and clustered into three groups, i.e. EC-WC, HN and SY. Environmental factors are the primary sources that drive the evolution of acoustic signals in a broad range of taxa, such as arthropods (Lampe et al. 2014), amphibians (Ryan et al. 1990), birds (Slabbekoorn and Smith 2002, Nemeth et al. 2013), and mammals (Mitani and Stuht 1998, Luo et al. 2014). Temperature, humidity, and other environmental factors influence directly the transmission of sounds so that they may drive differentiation of acoustic signals across different environments. In this study, although a significant correlation was found between echolocation pulses and temperature in Mantel tests, the correlation was not significant after removing the effect of morphological variation. An unexpected positive correlation between f_{peak} of echolocation pulses and temperature revealed by linear regression model suggested that temperature did not directly affect the acoustic characteristics of echolocation pulses of *H. armiger*. Similarly, although a significant correlation was found between acoustic and latitudinal distances, the correlation was not significant after controlling for temperature. Indeed, the f_{peak} of echolocation pulses did not gradually vary with latitude as shown in linear regression analysis, suggesting that the correlation between echolocation pulses and latitude did not indicate an effect of ecological conditions varying with latitude. These results indicate that the variation of echolocation pulses was likely not the result of direct ecological selection. Nevertheless, although climatic factors are one of the most important sources that can drive acoustic divergence, other factors such as ambient noise and vegetation types (Morton 1975, Dingle et al. 2008, Nemeth et al. 2013, Lampe et al. 2014) may also drive the evolution of acoustic signals. Future research is necessary to determine the role of those factors in the processes driving divergence in echolocation pulses.

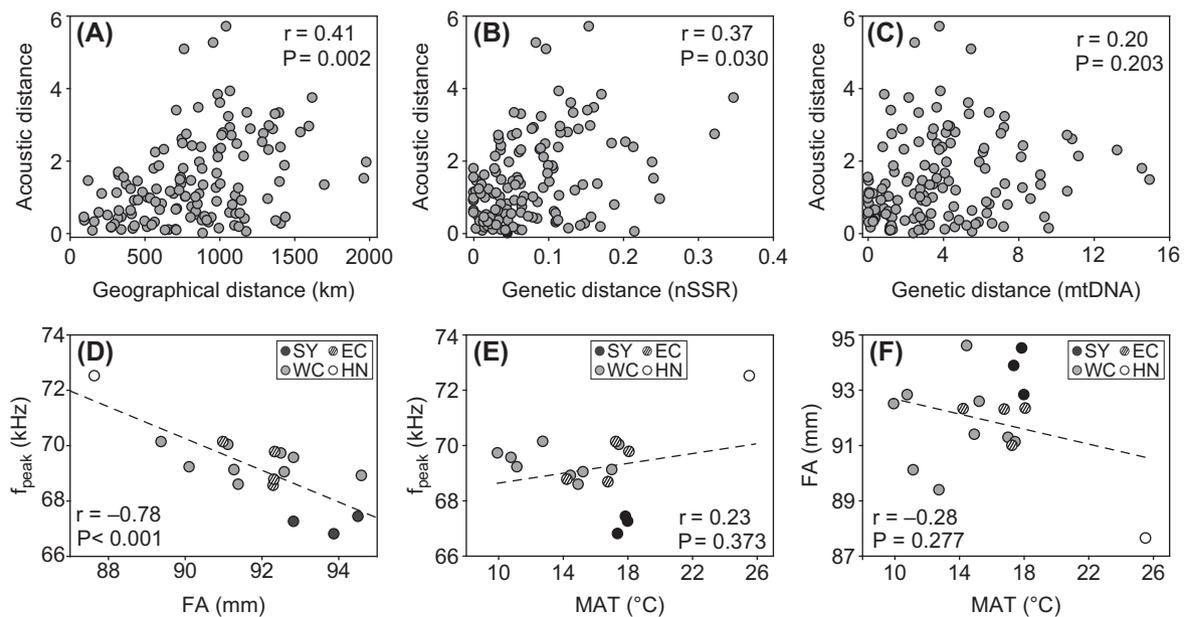


Figure 4. Scatter plots and results of Mantel tests for population differences in peak frequency (f_{peak}) of echolocation pulses of *Hipposideros armiger* versus geographical (A) and genetic distance ($F_{\text{ST}} / (1 - F_{\text{ST}})$) based on nSSR (B) and mtDNA (C). Scatter plots and results of linear regression analyses for f_{peak} of echolocation pulses versus lengths of forearm (FA) (D) and Mean annual temperature (MAT) (E) as well as for FA versus MAT (F). Samples of *Hipposideros armiger* were collected from eastern China (EC), western China (WC), southern Yunnan (SY) and Hainan (HN).

In contrast, both Mantel and partial Mantel tests showed a significant correlation between acoustic and morphological distance, and the f_{peak} of echolocation pulses was significantly negatively correlated with FA. These results reflect the fact that vocal apparatuses produce incrementally low frequencies with increase in body size, as observed in many vertebrate animals (reviewed by Taylor and Reby 2010). This has also been demonstrated for echolocation pulses in species belonging to five families of microchiropteran bats (Jones 1999), but is usually not supported at the intraspecific level (Francis and Habersetzer 1998, Guillén and Ibanez 2000, Chen et al. 2009, Jiang et al. 2010a, 2013). The population differences in body size (FA) of *H. armiger* were significantly correlated with climatic (MAT) distance, but not significantly correlated with genetic distance, suggesting that morphological variation was a result of adaptation to local ecological conditions, but not due to phylogenetic history. Linear regression analysis showed a negative but insignificant correlation between FA and MAT. This suggests that there was some tendency for larger body size in colder conditions. However, the insignificant and weak correlation indicated that the effects of MAT on body size were not able to explain most morphological variation in *H. armiger*. Other environmental factors, such as habitat and food availability and quality might exert selection on body size of individuals (Yom-Tov and Geffen 2006, Nash et al. 2014). Nevertheless, these results suggested the geographical variation of echolocation pulses emerged partly as a result of morphological variation which was likely due to adaptation to local environments, supporting the indirect ecological selection hypothesis (i.e. epiphenomenon hypothesis, Andrew 1962). Similar evolutionary processes have been observed in other taxa. For example, selection of beak size for feeding has resulted in the differentiation of acoustic features of song in swamp sparrows (Ballentine and Pfennig 2006).

Cultural drift is another primary force that could drive differentiation of acoustic signals (Slater 1986, Wilkins et al. 2013). Behaviour research has demonstrated the existence of vocal learning in several bat species, such as *Phyllostomus hastatus* (Boughman 1998) and *Saccopteryx bilineata* (Knörnschild et al. 2010). It has also been suggested that cultural drift contributed partly to the geographical variation of echolocation pulses in *Rhinolophus cornutus* (Yoshino et al. 2008), *R. monoceros* (Chen et al. 2009), and *R. ferrumequinum* (Sun et al. 2013). Therefore, cultural drift may be an important force driving vocal geographical variation in bats. The cultural drift hypothesis predicts a positive correlation between acoustic and geographical distance. In our study, a highly significant relationship was found between them after removing the effect of morphological variation, indicating that some acoustic differentiation was independent of body size and supporting random cultural drift in echolocation pulses of *H. armiger*.

Although significant genetic differentiation was found among populations, our genetic data did not support a genetic drift hypothesis for acoustic divergence in *H. armiger*. The genetic drift hypothesis predicts a positive correlation between acoustic and genetic distance (Podos and Warren 2007, Campbell et al. 2010). However, the population differences in echolocation pulses of *H. armiger* were not significantly correlated with either mtDNA or nSSR based

genetic distance. Moreover, both genetic markers revealed a remarkable split between EC and WC population groups (Lin et al. 2014), but there was no significant differentiation in echolocation pulses between the two groups. Similarly, although deep genetic splits were observed among the populations in SY (Lin et al. 2014), the echolocation pulses of them clustered into the same group in the MDS analysis. In contrast, the population in HN had a closed genetic relationship with the EC populations based on mtDNA data but with the WC populations based on nSSR (Lin et al. 2014); however, they exhibited great differentiation in echolocation pulses between those of both, EC and WC. In conclusion, the patterns seen in the genetic structure were inconsistent with the patterns seen in the geographical variation of echolocation pulses in *H. armiger*. In fact, the genetic drift hypothesis is not supported in many other bat species such as *R. cornutus*, *R. monoceros*, *R. ferrumequinum* and *Myotis davidii* (Jiang et al. 2013).

Echolocation signals are important for orientation and prey capture for echolocating bats. Divergence in the acoustic structure of echolocation pulses may affect the precision for target localization and result in resource partitioning of prey (Aldridge and Rautenbach 1987). Indeed, pulse parameters, e.g. sound frequencies (e.g. peak frequency, maximum frequency, and minimum frequency) vary substantially (>6 kHz) within individuals in many bat species, such as *Myotis bechsteinii* (Siemers and Kerth 2006) and *M. macrodactylus* (Luo et al. 2012), suggesting that the variation in characteristic frequency of echolocation pulses within a certain range does not influence the species' ecological adaptation. The variation of echolocation pulses (66.80~72.51 kHz) in *H. armiger* leads to a ca 0.4 mm variation of wavelengths (assuming a typical speed of sound of 340 m s^{-1} , an ambient temperature of 22°C , and 80% humidity) that is smaller than would be expected if the bats forage on prey of different sizes. Similarly, a 5–7 kHz variation of echolocation pulses in *R. monoceros*, *R. ferrumequinum* and *H. larvatus* (Jiang et al. 2010a) that does not impact prey detection theoretically has also been predicted. Moreover, echolocation pulses could serve an intraspecific communication function in bats (Jones and Siemers 2011). In some birds, population-specific acoustic traits facilitate recognition of individuals within populations while divergence in traits would affect genetic exchange among populations (Baker and Mewaldt 1978, MacDougall-Shackleton and MacDougall-Shackleton 2001). It is still unknown whether acoustic differences would reduce gene flow among populations in bats. In our study, both mtDNA and nSSR based genetic distance showed a pronounced significant correlation with geographical distance, indicating gene flow between populations in south China. This conclusion is supported by the results reported in Lin et al (2014) that substantial gene flow occurred among geographical groups of *H. armiger*. Removing the effects of acoustic distance did not significantly alter the relation between genetic and geographical distances. Taken together, these results suggest that divergence in echolocation vocalizations might not obviously reduce gene flow between populations in *H. armiger*.

In summary, our study showed significant geographical variation in vocalizations used for echolocation by *H. armiger* in south China. We propose this variation results

in part from a combination of factors, namely morphological adaptation and cultural drift. Accordingly, our results support the indirect ecological selection and cultural drift hypotheses, but do not support the direct ecological selection or genetic drift hypotheses. We conclude that the acoustic divergence of vocalizations used for echolocation by *H. armiger* is highly unlikely to either impact their ecological adaptations or be a barrier to gene flow between geographically dispersed populations.

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Supplementary material (available online as Appendix oik.01604 at <www.oikosjournal.org/readers/appendix>). Appendix 1.