Variation in the resting frequency of *Rhinolophus pusillus* in Mainland China: Effect of climate and implications for conservation

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This study describes variation patterns in the constant frequency of echolocation calls emitted at rest and when not flying (“resting frequency” RF) of the least horseshoe bat, *Rhinolophus pusillus*, on a broad geographical scale and in response to local climatic variables. Significant differences in RF were observed among populations throughout the species range in Mainland China, and this variation was positively and significantly related to climate conditions, especially environmental humidity, but the variability was only weakly associated with geographical distance. Sex dimorphism in the RF of *R. pusillus* may imply that female and male might keep their frequencies within a narrow range for sex recognition. Moreover, bats adjusted resting frequency to humidity, which may imply partitioning diet by prey size or the influence of rainfall noise. The results indicate that bats adjust echolocation call frequency to adapt to environmental conditions. Therefore, environmental selection shape the diversity of echolocation call structure of *R. pusillus* in geographically separated populations, and conservation efforts should focus on changes in local climate and effects of environmental noise.

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I. INTRODUCTION

Among the different variations observed, geographical variation in acoustic signals are a very valuable material for clarifying a wide variety of factors shaping the evolution and divergence of communication systems and for testing fundamental hypotheses about the evolution of behavior (Wilczynski and Ryan, 1999). The geographic differences could promote population divergence and ultimate speciation because they are related to variability in mating signals, communication and resource use (Kingston and Rossiter, 2004; Patten *et al.*, 2004).

The primary functions of echolocation in bats are object detection and localization (Griffin, 1958; Jones and Holderied, 2007). However, mounting evidence suggests that echolocation is important for acoustic communication as well (Ma *et al.*, 2006; Chiu *et al.*, 2008; Jones, 2008; Kazial *et al.*, 2008; Yovel *et al.*, 2009). Such acoustic signals must relay different information and thus have a high degree of plasticity (Obriest, 1995). As a consequence, geographic variations in intraspecific communication signals between populations are expected to be common.

In geographically separated populations, variability in bat echolocation calls has been observed in response to a variety of conditions, including geographical barriers (O’Farrell *et al.*, 2000; Davidson and Wilkinson, 2002), morphological differences between populations (Heller and von Helversen, 1989; Parsons, 1997; Francis and Habersetzer, 1998; Barclay *et al.*, 1999; Guillén *et al.*, 2000; Law *et al.*, 2002; Aspetsberger *et al.*, 2003; Yoshino *et al.*, 2006; Armstrong and Coles, 2007), sex dimorphism in call structure (Jones *et al.*, 1992; Jones *et al.*, 1993; Francis and Habersetzer, 1998; Guillén *et al.*, 2000), character displacement or release (Guillén *et al.*, 2000; Russo *et al.*, 2007), humidity (Guillén *et al.*, 2000), vegetation types (Barclay *et al.*, 1999; Denzinger *et al.*, 2001), habitat acoustics (Gillam and McCracken, 2007), and genetic and cultural drift (Guillén *et al.*, 2000; Jones and Holderied, 2007; Yoshino *et al.*, 2008; Chen *et al.*, 2009).

Although some factors have been shown to explain variations in the ultrasonic frequency of bat echolocation calls within species across geographically dispersed populations, both the cause and the meaning of such differences remain little understood, especially with in bats of the families Hipposideridae and Rhinolophidae. These bats produce calls dominated by an extremely stable constant frequency

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(CF) tone that is matched to their acoustic fovea (Schnitzler *et al.*, 1976; Schuller and Pollak, 1979; Suga *et al.*, 1987; Kingston *et al.*, 2001). The differences in the CFs between populations in some horseshoe and roundleaf bats may be promoted by geographical barriers, such as in *Rhinolophus ferrumequinum* (Taniguchi, 1985; Heller and von Helversen, 1989; Huihua *et al.*, 2003), *Hipposideros cervinus* (Francis and Habersetzer, 1998), and *Hipposideros ruber* (Guillén *et al.*, 2000). In addition, recent studies indicated that mother-offspring transmission of CF (see Jones and Ransome, 1993) combined with a restricted dispersal of females between regions and small effective population size may maintain intercolony acoustic differences in *Rhinolophus cornutus pumilus* even within a relatively small geographical area (Yoshino *et al.*, 2008) and *R. monoceros* (Chen *et al.*, 2009).

So far, however, few studies found an effect of climate on geographical variation in the frequency of bat echolocation calls. Bats have been identified as an important bioindicator taxa for measurable responses to climate change (Jones *et al.*, 2009). Climate change can be related to shifts in bat numbers or activity (Jones *et al.*, 2009). Echolocation call structure also may have been influenced by climate conditions. Body temperature, which is to a certain degree affected by ambient temperature, is associated with call frequency in some species (Huffman and Henson, 1991). Additionally, Guillén *et al.* (2000) suggested that humidity can influence the call frequency of *H. ruber* through its effect on atmospheric attenuation of acoustic signals. In the present study, we therefore hypothesized that climatic factors would influence geographic variation in call frequency of horseshoe bats, especially at a broad geographical scale.

In this paper, we report intraspecific patterns of variation in the resting frequency (RF) of *Rhinolophus pusillus* across Mainland China and attempt to identify factors underlying this variation in RF. The results of our geographical analysis led us to test for the existence of: (1) a constraint of body size on RF, (2) sexual dimorphism of RF, (3) variation of RF associated with geographical distances, and (4) adaptation of RF to local climate conditions and implication for conservation.

II. MATERIALS AND METHODS

A. Bat species and geographical distribution

The least horseshoe bat, *Rhinolophus pusillus*, is one of the smallest rhinolophids in the world, and is widely distributed in the Indomalayan region (Corbet and Hill, 1992; Csorba *et al.*, 2003; Simmons, 2005). In Mainland China, the species is found in South and Southwest China, including the provinces of Xizang, Sichuan, Guizhou, Fujian, Guangdong, Guangxi, Yunnan, Hainan (Wang, 2003; Simmons, 2005). It has recently also been recorded in some provinces of Northern China, such as Beijing, Hubei, Jiangxi and Shandong (Zhang *et al.*, 2007). We focused our collection efforts mainly on South China, but also included some samples from the North China.

FIG. 1. Study area showing sampling localities of *Rhinolophus pusillus* analyzed in this study.

B. Data collection and sound analysis

A total of 199 bats were collected from June 2006 to September 2008 at 11 locations, covering most of the distribution range in Mainland China (Fig. 1). At each site, we captured bats at caves using mist nets. To control for possible effects of age (Jones and Ransome, 1993; Masters *et al.*, 1995), only the morphology and the calls of adult bats were measured and recorded. A bat was identified as adult when the phalangeal epiphyses were fused with the diaphyses (Racey, 1974). For each bat, we measured forearm length (FAL; to within ± 0.01 mm) using digital calipers and determined its sex. For each location, we also determined latitude, longitude and elevation using GPS (eTrex Vista).

Bat echolocation calls were recorded with a real-time ultrasonic detector (UltraSoundGate 116, Avisoft Bioacoustics, Berlin, Germany) positioned approximately 30 cm in front of the hand-held bats, and sounds stored on a laptop computer. The sampling frequency was 441 kHz. The 'resting frequency (RF)' that the bats emitted under these recording conditions is the individually characteristic and stable CF portion of the echolocation pulses emitted by the motionless bat, which matches the bat's 'acoustic fovea' (Schuller and Pollak, 1979).

From these recordings, we selected high-quality calls from call sequences of each individual to determine their resting frequency according to the following criteria: (1) initial calls within a call series were not considered for analysis, because such calls may show transient, lower frequency values before reaching the final RF level (Siemers *et al.*, 2005); (2) when calls were emitted in groups (doublets, triplets, etc.), only the second call per group was chosen (Russo *et al.*, 2001); (3) only calls with CF portions longer than 10 ms were analyzed. We obtained acoustic measurements from spectrograms using a 1024-point fast Fourier transform (94% overlap, spectral resolution: 195 Hz). The bats emitted typical rhinolophid echolocation calls, composed of three harmonics, with the first and third harmonic being weaker than the second (Fig. 2). From each individual, we selected 30–40 of the highest-quality calls and measured the frequency of the

FIG. 2. Sonogram (a) and power spectrum (b) of a typical echolocation call of *Rhinolophus pusillus*. Maximum energy is in the second harmonic around 105 kHz, which is used as the information carrier.

CF component in the dominant second harmonic using Avisoft SasLab Pro (Avisoft Bioacoustics, Berlin, Germany).

Climatic differences in habitats can be reflected in differences in mating songs in a song bird (Ruegg *et al.*, 2006), and echolocation call frequency may be correlated with climate conditions (Guillén *et al.*, 2000). Therefore, we also included climatic factors to describe environmental variation across our eleven locations. To test for correlations between observed call frequency and climate conditions at different locations, we obtained climate information for each collection site from the China Meteorological Data Sharing Service System (<http://cdc.cma.gov.cn/shuju>), including mean annual temperature (MAT), mean annual rainfall (MAR), mean annual atmospheric pressure (MAAP), and mean annual wind velocity (MAWV) (Table I). In the present study, the geographic distance between the sampled localities and the weather stations ranged from 5 to 40 km.

C. Statistical analysis

We conducted a univariate one-way ANOVA of the averaged call frequency values obtained at each location to determine if call frequencies differed between locations. In this analysis, data from females and males were initially tested separately, and then combined for each site. We performed a *t* test to evaluate gender differences in RF in each

location. The same approach was used to determine FAL differences between the sexes at each location and differences between locations.

Because the distribution of both RF and FAL did not depart significantly from normality (Kolmogorov–Smirnov test, $P > 0.05$), we conducted general linear models (GLM) for the analyses with type III sums of squares to develop the test in the SPSS statistical package. Full models were built initially, but they were subsequently simplified by removing non-significant terms.

Variation in the RF related to the sexual composition of populations and geographical structure found at the locations was studied with a linear model that included SEX and LOCATION as fixed effects. Since changes in RF may be promoted by variation in FAL, we used FAL as reference for assessing the importance of variation in RF. Therefore, a GLM with the same structure as the one presented above was selected for studying variation in FAL. In addition, an ANCOVA model was used to determine the effects of FAL to changes in RF. In this model, the means of RF and FAL per location (Table II) were the dependent variable and covariate, respectively, and LOCATION and SEX were the main fixed effects.

To assess whether differences in RF were related to geographic distance, we first calculated a dissimilarity matrix of acoustic distances using RF differences in kHz between locations. We then calculated a geographical distance matrix from the latitude and longitude of each location, and compared the acoustic and geographical distance matrices using a nonparametric Mantel test of matrix association (Mantel, 1967; Schneider *et al.*, 2000) in Arlequin (Schneider *et al.*, 2000). The Mantel test was performed with 1000 permutations.

To test if climate conditions may have influenced echolocation call frequency, we first implemented a principal component analysis on the four climate variables (mean annual temperature, MAT; mean annual rainfall, MAR; mean annual atmospheric pressure, MAAP; mean annual wind velocity, MAWV; see above and Table I), and used the first two PC factor scores to obtain a matrix of Euclidean distances. This climate matrix was then compared to the previously

TABLE I. Latitude, longitude, and elevation of collection sites (listed from North to South) and corresponding climate data. MAT: mean annual temperature, MAR: mean annual rainfall, MAAP: mean annual atmospheric pressure, MAWV: mean annual wind velocity.

Location	Latitude (°N)	Longitude (°E)	Elevation (m)	MAT (°C)	MAR (m)	MAAP (kPa)	MAWV (m/s)
Tongbai, Henan	32.81	113.40	250	15.3	1.11	100.33	2.4
Yixing, Jiangsu	31.25	119.78	59	15.5	1.6	101.62	2.5
Jingxian, Anhui	30.71	118.41	73	16.8	1.47	101.41	3.1
Hangzhou, Zhejiang	30.12	120.03	165	16.5	1.45	101.17	2.3
Wulong, Chongqing	29.27	107.84	350	14.9	1.33	93.96	0.8
Anshun, Guizhou	26.24	105.97	1368	15.4	1.12	89.39	2.1
Pingtang, Fujian	25.45	119.72	43	19.8	1.39	100.53	2.6
Xingan, Guangxi	25.41	110.68	417	18.9	1.92	99.51	2.5
Shaoguan, Guangdong	24.77	113.56	147	20.4	1.58	100.66	1.5
Hekou, Yunnan	22.83	103.57	123	18.7	0.86	86.85	3.3
Nanning, Guangxi	22.82	108.71	100	21.8	1.31	99.79	1.3

TABLE II. Resting frequency (RF) in echolocation calls and forearm length (FAL) of adult female (F) and male (M) *Rhinolophus pusillus* from 11 locations in China. Values are given as mean $\pm$ SD. The results of the T -test for sexual difference are shown as DIF. Significant levels are ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; and —, $P > 0.05$. In addition, the results of one-way ANOVA for difference between locations.

Location	N		RF (kHz)			FAL (mm)		
	F	M	F	M	DIF	F	M	DIF
Tongbai, Henan	10	8	106.72 $\pm$ 0.47	106.65 $\pm$ 0.42	*	40.46 $\pm$ 0.32	39.71 $\pm$ 0.41	*
Yixing, Jiangsu	6	12	105.85 $\pm$ 0.49	107.27 $\pm$ 1.47	***	39.61 $\pm$ 0.36	38.31 $\pm$ 0.94	—
Jingxian, Anhui	13	6	109.75 $\pm$ 0.80	110.03 $\pm$ 0.10	***	39.68 $\pm$ 0.85	39.49 $\pm$ 0.38	—
Hangzhou, Zhejiang	5	5	108.16 $\pm$ 0.90	107.12 $\pm$ 0.16	**	39.51 $\pm$ 1.47	36.22 $\pm$ 1.37	**
Wulong, Chongqin	6	8	107.54 $\pm$ 0.55	105.86 $\pm$ 1.03	***	36.37 $\pm$ 2.14	36.31 $\pm$ 0.96	—
Anshun, Guizhou	6	18	107.22 $\pm$ 0.14	104.44 $\pm$ 0.13	***	37.04 $\pm$ 0.27	37.41 $\pm$ 0.76	—
Pingtian, Fujian	10	11	109.75 $\pm$ 0.26	107.15 $\pm$ 1.10	***	36.51 $\pm$ 1.83	36.55 $\pm$ 0.87	—
Xingan, Guangxi	10	6	113.84 $\pm$ 0.13	110.54 $\pm$ 0.37	***	37.60 $\pm$ 0.55	37.17 $\pm$ 1.26	—
Shaoguan, Guangdong	6	6	109.88 $\pm$ 0.11	109.28 $\pm$ 0.14	***	38.05 $\pm$ 0.06	35.53 $\pm$ 0.12	***
Hekou, Yunnan	10	16	104.12 $\pm$ 0.44	103.95 $\pm$ 0.91	***	37.50 $\pm$ 1.00	36.72 $\pm$ 1.06	—
Nanning, Guangxi	11	10	108.19 $\pm$ 0.91	105.96 $\pm$ 0.78	***	37.08 $\pm$ 0.81	35.00 $\pm$ 2.35	*
One-way ANOVA	93	106	$F_{10}=1796.72$ $P < 0.001$	$F_{10}=1060.82$ $P < 0.001$		$F_{10}=9.31$ $P < 0.001$	$F_{10}=8.58$ $P < 0.001$	
One-way ANOVA	199		$F_{10}=1152.46$, $P < 0.001$			$F_{10}=13.70$, $P < 0.001$		

calculated dissimilarity matrix of acoustic distances by a Mantel test as described above. Additionally, a stepwise multiple regression model was performed to test the relationship between RF and climate variables with variables entered and removed at the 0.05 and 0.10 significance level, respectively. In this analysis, RF of females, males, and all individuals (both sexes) was the dependent variable, respectively, and climatic factors were the independent variables.

III. RESULTS

From the 11 sampled locations, we analyzed 6077 calls from 199 individuals with an average of 30 calls per bat (range 22–40 calls/bat). For the entire sample, RF ranged from 104.10 to 113.90 kHz (SD=1.58) in females and from 101.50 to 111.30 kHz (SD=1.78) in males. FAL ranged from 31.71 to 41.51 mm (SD=1.77) in females and from 31.00 to 40.12 mm (SD=1.49) in males. For RF as well as FAL and for both sexes, the variation within locations was generally smaller than between locations (Table II).

The RF values of the bats differed significantly between locations for both genders (ANOVA: $F_{10}=1796.72$, $P < 0.001$, in females; $F_{10}=1060.82$, $P < 0.001$, in males; $F_{10}=1152.46$, $P < 0.001$, for all samples) (Fig. 3). There were also significant differences in FAL between locations (ANOVA: $F_{10}=9.31$, $P < 0.001$, in females; $F_{10}=8.58$, $P < 0.001$, in males; $F_{10}=13.70$, $P < 0.001$, for all samples) (Table II). For each site, the mean value of RF was consistently higher in females than in males except for Yixing in Jiangsu province and Jingxian in Anhui province, and this difference was statistically significant for each site (Fig. 3). Similarly, mean FAL was significantly larger in female than in male bats in four locations (Table II).

LOCATION and SEX had a significant effect on RF (Table III). There were significant differences between locations with colonies in Xingan (Guangxi province) and Hekou (Yunnan province) showing the highest and lowest RF val-

ues, respectively (Table II; Fig. 3). There was a distinct sexual dimorphism in RF: female used higher pitched calls in all locations except at Yixing in Jiangsu and Jingxian in Anhui, where the dimorphism was reversed (Table II; Fig. 3). Moreover, the prevalence of this dimorphism changed among populations (SEX $\times$ LOCATION term significant) (Table III).

Similarly, LOCATION and SEX had a significant effect on FAL, although to a lesser degree than for RF (Table III). Females had significantly longer forearms in four locations, but there were no significant difference in the other seven locations (Table III).

FIG. 3. Variation in resting frequency (RF) of echolocation calls among individuals of *Rhinolophus pusillus* between locations. Each triangle indicates the mean call frequency of an individual, and bars represent mean $\pm$ SD. The sample sizes were showed in Table II. Abbreviation: TH, Tongbai, Henan; YJ, Yixing, Jiangsu; JA, Jingxian, Anhui; HZ, Hangzhou, Zhejiang; WC, Wulong, Chongqin; AG, Anshun, Guizhou; PF, Pingtian, Fujian; XG, Xingan, Guangxi; SG, Shaoguan, Guangdong; HY, Hekou, Yunnan; NG, Nanning, Guangxi. The arrangement of these locations from left to right is according to latitude from North to South.

TABLE III. Effect of SEX and LOCATION on the resting frequency (RF) and forearm length (FAL) of *Rhinolophus pusillus* in China, respectively. Tests were built with type III sum of squares. SEX and LOCATION were considered as fixed effect. The two models are significant and explain a large proportion of data variance ($F_{21}=1493.861$, $P<0.001$, $r^2=0.838$, in RF; and $F_{21}=10.545$, $P<0.001$, $r^2=0.654$, in FAL).

Effect	RF				FAL			
	df	MS	F	P	df	MS	F	P
SEX	1	788.787	1357.223	<0.001	1	25.437	21.08	<0.001
LOCATION	10	1004.336	1728.117	<0.001	10	17.883	14.82	<0.001
SEX×LOCATION	10	166.354	286.237	<0.001	10	3.395	2.814	<0.01
ERROR	6055	0.581			177	1.207		

Forearm length cannot explain variation in mean RF across locations (FAL: $F_1=1.56$, $P=0.246$) (Table IV). Although the FAL×SEX term had a significant effect on RF, this was promoted only by SEX because FAL did not have a significant effect on RF (Table IV).

In our study, there was a weak relationship between differences in RF and geographical distances between locations although they lacked sufficient statistical power to detect it [Mantel test: $r=0.18$, $P=0.09$; Fig. 4(a)].

Interestingly, however, resting frequency differences were positively and significantly correlated with climate conditions [Mantel test: $r=0.45$, $P=0.007$; Fig. 4(b)]. From the four climate variables, multiple regression models showed that only MAR had a positive and significant effect on call frequency of females, males and all individuals (Fig. 5). Therefore, the other three climatic variables were removed in this analysis.

IV. DISCUSSION

In rhinolophid and hipposiderid bats, females normally call at a higher frequency than males (Neuweiler *et al.*, 1987; Jones *et al.*, 1992; Guillén *et al.*, 2000; Yoshino *et al.*, 2006; Yoshino *et al.*, 2008), but there are exceptions to this rule (Jones, 1995; Pedley, 2004), as was also evident from our data for RFs in the least horseshoe bat, in which males broadcasted higher RFs than females at two locations, i.e., in Yixing (Jiangsu province) and Jingxian (Anhui province) where (Table II and Fig. 3). Gender differences in RF may be based upon sex dimorphism in body size rather than directly gender. In our study, SEX had a significant effect on RF and FAL, respectively (Table III), whereas FAL had no significant effect on RF despite the fact that the FAL×SEX term had a significant effect on RF (Table IV). Moreover, females

of *R. pusillus* used higher pitched sounds than males (except for the two locations mentioned above; Table II and Fig. 3), but there was no significant difference between female FAL and male FAL. In addition, in only 4 of the 11 locations, there was a significant difference in FAL between the sexes (Table II). As a result, sex dimorphism in RF of *R. pusillus* does not appear to be associated with FAL. In addition, many studies also showed that intraspecific shifts in the CF value were independent of differences in body size (Neuweiler *et al.*, 1987; Jones *et al.*, 1992; Jones *et al.*, 1994; Francis and Habersetzer, 1998; Guillén *et al.*, 2000). Therefore, it ap-

FIG. 4. Relationship of RF difference with (a) geographical distance and (b) climate Euclidean distance. Points represent all possible pairwise comparisons of the 11 collection sites.

TABLE IV. Effect of SEX, LOCATION and FAL on the resting frequency (RF) of *Rhinolophus pusillus* in China. Tests were built with type III sum of squares. SEX and LOCATION were considered as fixed effect, and FAL was covariate. The model is significant and explains a large proportion of data variance ($F_{13}=13.211$, $P<0.001$, $r^2=0.883$).

Effect	df	MS	F	P
SEX	1	5.514	8.420	<0.05
LOCATION	10	10.184	15.551	<0.001
FAL	1	1.024	1.564	0.246
FAL×SEX	1	4.98	7.655	<0.05
ERROR	8	0.655

FIG. 5. Relationships between mean annual rainfall (MAR) and resting frequency (RF).

pears that body size does not constrain geographic variation of RF in these bats.

Sexual dimorphism in echolocation calls may have an important social function by signaling the sex of the caller, and therefore promote mate recognition and reproductive success (Guillén *et al.*, 2000). Kazial and Masters (2004) showed that echolocation calls of bats can be used for sex-recognition, but direct empirical evidence showing that call frequency is a critical cue for sex recognition is virtually absent. Here, we found that the mean RF between females and males in each location was distinctly different (Table II and Fig. 3), which may indicate that females and males at each site keep their frequencies within a narrow range for sex recognition.

The substantial variation in RF of *R. pusillus* that we found here corroborates reports in other rhinolophids (Guillén *et al.*, 2000; Yoshino *et al.*, 2006; Chen *et al.*, 2009). We observed significant differences in RF between locations for females, males as well as all samples combined (Tables II and III). These differences were only weakly correlated with geographical distance between locations (Fig. 4). Similar to our results, several reports suggested that geographical distance cannot, however, always and completely explain the variation in call structure of bats (Guillén *et al.*, 2000; Murray *et al.*, 2001; Law *et al.*, 2002; Aspetsberger *et al.*, 2003; Armstrong and Coles, 2007; Gillam and McCracken, 2007).

Acoustic divergence in RF by more than 5 kHz between populations of CF rhinolophoid and hipposiderid bats separated by geographical barriers may be common (Heller and von Helversen, 1989; Francis and Habersetzer, 1998; Guillén *et al.*, 2000). Some populations that are geographically separated by large distances also show large differences in RF (e. g. *R. ferrumequinum* between Europe and Japan, Heller and von Helversen, 1989; *Hipposiders fulvus* between India and Malaysia; Jones *et al.*, 1994). In this study, although geographical distance was only weakly associated with RF difference between locations, we cannot completely rule out a potential impact of geographical distance for the following reasons: First, numerous high mountain ranges form a network of barriers within Mainland China, which may restrict

gene flow. Second, least horseshoe bats have short, broad wings suitable for maneuverable flight but not for long-distance migration (Maeda, 1978). This may severely restrict seasonal migration in *R. pusillus*. Finally, the geographic distance between sampling localities were so large (in some cases over 1000 km) that vicariance may obscure any pattern of geographical distance mediated by dispersal. This so far suggests that genetic or cultural drift and natural selection could play an important role in promoting geographical variation in RF of *R. pusillus*.

Cultural drift has been suggested to yield variations in call frequency in the maternal transmission hypothesis. Cultural learning of call frequencies has been reported in the genus *Rhinolophus*, determining the fine-tuning of call frequencies in part by vertical transmission from mother to offspring (Jones and Ransome, 1993), a view subsequently supported by Chen *et al.* (2009) and Yoshino *et al.* (2008). Yoshino *et al.* (2008) suggested that call frequency difference of on average 5–8 kHz in *R. cornutus* across its area of distribution have developed through random cultural drift. Although in our study, the mean call frequency difference in *R. pusillus* was within a similar range and females exhibited a restricted dispersal between regions, differences in RF was not likely subject to cultural drift because of the three following reasons. First, variation in RF lacked any geographical pattern (Fig. 3), which is different from a cultural drift, in which variations should be geographically directed. Second, RF differences were only very weakly correlated with geographical distance. Finally, numerous high mountain ranges separating the different, distant locations paired with a limited flight capability of *R. pusillus* should make female dispersal between locations unlikely at a geographic scale as wide as seen in our study. However, only by determining a direct genetical basis for RF can this question be resolved.

As an alternative hypothesis, natural selection will adapt a population to a particular local environmental condition but immigrants from other populations will introduce genes adapted to other conditions (Slatkin, 1987). A number of studies demonstrated that differences in the structure of bat echolocation calls can occur in response to a variety of environmental conditions, including humidity (Jones *et al.*, 1993; Guillén *et al.*, 2000), vegetation types (Barclay *et al.*, 1999; Denzinger *et al.*, 2001) and habitat acoustics (Gillam and McCracken, 2007). Our own results also revealed that a particular climatic condition, i.e., MAR, was significantly and positively correlated with RF in *R. pusillus*. This implies that environmental selection plays an important role in shaping the geographic pattern of RF of *R. pusillus*, especially at a wide geographic scale.

Interestingly, we found that RF was positively correlated with the mean annual rainfall (MAR) for each sex and all individuals (Fig. 5). This is opposite to what would be expected since higher humidity should favor lower frequencies because of the lower attenuation for signals with longer wavelengths (Lawrence and Simmons, 1982; Hartley, 1989). This inverse correlation was reported by Guillén *et al.* (2000), who showed that MAR was negatively correlated with call frequency in *H. ruber*. The unexpected positive correlation between high humidity and high call frequency in

our observations may have three possible causes. First, Remmert (1981) showed that moist environments contain a greater number and, on average, smaller insects than dry environments. This suggests that *R. pusillus* may forage on smaller insects by using a higher RF in wetter locations. However, the range of CF frequencies recorded in *R. pusillus* corresponds to wavelengths between 3.03 and 3.33 mm, assuming a sound speed of 340 m/s, a temperature of 22 °C, and a relative humidity of 80%. Such small differences in wavelength, however, seem inappropriate to interpret the intraspecific variations as a basis for partitioning diet by prey size (Russo *et al.*, 2001). Second, Chen *et al.* (2009) suggested for different populations of *Rhinolophus monoceros* in Taiwan, that any correlation between humidity (ranging between 76% and 86%) and call frequency may merely represent an artifact based on differences in the genetic population identity. In our study, however, the differences in MAR between sampling localities are so large (range between 0.86 m and 1.92 m; Table 1) that the effect of population identity seems unfeasible. Finally, in general, a larger MAR corresponds to higher humidity (Guillén *et al.*, 2000), but noise effects induced by the rainfall have so far always been neglected. It is therefore tempting to speculate that noise caused by rainfall may also interfere with the bats' echolocation performance. There is mounting evidence implicating environmental noise in changes of echolocation call frequency in frequency modulated (FM) bats (Rydell *et al.*, 1999; Gillam and McCracken, 2007; Schaub *et al.*, 2008) and even constant frequency (CF) bats (Hage *et al.*, 2009). However, obviously only further experimental examination will help in answering this paradoxical relation between call frequency and humidity.

Bats are sensitive to the effects of environmental stressors, such as direct exploitation, habitat loss and climate change (Jones *et al.*, 2009). Global climate change, like extremes of drought, heat, cold and precipitation, are linked to changes in bat numbers or activity (Jones *et al.*, 2009). Therefore, local climatic conditions may influence physiological reactions of bats and thus lead to changes in the echolocation behavior of bats, which ultimately impacts bat numbers and even their survival. Echolocation calls in bats, possessing both echolocation and communication functions (Ma *et al.*, 2006; Jones, 2008), are the basis for survival and reproduction. Numerous evidence showed that foraging behavior and acoustic communication signals of bats were altered by ambient noise, such as noise caused by wind, rain, insect sounds, or anthropogenic noise (Arlettaz *et al.*, 2001; Gillam and McCracken, 2007; Jones, 2008; Schaub *et al.*, 2008). In the present study, RFs in *R. pusillus* apparently adapted to local climatic conditions, especially MAR, underscoring the importance for conservation efforts to also focus on local climate changes and environmental noise.

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