



Geographic variation and discrimination of social calls in male great himalayan leaf-nosed bats: do functionally similar calls have similar patterns?

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Abstract

Geographical variation in animals' acoustic signals has received much attention. However, few studies have compared the patterns and underlying selective forces driving geographical divergence of vocalizations with similar and different functions within the same species. Also, the social consequences of geographical divergence in acoustic signals are still rather poorly understood. Here we recorded three types of social calls of male great Himalayan leaf-nosed bats (*Hipposideros armiger*) across eight colonies in China. Two calls share similar functions and the third has a function distinct from the other two. We examined the patterns and causes of geographical variation of each of these calls. We found that all three calls had significant geographic variation with similar patterns of spatial variation. Only one of the two social calls with similar functions was found to be affected by genetic drift, while the other two calls were not affected by selection, drift, or morphological constraints. Furthermore, we found that bats could discriminate between vocalizations of their own colony and those of an allopatric colony. Overall, these results suggest that acoustic signals with similar functions may be shaped by different driving forces and acoustic signals with different functions may exhibit similar geographical patterns. This study expands our limited knowledge of the patterns of geographical variation of vocalizations emitted at different emotional states and highlights the importance of comparing simultaneously patterns and causes of geographical divergence of vocalizations with similar and different functions.

Significance statement

This study provides the empirical evidence suggesting that calls with a similar function may fail to co-vary and alternatively calls with a different function may co-vary. Moreover, we show that bats can discriminate between calls of their own colony from those of a foreign colony. These results expand our limited knowledge of the patterns of geographical variation of vocalizations uttered under different emotional states and the nature of functional drivers that influence the evolution of communicative systems.

Keywords Acoustic signals · Bat · Covariation · Call function · Vocal discrimination

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Introduction

Geographical variation in acoustic signals is widespread in the animal kingdom (Lameira et al. 2010; Zhang et al. 2022) and may arise from different evolutionary driving forces, including ecological selection, cultural drift, genetic drift, morphological constraints or a combination of these factors (reviewed in Jiang et al. 2015). Ecological selection is a main driver of acoustic divergence among allopatric populations since sound transmission is affected by local ecological factors such as temperature, humidity and ambient noise (Jiang et al. 2010a, b; Sun et al. 2013; Luo et al. 2014). Variation in acoustic signals can also be caused by cultural drift which generates random variations between signal variants that have been learned (Slater 1986). Genetic drift can result in geographical variation in vocalizations due to random changes in the genetic loci related with vocal structure, brain neuroanatomy or vocal ontogeny (Podos and Warren 2007). Moreover, spectral parameters of acoustic signals can be influenced by an animals' morphological size. Generally, large individuals tend to have larger lung volumes and longer vocal folds, permitting them to emit relatively long-duration and low-frequency sounds compared to small individuals (Fitch and Hauser 2003).

Geographical variation in acoustic signals may affect signal recognition and discrimination among reproductive individuals from different populations, leading to assortative mating, reproductive isolation, and speciation (Wilkins et al. 2013). This has been shown in many taxonomic groups, i.e., insects, frogs, birds, and mammals (Henry 1994; Boul et al. 2007; Braune et al. 2008; de Oliveira Gordinho et al. 2015). Before determining the consequences of geographical divergence in animal acoustic signals, the primary question that needs to be addressed is whether animals can discriminate differences in acoustic signals among allopatric populations (Milligan and Verner 1971). Sound playback experiments are often used to solve this question by observing individual responses to playbacks of signals from their own population and from allopatric populations (Charrier et al. 2013; Lin et al. 2016; Ahonen et al. 2014).

Acoustic signals with distinct functions have been hypothesised to exhibit discordant geographical distributions due to different evolutionary pressures that affect signal evolution (Byers 1996; Baker 2011; see Armbruster and Schaeffer 1996 for a general discussion). This hypothesis has been substantially tested in bird species. In several species of birds with multiple song types, song types that have similar functions tend to have similar patterns of geographical variation (Byers 1996; Kroodsma 1996; Beebe 2002; Soha et al. 2016), and vocalizations with different functions may vary discordantly among populations (Baker 2011). Previous studies primarily focused on multiple vocal types

produced in different social contexts. In contrast, little is known about the patterns of geographical variation of the same vocal type emitted at different physiological/emotional states. An emotion is a complex, subjective experience that arises in response to internal or external stimuli and is characterized by a range of psychological and physiological reactions (Briefer 2012). Emotions play a vital role in an animal's life and receivers may benefit from a caller's vocalizations that encodes emotional state, because they can make appropriate decisions based on the signaled information (Briefer 2012, 2018). Moreover, few investigations have compared patterns of geographical divergence of social calls with both similar and different functions (Baker 2011).

Echolocating bats are known to produce two types of sounds for activity in dark environments, namely echolocation calls and social calls (Gillam and Fenton 2016). There are three main differences between them: (1) function: echolocation calls are to sense the environment such as orientation and prey detection, while social calls are to influence another animal; (2) receiver: the receiver of the echolocation calls is the senders themselves while the receiver of the social calls is other individuals; (3) structure: echolocation calls are typically high-pitched and short in duration while social calls exhibit more complex and diverse acoustic features (Chaverri et al. 2018; Luo et al. 2019). To the best of our knowledge, only five studies investigated geographical variation in social calls (Boughman and Wilkinson 1998; Esser and Schubert 1998; Davidson and Wilkinson 2002; Montero et al. 2018; Sun et al. 2020) and two of them have explored underlying driving forces of acoustic divergence in social calls in bats (Montero et al. 2018; Sun et al. 2020). Only one report on the Spix's disc-winged bats (*Thyroptera tricolor*), compared the patterns and causes of geographical variation of two contact calls (INQ: inquiry call; LUM: long upward modulated call) that had similar functions (Montero et al. 2018). Montero et al. (2018) found that both spatial and genetic factors are the main drivers of acoustic differentiation in LUM calls while these factors do not explain the acoustic divergence of INQ calls for the entire population. Note that social calls could potentially be emitted by individuals in different emotional states but could nonetheless have similar functions. Bat's calls could also be emitted in different social contexts and therefore have different functions. The details of these associations are poorly known even within populations. As such, the geographical patterns across populations are to date at best unclear.

The great Himalayan leaf-nosed bat, *Hipposideros armiger*, is a nocturnal and highly social species with a polygynous mating system (Yang 2011). Hundreds of individuals roost in caves where they maintain a minimum distance of 10–15 cm from each other during day-time roosting (Sun et

al. 2019). They defend their day-roost territory using broadband social calls (Sun et al. 2018, 2021). Among the social calls emitted by these bats, two call types are particularly common: bent upward frequency modulated calls (bUFM; Fig. 1a, b; Sun et al. 2018) and stepped upward frequency modulated calls (sUFM; Fig. 1c; Sun et al. 2021). The bUFM calls are produced during a confrontation between two bats that are perched close to each other, accompanied by agonistic acts (Video S1; Sun et al. 2018). The bUFM calls are divided into two subtypes (i.e., L-bUFM and H-bUFM) that correlate with a bat’s level of aggressive intensity (Sun et

al. 2018). We interpret the differential use of these calls as an index of emotional state. L-bUFM calls are emitted at low aggression intensity levels: bared teeth, slightly raised body and/or wings, and H-bUFM calls are emitted at high aggression intensity levels: rapidly flapping wings, punching and/or biting (Sun et al. 2018). In contrast, the sUFM calls are emitted by a roosting individual when a free-flying bat approaches (Video S2; Sun et al. 2021). Thus, the two types of calls have different functions: bUFM calls serve the function of territorial defense while sUFM calls convey warning information (Zhang et al. 2021).

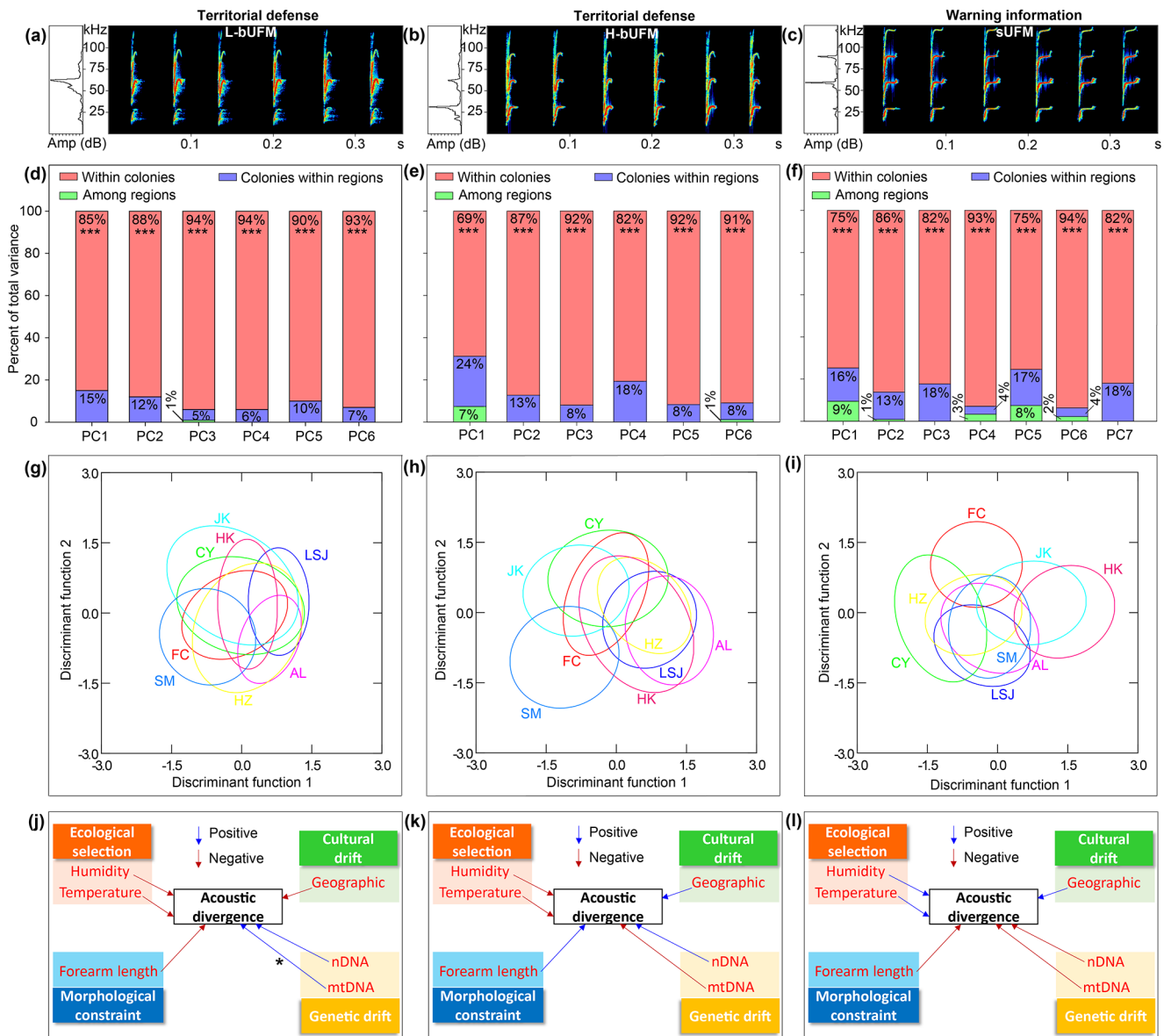


Fig. 1 Sound spectrograms of three call types, pattern and driving forces of geographical variation of three call types in *H. armiger*. The Spectrograms of (a) L-bUFM (bent upward frequency modulated), (b) H-bUFM and (c) sUFM (stepped upward frequency modulated) calls. The acoustic variation of (d) L-bUFM, (e) H-bUFM, and (f) sUFM calls at three levels: the region level, the colony level, and the individual

level. 50% confidence ellipses obtained from discriminant function analyses of (g) L-bUFM, (h) H-bUFM, and (i) sUFM calls. The summary of the effects of ecological selection, cultural drift, genetic drift, and morphological constraint on acoustic divergence for (j) L-bUFM, (k) H-bUFM, and (l) sUFM calls. * $P < 0.05$. Definitions of locality abbreviations are provided in Table 1

Table 1 Colony location, number of calls, temperature, humidity and mean forearm length of bats

Region	Colony	Longitude (°)	Latitude (°)	N_{H-bUFM}	N_{L-bUFM}	N_{sUFM}	MAT (°C)	MARH (%)	FL (mm)
Eastern China (EC)	Fanchang (FC)	118.30	31.05	100	100	100	13.0	90	95.50 ± 2.62 (24)
	Lenshuijiang (LSJ)	111.57	27.75	100	100	100	19.5	99	93.19 ± 2.99 (15)
	Chongyi (CY)	114.10	24.50	100	100	100	25.4	92	94.03 ± 2.49 (33)
Western China (WC)	Anlong (AL)	105.54	25.28	100	100	100	21.4	77	94.06 ± 1.86 (24)
	Jiangkou (JK)	108.83	27.68	100	100	100	17.8	99	95.79 ± 2.29 (34)
	Hanzhong (HZ)	107.03	32.85	100	100	100	17.5	79	96.45 ± 2.24 (96)
Southern Yun-nan (SY)	Simao (SM)	100.71	22.61	100	100	100	18.5	95	95.81 ± 2.45 (88)
	Hekou (HK)	103.91	22.74	100	100	100	21.9	99	95.16 ± 1.75 (46)

N_{H-bUFM} : the number of H-bUFM (bent upward frequency modulated) calls analysed. N_{L-bUFM} : the number of L-bUFM calls analysed. N_{sUFM} : the number of sUFM (stepped upward frequency modulated) calls analysed. MAT: mean annual relative humidity. MARH: mean annual temperature. FL: Forearm length. The numbers in parentheses of FL represent the number of male individuals measured

The aims of this study were to (1) compare the patterns and evolutionary driving forces of geographical variation of *H. armiger* social calls (L-bUFM, H-bUFM and sUFM), and (2) to understand the level of perception of geographical variation in sUFM calls. We only examined the perceptual discrimination abilities for sUFM calls because bUFM calls were tested in our previous study (Sun et al. 2020). First, because functionally similar calls may be shaped by similar evolutionary driving forces and functionally different calls may be shaped by different developmental pathways (Barker 2011), we hypothesized that L-bUFM and H-bUFM call properties would show similar geographical distributions. In contrast, the geographical patterns of sUFM calls should differ from those of L-bUFM and H-bUFM calls. As a test of the hypothesis, we predicted that: (1) the geographical pattern in the distribution L-bUFM and H-bUFM call properties would be similar at three levels (i.e., regional level—between regions; colony level—between colonies within a region; call level—between calls within a colony), and the geographical pattern at all three levels of sUFM call properties should diverge from the geographical pattern of the two bUFM calls; (2) the driving forces of geographical variation in acoustic signals between L-bUFM and H-bUFM calls would be similar, whereas the driving forces of geographical variation in sUFM calls would not be similar to those of bUFM calls. Finally, our previous study showed that *H. armiger* can discriminate between bUFM calls from its own colony and those from a colony from a different region (Sun et al. 2020). We hypothesized that *H. armiger* would also discriminate sUFM calls of its own colony from those of a foreign colony. We predicted that *H. armiger* would display pronounced responses after playback stimuli were switched from sUFM calls from their own colony to those from allopatric colonies in a habituation-dishabituation experiment. Moreover, we predicted that dishabituation would be stronger when a bat is presented with calls from a colony from a different region compared to calls recorded from a colony

within the same region as the home colony of the responding bat.

Methods

Experiment 1: geographical variation of social calls

Sound collection

Bats' bUFM calls were obtained from previous recordings (Sun et al. 2020). Briefly, in April–June of 2015–2016, Sun et al. (2020) captured 80 adult males from eight colonies (10 males per colony) in China on average 896 km apart from each other (270–1978 km between adjacent sties). We only sampled males because their vocalizations play seminal roles in the mating and competitive activities of many bat species (Behr et al. 2006; Fernandez et al. 2014; Zhao et al. 2018). The eight colonies are Simao (SM), Hekou (HK), Hanzhong (HZ), Jiangkou (JK), Anlong (AL), Fanchang (FC), Lengshuijiang (LSJ) and Chongyi (CY) (Fig. 2a; Table 1). For each colony, bats were introduced into a cage for acoustic and behavioral recording for six days. Our previous study showed that the production of these bUFM calls was not influenced by the stress of being in a non-natural environment (cage) (Sun et al. 2018). Vocalizations were recorded with an Avisoft UltraSound-Gate 116 H (Avisoft Bioacoustics, Glienicke, Germany), connected to a condenser ultrasound microphone (CM16/CPMA, Avisoft Bioacoustics) at a sampling rate of 375 kHz and 16-bit resolution. Simultaneously, an infrared camera (HDR-CX 760E; Sony Corp., Tokyo, Japan) was used to record territorial behavior. Our previous study showed that male *H. armiger* emitted two versions of the bent-upward frequency modulation (bUFM) syllable calls during roost territory defence, with each version indicating a different level of aggressiveness (Sun et al. 2018). H-bUFM syllables produced during high aggression intensity interactions had

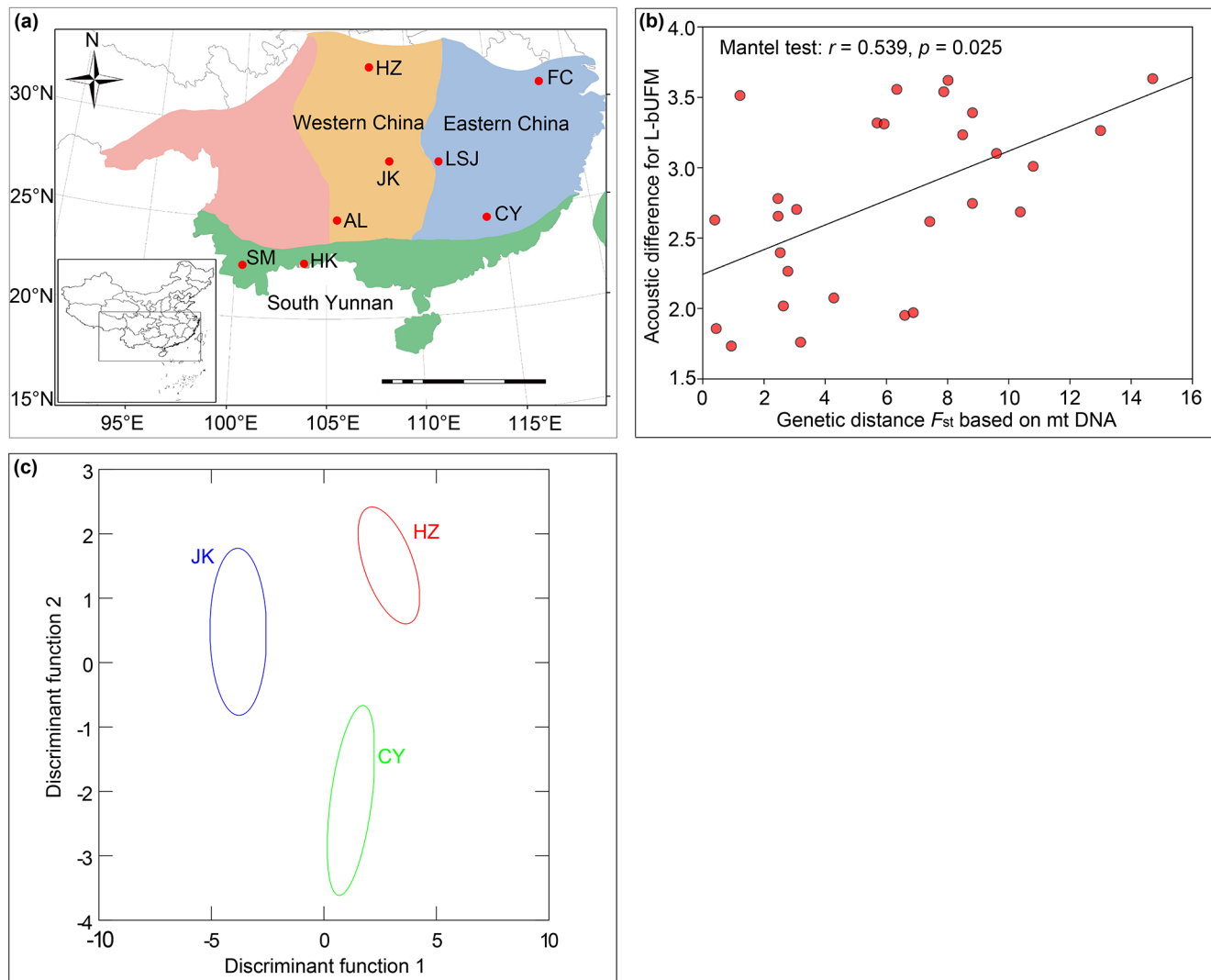


Fig. 2 (a) Map of sampling localities for *H. armiger* in three zoological regions of southern China. Definitions of locality abbreviations are provided in Table 1; (b) Pairwise acoustic differences for L-bUFM (bent upward frequency modulated) vs. genetic distance based on mtDNA;

(c) Ellipses showing the 50% confidence interval obtained from a discriminant function analysis of four principal component factor scores measured from sUFM (stepped upward frequency modulated) calls. Definitions of locality abbreviations are provided in Table 1

lower minimum frequencies and wider bandwidths, compared to the L-bUFM syllables produced during low aggression intensity interactions (Sun et al. 2018). Both bUFM syllable call types were recorded.

Bats' sUFM calls from the above eight colonies were recorded in April–June of 2015–2016. We recorded bats' sUFM calls in caves between 16:30 and 20:00. Our previous observations in the field and in the lab showed that the sUFM calls are produced by a roosting male resident when a free-flying male intruder approaches (Video S2; Sun et al. 2021). The sUFM calls were recorded with the same equipment and sound parameter levels used to record bUFM calls. The microphone used to record sUFM calls was placed 3–7 m away from the bat colonies. The location of the microphone was changed daily to minimize the possibility of getting a

recording from the same group of bats every time. Vocalizations were recorded every 1–2 days, making a cumulative record of 8 to 10 days per colony.

Sound analysis

We analyzed vocalizations using Avisoft-SASLab Pro (version 5.2; R. Specht, Avisoft Bioacoustics). Following Kanwal et al. (1994), we defined a syllable as the smallest discrete part of a vocalization and a call as the simplest emission of a whole vocalization consisting of one or more syllables. Following Sun et al. (2018), we used 81 ms as a lower threshold for inter-call interval of bUFM calls. To reduce pseudoreplication in our analyses, only one high-quality (signal-to-noise rate > 30 dB) syllable was selected

from each call sequence. Note that bUFM calls are given in a series of short bouts (Fig. S1a). For each individual, 10 syllables were selected for analysis. We measured acoustic parameters of H-bUFM calls from the first harmonic and L-bUFM calls from the second harmonic because the majority of the H-bUFM syllables energy is distributed in the first harmonic and the majority of energy in the L-bUFM syllables is distributed in the second harmonic (Fig. 1a, b; Sun et al. 2018). Taken together, 100 H-bUFM calls and 100 L-bUFM calls were selected for analysis per colony for a total of 800 calls (Table 1).

Roosting males typically emit only one sUFM vocalization composed of a series of syllables when a free-flying male approaches the roosting male (see Fig. S1b). As with bUFM calls, only one high-quality (signal-to-noise ratio > 30 dB) syllable from each call was selected to avoid pseudoreplication. Because most energy is put into the second harmonic for sUFM syllables (Fig. 1c), we measured each syllable in the second harmonic. Taken together, 100 sUFM calls were selected for analysis per colony for a total of 800 calls (Table 1).

We normalized each syllable to an amplitude of 0.75 V before taking any acoustic measurements. Estimates of frequency parameters were made from spectrograms using a 1024-point fast Fourier transform (Hamming window; 75% frame size; 93.5% overlap; frequency resolution: 0.244 kHz; temporal resolution: 0.256 ms). Measurements of temporal parameters were made from the oscillograms. We measured all frequency parameters at a threshold of 20 dB below the peak spectral amplitude. To describe H-bUFM, L-bUFM and sUFM calls, 23 acoustic parameters were measured including one temporal parameter, one vocal rate and 21 spectral parameters (see Table S1 for the description of the measured variables).

Driving forces for acoustic divergence

To explore the determinants of geographical variation in social calls, we characterized each site using site-specific climate, geographic location, genetic properties (see below), and an individuals' forearm length (an estimate of body size). More explicitly, the mean annual relative humidity (MARH) and mean annual temperature (MAT) for each colony across the years 1971–2010 were obtained via the package RNCEP (Kemp et al. 2012) in R v. 4.2.2 (R Development Core Team 2022). We used GPS (eTrex Vista, Beijing, China) to collect the latitude and longitude of each sampling locality, and then calculated the geographical distance matrices between all sampled localities. We used genetic data of the eight bat colonies from Lin et al. (2015), including partial mitochondrial control region (358 bp), mitochondrial cytochrome b (1140 bp) sequences, and

seven microsatellite loci (nSSR: PE4, P541, P6D12, PT5B2, TE2, CHANG242, and BAM09). Genetic distances among colonies were quantified by calculating pairwise $F_{ST}/(1-F_{ST})$ respectively for concatenated mt DNA sequences and nSSR in Arlequin version 3.5 (Table S2; Excoffier et al. 2005). Finally, we measured the forearm length with a digital vernier caliper (accuracy ± 0.01 mm; 111–101 V-10G, Guanglu Ltd., Shenzhen, China).

Experiment 2: discrimination of sUFM calls

We chose HZ, JK and CY colonies to examine whether *H. armiger* could discriminate sUFM calls from their own colony and those from an allopatric colony. These three colonies were chosen for two reasons. First, there were significant differences in acoustic parameters among the three colonies (Fig. S2). Second, the HZ and JK colonies come from western China and have measurable levels of gene flow between them (Lin et al. 2015). The CY colony comes from eastern China and has a significant genetic differentiation with HZ and JK colonies. Therefore, these three sites provide a test of bat perceptual discrimination abilities of sUFM calls from both nearby and distant colonies.

Sound and behavioral recording and analysis

In 2018 and 2022, we recorded the sUFM calls from HZ, JK and CY colonies in the laboratory. See supplementary materials for detailed recording process. We also measured 23 acoustic parameters using the same measurements as above to describe differences in acoustic parameters between the HZ, JK and CY colonies.

Collection and husbandry of bats

We conducted reciprocal playback experiments on the HZ, JK and CY colonies. In April–June of 2022, 60 adult male *H. armiger* were captured from these three colonies (20 bats per colony). Captured bats from each colony were housed in a flight cage (4.4 m long \times 1.5 m wide \times 1.8 m high) in a local husbandry room (6 m long \times 5 m wide \times 2 m high). The temperature and humidity in the husbandry room were maintained at around 23°C and 60%. An astronomical light timer was used to maintain a 12-h dark/light cycle (dark: 2000–0800 h; light: 0800–2000 h). Bats were given *Zophobas morio* larvae and fresh water, including vitamin and mineral supplements *ad libitum*. All bats were marked with metal rings (Porzana Ltd, East Sussex, UK) on their forearms for individual identification.

Playback stimuli construction

We used Avisoft-SASLab Pro 5.2 to create seven playback files for the HZ, JK and CY colonies, respectively. Each playback file was 60 s long. Playback files were created by randomly mixing calls from five different individuals which belonged to the same colony. At least three of the five individuals were different between any two playback files. To create a playback file, five high-quality sUFM calls (SNR > 30 dB) were selected from each of the five individuals (Table S3, S4, S5). A total of 25 sUFM calls were included in each playback file. The sUFM calls in the playback files were separated by silent intervals of 0.51–38.34 s, mimicking the natural call intervals during approaching encounters (Sun et al. 2021). This design ensures that tested bats recognized playback sUFM calls by colony instead of by individual. We normalized each playback file (250 kHz sampling rate and 16-bit resolution) so that the peak amplitude of the weakest call was about –30 dB. All playback files were high-pass filtered at 2 kHz.

Habituation-dishabituation experiments

Habituation-dishabituation playback experiments were conducted from 20:00 to 06:00 h in a flight cage (2.1 m long × 1.5 m wide × 1.8 m high) lined with sound-absorbing foam (Fig. S3). Bats could fly freely in the flight cage. We placed an infrared camera, an ultrasonic loudspeaker (Ultrasonic Dynamic Speaker, Avisoft Bioacoustics, Glienicke, Germany) and a condenser ultrasound microphone on the sound-absorbing foam board. The loudspeaker and microphone were connected to an ultrasound playback interface (UltraSoundGate player 116) and an ultrasound recording system (Avisoft UltraSoundGate116H), respectively. The bat was hanging in the testing cage and the distance between the tested bat and the microphone, loudspeaker and camera was 1.5 m.

After the tested bat was positioned, a habituation file from their own colony was broadcast until the tested bat habituated (i.e., no echolocation calls, and no body, head, leg or wing movement). After the bat remained habituated for 60 s, we broadcast another file from the same or different colony. After the bat dishabituated for 60 s, we broadcast a control stimulus which consisted of 300 ms pink noise to determine if the bat focused on the playback file rather than being distracted by any sound or started sleeping. We also recorded the behavior of bats for 60 s after dishabituation files were switched off. Behaviors included echolocation calls and head and body movements. If the tested bat responded to the dishabituation and control stimuli, we considered this as evidence that the tested bat could discriminate between the habituation and dishabituation file. If the

tested bat failed to respond to dishabituation stimulus but responded to the control stimulus, we took this as indication that the tested bat did not discriminate between the habituation and dishabituation file.

Each bat was tested with three different types of combinations of habituation and dishabituation file (e.g., for JK colony, JK-JK', JK-CY and JK-HZ; note: JK' is a JK call recorded from a different individual) and was presented randomly with one combination every day. For example, the JK-JK' combination was used as the control group (including 42 unique combinations), and JK-CY or JK-HZ combinations were used as the experimental group (including 49 unique combinations). To avoid pseudoreplication (McGregor 1992), each bat was tested once and received a unique combination of the three playback types (JK-JK', JK-CY and JK-HZ). For each colony, we tested 20 males that generated 60 unique combinations of playback stimuli (three for each of 20 bats). Therefore, a total of 180 playback stimuli were used for 60 tested bats among the three colonies.

Following our previous study (Sun et al. 2020), we counted the number of echolocation calls, head nods and body movements of each bat. Echolocation calls were recorded when a bat produced an echolocation pulse. Head nods were recorded when a bat turned its head to its chest. Body movements were recorded when a bat expanded its wings or rotated its body. To minimize observer bias, all behavioral recording and data analysis were done blind.

Statistical analysis

We used raw data of all acoustic parameters to quantify the acoustic differences between calls within a colony, between colonies within a region and between the three zoological regions (south Yunnan; western China and eastern China; see Fig. 2a). Except for the maximum frequency at the start location of the syllable for L-bUFM, disttymax and minimum frequency at the centre location of the syllable for sUFM (see Table S1), the variables were not normally distributed (Kolmogorov–Smirnov; $P < 0.05$).

We tested whether acoustic parameters for each call type differed between colonies as follows: we first performed a principal component (PC) analysis for the 23 acoustic parameters. We extracted six PCs (with eigenvalues > 1) for H-bUFM calls that explained 81.86% of the total variance (Table S6), six PCs (with eigenvalues > 1) for L-bUFM calls that explained 81.14% of the total variance (Table S7), and seven PCs (with eigenvalues > 1) for sUFM calls that explained 80.52% of the total variance, respectively (Table S8). Subsequently, we conducted Kruskal-Wallis tests for each call type to examine whether each of these PC factor scores of call parameters differed between colonies.

For each call type, we performed a discriminant function analysis (DFA) using the above PC factor scores to determine whether calls could be correctly classified as belonging to the sampled colony. Subsequently, we performed a two-tailed binomial test to determine whether the observed percentage of correct classification was higher than the percentage assuming a random classification.

For each call type, we performed a univariate nested ANOVA based on each PC factor score to examine the differences between calls within a colony (call level), between colonies within a region (colony level) and between regions (region level). This analysis was used to determine the percentage of total variance in each PC which was attributed to region, colony or call differences.

While L-bUFM and H-bUFM calls share the same general function, the latter are emitted under higher aggressive intensity levels than the former. This pattern would indicate that emotion may play a role in the general expression of bUFM calls if between-individual differences in H-bUFM calls were greater than individual differences in L-bUFM calls. To examine whether there were larger inter-individual differences in H-bUFM than in L-bUFM calls, we calculated between-individual coefficients of variation (CV) for each acoustic parameter of L-bUFM and H-bUFM calls. The CV is calculated as $CV = 100(1 + 1/4n)(SD/\bar{x})$ (Robison et al. 1993). In this formula, n represents the sample size of vocalizations, SD the standard deviation of the sample, and \bar{x} the mean value. Larger values of CV indicate higher inter-individual variability.

We calculated the difference in acoustic parameters (as indexed using the PC scores discussed above) for all pairs of colonies. Note that these parameters are derived from colony averages, unlike the parameters discussed above that included call variation. We also generated differences for all pairs of ecological and geographical properties of those colonies. These colony property differences included relative climate (relative humidity and temperature), GPS location, genetic properties (F_{st}), and average size of bats in the colony (based on forearm length). We first performed PC analysis on the colony average values of the 23 acoustic parameters. We extracted three PCs (with eigenvalues > 1) for H-bUFM calls that explained 92.43% of the total variance (Table S9), four PCs (with eigenvalues > 1) for L-bUFM calls that explained 93.06% of the total variance (Table S10), and four PCs (with eigenvalues > 1) for sUFM which explained 93.44% of the total variance (Table S11). Subsequently, we used the PC factor scores of each call type to calculate the acoustic Euclidean distance matrix between colonies. Simultaneously, we calculated a climate distance matrix, a geographical distance matrix, a genetic distance matrix, and a forearm length distance matrix using SPSS (version 20.0; SPSS Inc., Chicago, IL, U.S.A.). Finally,

we tested the relationships between the acoustic Euclidean distance matrix and climatic (relative humidity and temperature), geographic, genetic, and morphological distance matrices using a Mantel test in PASSaGE version 2 with 999 permutations (Rosenberg and Anderson 2011).

To test whether acoustic parameters of sUFM calls recorded in the laboratory differed between the HZ, JK and CY colonies, we first performed a PC analysis on the 23 acoustic parameters. We extracted four PCs (with eigenvalues > 1), which explained 89.97% of the total variance (Table S12). Then, we conducted Kruskal-Wallis tests to examine whether each of the four PC factor scores of call parameters differed between colonies. To determine whether sUFM calls from these three colonies could be correctly classified as the sampled colony, we performed a DFA on the four PCs. Then, a two-tailed binomial test was used to examine whether the observed percentage of correct classification was higher than the percentage assuming a random classification ($1/3 = 33.30\%$).

Pearson chi-square tests were used to examine whether there were significant differences in the proportion of bats that showed any response among the three playback types (e.g., for JK colony, JK-JK', JK-CY and JK-HZ). If there were significant differences, Pearson chi-square tests were further used to determine which playback combinations differed.

We evaluated the potential for habituation and dishabituation using three behavioral measures: number of nods, number of body movements and number of echolocation calls. These three measures were strongly correlated with each other. We therefore ran a principle component analysis on all three behavioral measures which generated a single significant PC axis. We then conducted a one-way ANOVA to compare the PC factor scores among the three types of playback combinations (e.g., for JK colony: JK-JK', JK-CY and JK-HZ) to test for the bat's detection of within-colony, within region, and across region differences in sUFM calls. If there were significant differences, Tukey's multiple-comparison tests were further used to determine which playback combinations differed from one another. We performed a Pearson correlation test to assess the relationship between geographic distances and the strength of responses (PC scores). Average PC scores were calculated for each type of playback combinations.

All statistical analyses were conducted in SPSS 20.0 and R 4.2.2. The significance level was set at $P < 0.05$, and a Bonferroni correction for P -values was used because of multiple tests (Sokal and Rohlf 1995).

Results

Experiment 1: geographical variation of social calls

Patterns of geographical variation of three types of social calls

For each call type, we analyzed 800 calls from eight colonies (100 calls per colony; Table 1 and Table S13). Kruskal-Wallis tests showed that there were significant differences in all PC factor scores of 23 parameters between the eight colonies (H-bUFM: $df=7$, $\chi^2=50.32$ – 181.90 , $P<0.0001$; L-bUFM: $df=7$, $\chi^2=35.66$ – 187.32 , $P<0.0001$; sUFM: $df=7$, $\chi^2=52.15$ – 169.44 , $P<0.0001$). Nested ANOVAs indicated that all PC factor scores differed significantly between colonies within regions (H-bUFM: $F_{5,792} = 9.68$ – 35.60 , $P<0.0001$, Fig. 1d; L-bUFM: $F_{5,792} = 6.11$ – 18.65 , $P<0.0001$, Fig. 1e; sUFM: $F_{5,792} = 5.07$ – 22.97 , $P<0.0001$, Fig. 1f), but not between regions (H-bUFM: $F_{2,5} = 0.09$ – 1.79 , $P=0.26$ – 0.92 , Fig. 1d; L-bUFM: $F_{2,5} = 0.54$ – 2.41 , $P=0.19$ – 0.61 , Fig. 1e; sUFM: $F_{2,5} = 0.34$ – 2.85 , $P=0.15$ – 0.73 , Fig. 1f). Even though call properties significantly differed between colonies, the majority of variation in all PC factor scores was attributed to differences between calls within a colony (H-bUFM: 68.96–92.43% of the total variation, Fig. 1e; L-bUFM: 85.00–94.26% of the total variation, Fig. 1d; sUFM: 74.74–93.61% of the total variation, Fig. 1f). The variation in all PC factor scores of colonies within regions (H-bUFM: 7.44–23.74% of the total variation, Fig. 1e; L-bUFM: 4.81–15.00% of the total variation, Fig. 1d; sUFM: 3.78–18.01% of the total variation, Fig. 1f) and calls within a colony (H-bUFM: 68.96–92.43% of the total variation, Fig. 1e; L-bUFM: 85.00–94.26% of the total variation, Fig. 1d; sUFM: 74.74–93.61% of the total variation, Fig. 1f) were more distinct than that between regions (H-bUFM: 0–7.30% of the total variation, Fig. 1e; L-bUFM: 0–0.93% of the total variation, Fig. 1d; sUFM: 0–9.47% of the total variation, Fig. 1f).

The DFA revealed that 37.0% of the individual H-bUFM calls (Table S14; Fig. 1h), 32.0% of the individual L-bUFM calls (Table S15; Fig. 1g), and 44.40% of the individual sUFM calls (Table S16; Fig. 1i) could be correctly assigned to their colony. The percentages of correct classification for all three call types were significantly higher than the percentage of random classification (chance level: $1/8=12.50\%$; binomial test: $P<0.0001$).

We tested whether the acoustic parameters of L-bUFM calls were less variable than the acoustic parameters of H-bUFM calls. If so, this pattern could be used as an index of increased motivation of signalers when they signal the more aggressive H-bUFM calls. We found that the proportion of bats that showed an increased CV for H-bUFM compared

to L-bUFM calls ($N=17$) was significantly higher than the proportion of bats that showed a decreased CV for H-bUFM compared to L-bUFM calls ($N=6$; Table S25, binomial test: $P=0.037$).

Factors affecting geographical variation in three social calls

Mantel tests indicated no significant correlation between mtDNA genetic distance and acoustic difference for H-bUFM calls ($r = -0.16$, $P=0.622$; Table S17; Fig. 1k) and sUFM calls ($r = -0.01$, $P=0.97$; Table S18; Fig. 1l), but showed a significantly positive relationship between mtDNA genetic distance and acoustic divergence of L-bUFM calls ($r=0.54$, $P=0.025$; Table S19; Fig. 1j). The L-bUFM relationship was still significant after controlling for geographic distance ($r=0.56$, $P=0.019$; Table S19; Fig. 2b). No significant relationships were found between acoustic distance matrices and climatic (temperature and relative humidity), geographical, and forearm length distance matrices across all colonies for H-bUFM (Fig. 1k; Table S17), L-bUFM (Fig. 1j; Table S19) and sUFM (Fig. 1l; Table S18).

Experiment 2: discrimination of social calls

Variation in sUFM calls recorded in the laboratory

For the HZ colony, we collected a total of 235 sUFM calls from 17 adult males (mean \pm SD: 13.82 ± 8.35 calls per bat; range 3–31; Table S20). For the JK colony, a total of 220 sUFM calls from 18 adult males (mean \pm SD: 12.22 ± 5.61 calls per bat; range 3–28; Table S20) were collected. For the CY colony, we recorded a total of 224 sUFM calls from 16 adult males (mean \pm SD: 14.00 ± 6.28 calls per bat; range 5–24; Table S20).

There were significant differences in all PC factor scores among the three colonies (Kruskal-Wallis: $df=2$, $\chi^2=7.02$ – 35.53 , $P<0.031$). The DFA revealed that 96.10% of the individuals could be correctly classified according to their colony (Table S21; Fig. 2c). The percentage of correct classification was significantly higher than the percentage of random classification (chance level: $1/3=33.33\%$; binomial test: $P<0.0001$).

Discrimination of the sUFM calls

A total of 180 playback tests were conducted on 60 individuals from HZ, JK and CY colonies. All 60 males showed behavioral responses both to the habituation files and to the control stimuli (Video S3). The PCA run on our three behavioral measures (nods, body movements and echolocation calls) generated one significant PC axis (eigenvalue = 1.929, proportion variance = 64.3%) with

the following loadings: number of nods=0.768, number of body movements=0.872, and number of echolocation calls=0.760.

For the HZ colony

In the control trials, 15 of 20 bats remained habituated after the playback files were switched from HZ to HZ' (Fig. 3a). In experimental trials, 19 of 20 bats and 18 of 20 bats showed obvious responses after the playback files were switched from HZ to JK and from HZ to CY (Fig. 3a), respectively. There were significant differences in the proportion of bats responding between HZ-HZ' and HZ-JK combinations (Pearson chi-square test: $\chi^2_1 = 20.42, P < 0.0001$; Fig. 3a), and between HZ-HZ' and HZ-CY combinations (Pearson chi-square test: $\chi^2_1 = 17.29, P < 0.0001$; Fig. 3a). No significant differences were found in the proportion of bats responding between HZ-JK and HZ-CY combinations (Pearson chi-square test: $\chi^2_1 = 0.36, P = 0.548$; Fig. 3a).

There were significant differences in the behavioral responses among the three types of playback combinations

(ANOVA: $F_{2,39} = 5.80, P = 0.006$; Fig. 3d). Tukey's multiple-comparison tests showed that behavioral responses in the HZ-HZ' trial were significantly lower than behavioral responses in the HZ-JK ($P = 0.004$) and the HZ-CY trials, respectively (Fig. 3d; $P = 0.024$), while there was no significant difference in the behavioral responses between the HZ-JK and the HZ-CY trials (Fig. 3d; $P = 0.589$).

For the JK colony

In the control trials, 14 of 20 bats remained habituated after changing the playback files from JK to JK' (Fig. 3b). In the experimental trials, 18 of 20 bats showed obvious responses after switching the playback files from JK to HZ and from JK to CY (Fig. 3b). There were significant differences in the proportion of bats responding to the JK-JK' compared to the JK-HZ trials (Pearson chi-square test: $\chi^2_1 = 15.00, P < 0.0001$; Fig. 3b), and to the JK-JK' compared to the JK-CY trials (Pearson chi-square test: $\chi^2_1 = 15.00, P < 0.0001$; Fig. 3b). No significant differences were found in the proportion of bats responding to the JK-HZ compared

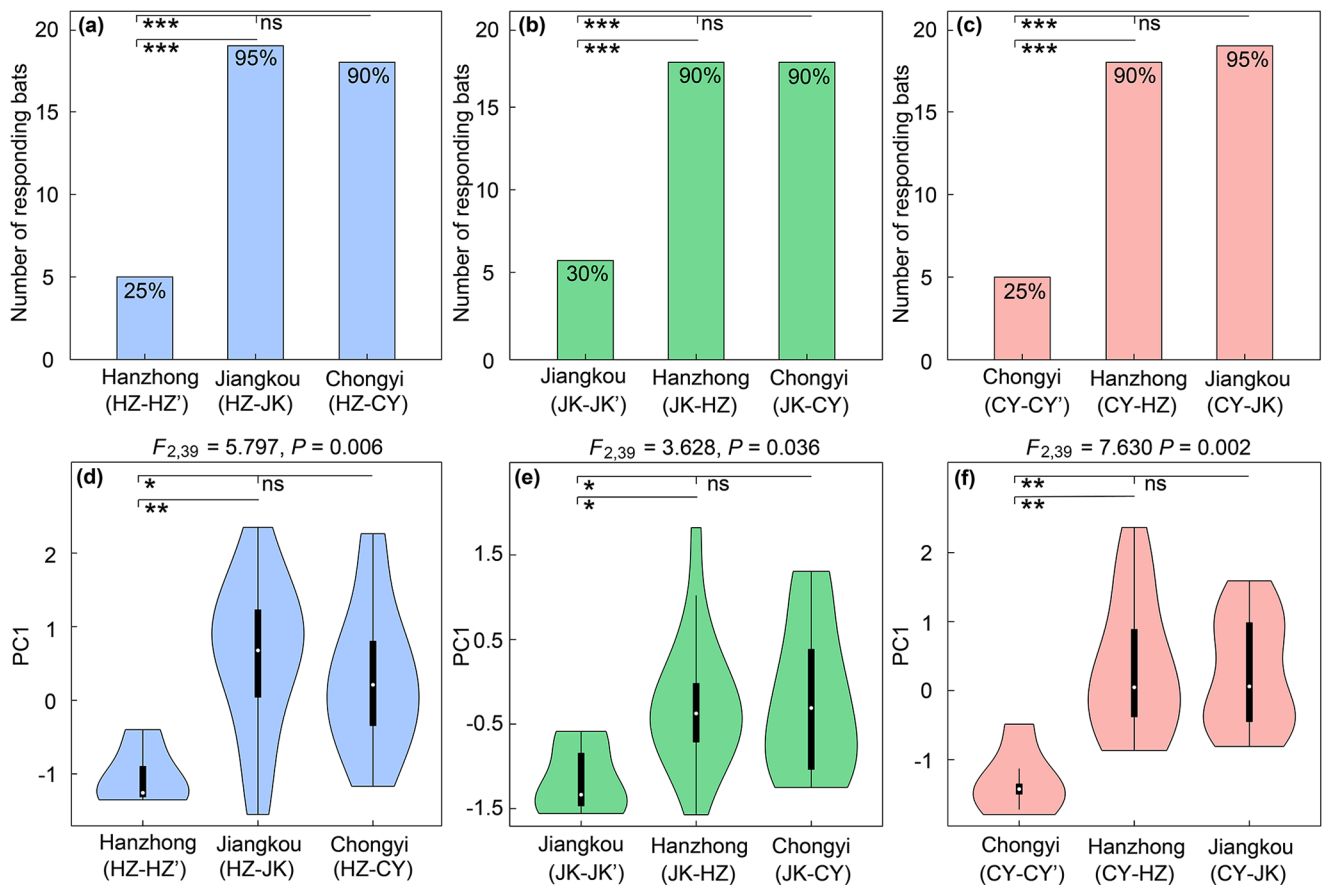


Fig. 3 Results of habituation-dishabituation playback experiment on *H. armiger* males from the Hanzhong (HZ), Jiangkou (JK), and Chongyi (CY) colonies. (a–c) Number of bats responding to control and experimental playbacks. (d–f) Violin plots of the principal component

of behavioral responses of bats responding to control and experimental playbacks. The box plots show the median and 25th and 75th percentiles; Statistical significance is based on post hoc Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

to the JK-CY trials (Pearson chi-square test: $\chi_1^2 = 0$, $P = 1$; Fig. 3b).

There were significant differences in the behavioral responses among the three types of playback trials (ANOVA: $F_{2,39} = 3.63$, $P = 0.036$; Fig. 3e). Tukey's multiple-comparison tests indicated that the behavioral responses to the JK-JK' trial were significantly lower than to the JK-HZ trials ($P = 0.046$) and to the JK-CY trials, respectively (Fig. 3e; $P = 0.037$). No significant differences were found in the behavioral responses to the JK-CY to the JK-HZ trials (Fig. 3e; $P = 0.991$).

For the CY colony

In the control trials, 15 of 20 bats remained habituated after the playback files were switched from CY to CY' (Fig. 3c). In the experimental trials, 18 of 20 bats and 19 of 20 bats showed obvious responses after the playback files were switched from CY to HZ and CY to JK, respectively (Fig. 3c). There were significant differences in the proportion of bats responding to the CY-CY' compared to the CY-HZ trials (Pearson chi-square test: $\chi_1^2 = 17.29$, $P < 0.0001$; Fig. 3c), and to the CY-CY' compared to the CY-JK trials (Pearson chi-square test: $\chi_1^2 = 20.42$, $P < 0.0001$; Fig. 3c). No significant differences were found in the proportion of bats responding to the CY-HZ compared to the CY-JK trials (Pearson chi-square test: $\chi_1^2 = 0.36$, $P = 0.548$; Fig. 3c).

There were significant differences in the behavioral responses among the three types of playback trials (ANOVA: $F_{2,39} = 7.63$, $P = 0.002$; Fig. 3f). Tukey's multiple-comparison tests indicated that the behavioral responses in the CY-CY' trials were significantly lower than that to the CY-HZ ($P = 0.002$) and to the CY-JK trials, respectively (Fig. 3f; $P = 0.003$). No significant differences were found in the behavioral responses to the CY-HZ compared to the CY-JK trials (Fig. 3f; $P = 0.953$). However, there was a significant positive correlation between the strength of response and geographic distance (Pearson correlation: $r = 0.818$, $N = 9$, $P = 0.013$).

Discussion

We found that all three types of social calls of male *H. armiger* exhibited significant geographical variation across eight colonies in China. We also found that variation in the spectral properties of both bUFM calls and sUFM calls have similar spatial patterns. These results fail to support the first hypothesis that calls with similar functions should follow similar spatial patterns whereas functionally dissimilar calls should follow dissimilar spatial patterns. Moreover, we found that genetic drift did not explain acoustic divergence

of H-bUFM and sUFM calls, but did account for a significant amount of acoustic variation of L-bUFM calls. These results also failed to support the second prediction of the first hypothesis that calls with similar functions have similar selective factors generating geographical variation and calls with different functions have different selective factors generating geographical variation. Finally, we found that a substantial number of individuals responded when playbacks were changed from calls of their own colony to those of allopatric colonies, which supported our second hypothesis that *H. armiger* can discriminate between sUFM calls of its own colony and those of an allopatric colony. To our knowledge, this is the first study exploring simultaneously the patterns and causes of geographical variation of social calls with similar and distinct functions in bats.

Patterns of geographical variation in three types of social calls

It has been hypothesised that vocalizations with similar functions should have similar geographical patterns (Nelson 2017; Baker 2011). In agreement with this expectation, we found that two calls that share similar functions (L-bUFM and H-bUFM calls) show similar spatial patterns. In particular, all acoustic parameters differed more between colonies within regions (6–24% of the total variation) than between regions (0–9% of the total variation). The significant variation in two bUFM calls among colonies within a region suggests that there are local dialects of these calls, which supports the colony password hypothesis (Feeles 1977). This hypothesis proposes that vocal dialects can be an indicator of colony identity and thus can facilitate the recognition of prospective intruders from foreign colonies (Feeles 1977). *Hipposideros armiger* bats from different colonies within a region are genetically similar and are normally able to interbreed (Lin et al. 2015). This indicates that there are frequent interactions between individuals from different colonies; thus, accurate recognition of individuals from nearby colonies may shape the local development of acoustic differences in the two bUFM calls. Similar phenomena can be found in intermediate leaf-nosed bats *Hipposideros larvatus* (Jiang et al. 2010a, b), Puerto Rican parrots *Amazona vittata* (Martínez and Logue 2020) and rufous-collared sparrow *Zonotrichia capensis* (Lougheed and Handford 1992).

Furthermore, we found that most of the relative variations for the two bUFM calls came from differences between calls within colonies (69–94% of the total variation; Fig. 1d, e). When determining the acoustic variation at the individual level, we found that most of the observed variation resulted from differences between individuals within colonies (28–69% of the total variation; Table S22, S23). The substantial variation in bUFM calls among bats suggests that these

calls play a role in individual recognition. This idea is supported by our previous study showing that male *H. armiger* L-bUFM and H-bUFM calls encoded significant individual signatures and that males were capable of discriminating individuals based on these calls (Sun et al. 2018).

Acoustic signals with distinct functions have been hypothesized to exhibit discordant geographical patterns (Armbruster and Schwaegerle 1996; Baker 2011; Nelson 2017). Contrary to this expectation, we found that sUFM calls, which convey warning information, have geographical patterns similar to those of the territorial defense calls (bUFM). All acoustic parameters of sUFM calls also differed more between colonies within regions (4–18% of the total variation) than between regions (0–9% of the total variation). The substantial variation in sUFM calls among colonies within a region may also indicate the presence of local dialects. Nonetheless, acoustic variation among HZ, JK and CY colonies was primarily attributable to differences between calls within colonies (75–94% of the total variation). When determining the acoustic variation among HZ, JK and CY colonies at the individual level, we found that most of the acoustic variation was attributed to differences between individuals within colonies (32.06–78.16% of the total variation; Table S24). The differences between individuals in vocal structures of sUFM calls indicate that the sUFM calls may play a seminal role in individual recognition and communicating information about the individual emitting the call. This view was supported by our previous study showing that sUFM calls of male *H. armiger* conveyed information about individual identity and that males could discriminate male individuals based on sUFM calls (Sun et al. 2021).

One possible interpretation for the similar geographical patterns results from the advantage for individual recognition of intruders. The great Himalayan leaf-nosed bat has a polygynous mating system where single males defend a harem of females and aggressive interactions between males are common (Yang 2011). Individual recognition of bUFM and sUFM calls could reduce unnecessary energy costs by facilitating intruder recognition and potentially reduce the number of aggressive encounters between males and/or prevent escalation of agonistic interactions to conflicts. For example, individual recognition would permit harem-holding males to recognize the status of other males and only directly attack nonharem-holding males that may pose a greater threat than harem-holding males (reviews in Clutton-Brock and Albon 1979). These benefits alone may account for the similar geographical patterns in bUFM and sUFM calls.

Selective factors driving geographical variation in three types of social calls

Acoustic signals with similar functions may be driven by similar evolutionary forces and vocalizations with different functions may experience different driving forces (Armbruster and Schwaegerle 1996; Byers 1996; Barker 2011). We found that the acoustic Euclidean distances of two bUFM calls were not related to climatic variables, geographical distance, or morphological difference, indicating that ecological selection, cultural drift and morphological constraints likely do not contribute substantially to the geographical variation in these calls. However, we found that the mitochondrial DNA genetic distance was significantly related to the acoustic Euclidean distance of L-bUFM calls, even after controlling for geographical distance. No such pattern was found for H-bUFM calls. This suggests that genetic drift does not drive the acoustic divergence of H-bUFM calls but may contribute to the acoustic divergence of L-bUFM calls. Covariance between genetic distance and acoustic variation in vocalizations has been documented in many species, such as frogs (Funk et al. 2009), birds (Irwin et al. 2008; Habel et al. 2014) and mice (Campbell et al. 2010). One possible explanation for the discordant driving forces between the two bUFM calls with similar functions is that genetic drift may have little influence on the acoustic divergence of H-bUFM calls when acoustic parameters are strongly influenced by emotional/physiological states. This view was supported by our results showing that emotion increased the variability of bUFM calls. The emotional state of the signaler causes tension and action of muscles used for vocalizations, thus affecting spectral parameters of the vocalizations (reviewed in Briefer 2012). Our previous study showed that at high levels of aggression, males *H. armiger* decreased the minimum frequency of bUFM calls and increased the frequency bandwidth (Sun et al. 2018). Similarly, Spix's disc-winged bats (*T. tricolor*) have two contact calls with similar functions that are nonetheless shaped by different evolutionary forces (Montero et al. 2018).

Similar to H-bUFM calls, we found that acoustic divergence in sUFM calls had no relationship with climatic variables, geographical distance, genetic distance or morphological difference, indicating that neither ecological selection, drift nor morphological constraints contribute substantially to the geographic divergence of sUFM calls. One potential explanation for this pattern is that strong selection favouring individual recognition within bat colonies may drive a majority of variation in H-bUFM calls and sUFM calls of *H. armiger*, and thus weaken the influences of ecological selection and drift. *H. armiger* has a polygynous mating system (Yang 2011). Therefore, selection on

clearly conveying a territory owner's identity should be particularly important in this species.

Discrimination of sUFM calls

We found that *H. armiger* could discriminate the acoustic differences between colony-specific and foreign sUFM calls. Colony-specific recognition coupled with individual recognition could mitigate energetic expenditure by preventing escalation to physical conflict during agonistic interactions. This benefit alone may drive local-scale differences in sUFM calls and may explain why ecological selection and drift failed to contribute significantly to acoustic divergence among colonies in *H. armiger*. More specifically, the ability of harem males to discriminate whether a male is from a foreign colony or its own colony may facilitate social ties between bats that have long-term associations within a colony (see Boughman and Wilkinson 1998). For example, conflicts between local and non-local males may be more intense than those between local males, given that non-local males may be a bigger threat in mate competition than local males. Given the costly nature of physical fighting [i.e., boxing and wrestling (Sun et al. 2019)], the ability to recognize opponents may influence the consequences of agonistic interactions among males and eventually affect how they allocate breeding effort. This is because the ability to discriminate opponents may allow males to compare their threat own levels or fighting ability with that of their opponents, and thus to decide whether to give up or continue competing for females. In this case, fighting withdrawal or escalation will impact energy allocation to reproduction. Male *H. armiger* may also cooperatively defend their colony to prevent non-resident males from mating with the female group. Long-term personal observations by CNS in the lab may support this view. We found that *H. armiger* males from the CY colony and males from the HZ colony roosted separately, and these two colonies of bats often fight to defend their roost territory when they were housed together in a large flight cage. Further study will need to examine whether there is cooperation in these colonies to defend females from intruders. At least in male greater sac-winged bats (*Saccopteryx bilineata*), resident males may cooperate in excluding non-resident males from their colony (Nagy et al. 2012).

A limitation of our study needs to be considered. Although our behavioral data provided the evidence that *H. armiger* males are capable of individual discrimination, the experimental paradigm used does not permit us to confirm that males have the ability to distinguish between individuals. Therefore, further playback experiments are needed to unequivocally demonstrate that receivers can both perceive

and utilize this information to make socially-relevant decisions (Tibbets and Dale 2007).

In conclusion, this study demonstrates that both bUFM calls with a similar function and the sUFM calls with a different function tend to co-vary across eight colonies, and the variation in calls among colonies is less than the substantial variation in calls between individuals within colonies. Nonetheless, male *H. armiger* have the ability to discriminate between sUFM calls from males from their own colony and those from an allopatric colony. Finally, genetic drift is a potential driving force for the evolution of L-bUFM calls but not for H-bUFM calls or sUFM calls. This research potentially lays the groundwork for expanding our limited knowledge of the patterns of geographical variation of vocalizations uttered under different emotional states and the nature of functional drivers that influence the evolution of communicative systems. Further playback experiments will need to examine whether the sex has an effect on individual recognition or whether there are sex-specific differences in these three types of social calls.

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Data availability The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Declarations

Ethical approval Our work adheres to the Guidelines for the Use of Animals in Research (ASAB/ABS, 2020), to the National Natural Science Foundation of China for experiments involving vertebrate animals, and were approved by the Hebei Normal University China (approval number: HEBTU-2022LLSC044).

Conflict of interest The authors declare that they have no competing interests.

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