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Original investigation

Variation in *Hipposideros pratti* in China based on morphology and mitochondrial genes

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ABSTRACT

Hipposideros pratti is mainly distributed in mainland China. Many geographical barriers, which might cause different populations of *H. pratti* to appear differentiation, exist in its distribution areas. Whether the differentiation having reached the levels for subspecies classification requires further study. Currently, there is not a comprehensive study for the external measurements and molecular sequences of *H. pratti*. The taxonomic status of *H. pratti* subspecies remains uncertain. To explore the differences and taxonomic status of *H. pratti*, we conducted a series of surveys on bats in four different areas of China from 2012 to 2015. We performed multivariate morphometric analyses using 16 external and 25 skull measurements and analysed sequence data of two mitochondrial genes (Cytb and COI). Scatter plots indicated that both external and cranial measurements of samples from the four geographical regions separated into two groups: specimens from Western mountain and plateau subregion in Central China (CW) and Southwest mountainous subregion in Southwest China (WS) gathered into a CW-WS group, and specimens from Eastern hilly and plain subregion in Central China (CE) and Min-Guang coastal subregion in Southern China (SM) gathered into a CE-SM group. The divergence between the CW-WS and CE-SM groups for the Cytb and COI genes were 1.6–2.4% and 1.9–2.3%, respectively, which reached the levels required for identifying subspecies classification according to previous studies. Moreover, in the phylogenetic tree based on Cytb and COI sequences, specimens from the regions CW and WS formed a clade, and specimens from the regions CE and SM formed another clade. Both the morphological and molecular results support the conclusion that *H. pratti* from the CW-WS group represents *H. p. pratti*, and the CE-SM populations should be termed *H. p. sinicus*.

Introduction

In the Oriental Region, *Hipposideros pratti* (Chiroptera: Hipposideridae) has a wide geographical distribution that includes Vietnam, southern China, Myanmar, Thailand and western Malaysia (Corbet and Hill, 1992; Hendrichsen et al., 2001; Koopman, 1993; Simmons, 2005). In mainland China, the species is widely distributed in 11 provinces in southern China (Smith and Xie, 2009; Wang, 2003). Based on paleozoological, natural and geographical conditions, the Oriental Region of China is partitioned into nine geographical areas (Zhang et al., 1997), of which five regions comprise the primary range of *H. pratti*. The geographical areas are characterized by numerous high mountains, deep valleys, broad rivers, and environmental heterogeneity, and they harbor many bat species (Li and Fang, 1999). Some bat species appear to have evolved morphological and genetic differentiation due to the geographical topology and environmental heterogeneity in South China (Lin et al., 2014; Lu et al., 2013; Mao et al., 2013; Sun et al., 2013; Zhou et al., 2005). Some of them have formed different subspecies, such as *Rhinolophus affinis*, which differentiated into *R. a. himalayanus*, *R. a. macrurus* and *R. a. hainanus* in the Oriental Region of China (Allen, 1940; Csorba et al., 2003; Wang, 2003; Zhang et al., 1997). A similar divergence occurred in *H. larvatus* and *H. pomona* (Zhao et al., 2014; Zhao et al., 2015). To date, some researchers consider that *H. pratti* in mainland China has no subspecies differentiation (Smith and Xie, 2009; Wang, 2003; Wang and Xie, 2009). However, many geographical barriers which might cause different populations of *H. pratti* to appear differentiation exist in its distri-

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bution areas. Whether the differentiation having reached the levels for subspecies classification requires further study.

In China, *H. pratti* are under serious threat because of cave exploiting for tourism, extensive using of pesticide, and the inclusion of bats in the diet (Bu et al., 2015). On the China Species Red List, *H. pratti* was listed in the near threatened (NT), closing to vulnerable (VU) (Wang and Xie, 2009). Studies of *H. pratti* in China have been on echolocation calls (Chen et al., 2002; Wei et al., 2011; Xu et al., 2014) and karyology (Gu, 2006; Wu et al., 2006; Zhang, 1985). However, there are no studies on subspecies differentiation of *H. pratti*. Obtaining external measurements and molecular data from multiple sites over such a large range can be very difficult; thus a nationwide comparison of external morphology and molecular sequences of *H. pratti* has not been performed. Therefore, whether there is subspecies differentiation of *H. pratti* in China is still unclear.

Classical identification of taxa on the basis of qualitative morphology utilizes an intuitive classification method, but it may not be suitable for some taxa with similar morphologies. The results of molecular phylogenetic analyses expressed in the form of a phylogenetic tree, which can reflect an organism's evolutionary relationship (Dai et al., 2005). To clarify the taxonomic status of the subspecies within *H. pratti*, we conducted a series of bat surveys in different areas of China from 2012 to 2015. External and skull characteristics of all specimens were compared. Two mitochondrial genes, Cytb and COI, were used to analyze molecular differences. By combining the results of morphological comparison and molecular analyses, we test whether the variation in *H. pratti* in different geographical areas reach that consistent with subspecies levels.

Material and methods

Material

Hipposideros pratti specimens were collected from eight roosts in four geographical regions. 1) Eastern hilly and plain subregion in Central China (CE): Xiushui County, Jiangxi Province and Youxi County, Fujian Province; 2) Western mountain and plateau subregion in Central China (CW): Nanzhao County, Xixia County and Neixiang County of Henan Province; 3) Southwest mountainous subregion in Southwest China (WS): Yanjin County of Yunnan Province; 4) Min-Guang coastal subregion in Southern China (SM): Jiaoling County, Guangdong Province. The collecting sites are shown in Fig. 1 and Table 1.

Bats were captured either by hand nets in roosts or mist nets at the entrance of caves. A total of 84 *H. pratti* specimens were obtained during the surveys. A 3-mm wing membrane biopsy punch was taken and preserved in 95% alcoholic solution for subsequent extraction of genomic DNA (Worthington and Barratt, 1996). Thirty of the 84 specimens were euthanased, preserved in 95% alcoholic solution and crania removed. The rest of the captured bats were released immediately after taking samples and measurements. Specimens were deposited in the College of Life Sciences, Henan Normal University, Xinxiang. All specimens were adult. All fieldwork abided by the Law of the People's Republic of China on Protection of Wildlife.

External and cranial measurements

Digital calipers (accuracy ± 0.01 mm) were used to measure 16 external and 25 skull variables (Bates and Harrison, 1997). The external measurements were taken as follows (shown in Table 2): HB: head and body length — from the tip of the snout to the anus, ventrally; TL: tail length — from the tip of the tail to the base adjacent to the anus; EL: ear length — from the lower border of the external auditory meatus to the tip of the pinna; FA: forearm length — from the extremity of the elbow to the extremity of the carpus with the wings folded; TIB: tibia length — from the knee joint to the ankle; HF: hind-foot length — from the extremity of the heel behind the os calcis to the extremity of the longest digit, not including the hairs or claws; CL: claw length — from the extremity of the longest digit to the tip of the claw; 3MT, 4MT and 5MT: length of the third, fourth and fifth metacarpals, respectively, from the extremity of the carpus to the distal extremity of the metacarpal; and 3D1P and 3D2P, 4D1P and 4D2P, 5D1P and 5D2P: length of the first and second phalanges of the third, fourth and fifth digits, respectively, from the proximal to the distal extremity of the phalange. Skull measurements were taken as follows (Table 3): GSL: greatest skull length — the greatest antero-posterior length of the skull between extremities regardless of anatomical structure; CBL: condylobasal length — from the exoccipital condyle to the alveolus of the first incisor; CH: cranial height — the distance between superior border (not including sagittal crest) of the braincase and the inferior orbital rim of the auditory bulla; NSH: nasal swelling height — the distance from the base of the anterior upper 2nd premolar to the superior border of nasal swelling; RW: rostral width — greatest width across the rostral; NW: nares width — width of nares from outer edge; BW: braincase width — width of the braincase at the posterior roots of the zygoma; MW: mastoid width — the greatest distance across the mastoid region; IOW: interorbital width — the shortest distance between the orbits; ZW: zygomatic width — the greatest width of the skull across the zygoma; ABL: length of auditory bulla — the maximal length of a single auditory bulla; ABG: greatest width of auditory bullae — the maximal distance of the two auditory bullae; ABD: distance between auditory bullae — the shortest distance between auditory bullae; CCL: condylo-canine length — the distance from the exoccipital condyle to the anterior margin of the canine alveolus; C^1-M^3: maxillary tooth-row length — distance from the anterior margin of the upper canine to the posterior face of the upper 3rd molar; C^1-C^1:upper C^1-C^1 (outer) — distance between the upper canines from the outer basal face; M^3–M^3: Maxillary M^3–M^3 (outer) — distance from the upper 3rd molar to a 3rd molar from basal buccal face; C_1–M_3: lower tooth-row length — distance from the anterior margin of the lower canine to the posterior face of the lower 3rd molar; ML: mandible length — distance from the most posterior part of the condyle to the most anterior point of the mandible, not including the incisros; OCG: greatest width of the occipital condyles — greatest distance across the occipital condyles; ONL: occipitonasal length — distance from the anterior margin of the nasal to the posterior face of the exoccipital condyle; PL: palatal length — distance from the anterior margin of the incisor alveolus to the posterior margin of the palate; BSL: basion-staphylion length — distance from the staphylion to the basion of the foramen magnum; MAW: width of mandible arthrosis — distance from the tip of the coronoid process to gonion laterale of the mandible; and ZH: maximal height of single zygoma — distance from the lowest point to the highest point of zygoma.

Statistical analysis

Sixteen external and 25 cranial variables of *H. pratti* were analysed statistically (Tables 2 and 3). Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance, using Kolmogorov–Smirnov and Levene tests, respectively. As all external measurements fitted normal distribution and the variances of the whole variable groups were equal, One-Way ANOVA was used to analyze the variation between the male and female. Stepwise discriminant function analysis was used to assess morphometric variation in *H. pratti* among four geographical regions by building scatter plots, meanwhile, the discriminant accuracy was calculated. Original and cross validation methods