Letter to the Editor

The Taxonomic Status of *Spermophilus* in the Plague Area of Dingbian County, Shaanxi Province, China*

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This study was conducted to define the taxonomic status of *Spermophilus* in the plague area of Dingbian County in Shaanxi Province, China, through the two-factor variance analysis of morphological characteristics, DNA barcoding, and chromosome karyotype analysis. The *Spermophilus* samples collected from Dingbian and Zhengxiang Baiqi Counties exhibited significant differences in their morphological measurements. All *Spermophilus* samples form two distinct branches in neighbor-joining (NJ) tree. One branch included the *Spermophilus* samples collected from Inner Mongolia, and the other branch included samples collected from the plague foci of Shaanxi Province and the Ningxia Region. The *Spermophilus* samples collected from Dingbian County had a chromosome number of 2*n* = 38 in 84.40% of all their cells. The *Spermophilus* species collected from the plague area of Dingbian County was categorized as *Spermophilus alashanicus* (*S.alashamicus*). The findings reported in this study are epidemiologically significant for monitoring plague in this region of west-central China.

All the species of *Spermophilus* are important host animals in an area affected by plague. Rodents are important host animals for several zoonoses that threaten public health worldwide. The plague area of Shaanxi Province is located in Dingbian County, Yulin City, China, where the species of *Spermophilus* has been categorized as *Spermophilus dauricus* (*S.dauricus*) over the years[1].

Traditionally, host animals are identified based on their morphological characteristics, assisted with chromosome karyotype technology. DNA barcoding, a taxonomic method that has been extensively used in recent years[2], also helps in the identification of morphologically conservative 'cryptic species'[3]. Currently, the most commonly used gene sequences for the classification of animals are the mitochondrial cytochrome C oxidase subunit I (*COI*) sequence[2] and the cytochrome b (*Cyt-b*) gene sequence[4].

Chromosome karyotype analysis is a key index that can be used to discriminate between *S. dauricus* and *S. alashanicus*. A previous study based on karyotype analysis from Russia reported that *S. dauricus* has a chromosome number of 2*n* = 36, while *S. alashanicus* in the southwest of Mongolia has a chromosome number of 2*n* = 38. The authors of that study insisted that *S. alashanicus* in the southwest of Mongolia is an independent species[5].

The plague area of Shaanxi Province has been affected three times by animal plague from 1987 to 2016. During the treatment and monitoring of the epidemic, it is often difficult to identify some host animals based on their morphological characters. To further define the taxonomic status of *Spermophilus* in the plague area of Shaanxi and the distribution of *S. alashanicus* and *S. dauricus* in Shaanxi, Inner Mongolia, and Ningxia, we collected *S. dauricus* samples from Keyou Zhongqi and Zhengxiang Baiqi in Inner Mongolia and *S. alashanicus* samples from Haiyuan County and analyzed them using morphological characteristics, DNA barcoding technology, and chromosome karyotype analysis.

The bodies and the organs of *Spermophilus* were collected from Dingbian County in Shaanxi Province, Haiyuan County, in the Ningxia Region, and from Keyou Zhongqi and Zhengxiang Baiqi in Inner Mongolia during the period from 2012 to 2016. The sample DB01-15 was obtained from Dingbian County, while the sample NX01-7 was collected from Haiyuan County. The samples NM49, NM50, NM52, and NM53 were collected from Keyou Zhongqi, while

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ZB01-17 was acquired from Zhengxiang Baiqi in Inner Mongolia. This study was approved by the Ethics Committee of Shaanxi Provincial Center for Disease Control and Prevention (No. SXCDC-2012SF001).

For the *Spermophilus* samples that were collected from Dingbian and Zhengxiang Baiqi, measurements of the external morphology (weight, body length, tail length, ear length, rear foot length, greatest length of skull, interorbital width, upper cheek teeth length, diastema length, length of auditory bulla, and width of auditory bulla) and those of the skulls were recorded (Supplementary Table S1 available in www.besjournal.com). The two-factor variance analysis of morphological characteristics was performed for 15 *Spermophilus* samples collected from Dingbian County (skull characters of 7 samples were measured) and for 17 *Spermophilus* samples collected from Zhengxiang Baiqi.

Gender and region were considered as independent variables, and morphological indexes were considered as dependent variables. No significant difference was observed in gender among the *Spermophilus* samples (Table 1). The *Spermophilus* samples collected from Dingbian and Zhengxiang Baiqi showed significant differences in body weight, body length, tail length, rear foot length, the ratio of tail length to body length, the ratio of interorbital width to greatest length of skull, upper cheek teeth length, and the ratio of upper cheek teeth length to diastema length ($P < 0.05$, Table 1). For individuals, we cannot judge accurately the types of *Spermophilus* based on morphological characters in Dingbian. However, in general, the *Spermophilus* samples collected from Dingbian and Zhengxiang Baiqi were significantly different.

Table 1. Two- factor Variance Analysis of Morphological Index Data

Independent Variable	Dependent Variable	III Sum of Squares	*df*	Mean Square	*F*	*P*
Gender	Weight (g)	879.007	1	879.007	0.416	0.526
	Body length (mm)	0.827	1	0.827	0.003	0.959
	Tail length (mm)	119.422	1	119.422	2.449	0.133
	Ear length (mm)	0.024	1	0.024	0.018	0.894
	Rear foot length (mm)	3.124	1	3.124	0.792	0.383
	Tail length/Body length	0.003	1	0.003	1.333	0.261
	Greatest length of skull (mm)	2.490	1	2.490	0.986	0.332
	Interorbital width/Greatest length of skull	0.000	1	0.000	0.515	0.481
	Interorbital width (mm)	0.754	1	0.754	0.926	0.347
	Upper cheek teeth length (mm)	0.278	1	0.278	1.161	0.294
	Diastema Length (mm)	0.358	1	0.358	0.393	0.537
	Upper cheek teeth length/Diastema Length	0.000	1	0.000	0.031	0.863
	Length of auditory bulla (mm)	0.031	1	0.031	0.075	0.787
	Width of auditory bulla (mm)	0.000	1	0.000	0.000	0.999
	Length of auditory bulla/Width of auditory bulla	0.001	1	0.001	0.081	0.778
Region	Weight (g)	12831.570	1	12831.570	6.071	0.022
	Body length (mm)	2425.937	1	2425.937	7.821	0.011
	Tail length (mm)	1074.895	1	1074.895	22.043	0.000
	Ear length (mm)	0.164	1	0.164	0.125	0.727
	Rear foot length (mm)	17.445	1	17.445	4.425	0.048
	Tail length/Body length	0.074	1	0.074	34.936	0.000
	Greatest length of skull (mm)	6.070	1	6.070	2.404	0.136
	Interorbital width/Greatest length of skull	0.003	1	0.003	9.403	0.006
	Interorbital width (mm)	3.236	1	3.236	3.974	0.059
	Upper cheek teeth length (mm)	2.282	1	2.282	9.531	0.006
	Diastema Length (mm)	3.204	1	3.204	3.514	0.075
	Upper cheek teeth length/Diastema Length	0.117	1	0.117	11.278	0.003
	Length of auditory bulla (mm)	0.066	1	0.066	0.162	0.691
	Width of auditory bulla (mm)	0.241	1	0.241	0.647	0.430
	Length of auditory bulla/Width of auditory bulla	0.011	1	0.011	0.741	0.399

Liver samples were collected and stored in 100% ethanol. Total genomic DNA of *Spermophilus* was obtained using the DNeasy Blood & Tissue Kits (Qiagen, Pudong, Shanghai, China) according to the manufacturer's instructions. The *COI* gene was amplified using the cocktail primer sets[6] under the following conditions: 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The cycle was repeated 35 times, with a final extension at 72 °C for 10 min. The Cyt-b genes were also amplified using the primers L14724 and H15915 as described by Irwin et al.[7]. Each polymerase chain reaction (PCR) cycle was carried out under the following conditions: 94 °C for 3 min, 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min. The cycle was repeated 30 times, with a final extension at 72 °C for 10 min. The cocktail primers and the Cyt-b primers are listed in Supplementary Table S2 (available in www.besjournal.com). All the amplified genes were directly sequenced in both directions.

The quality of the sequencing peak pattern was observed and evaluated using the Chromos software. When the quality was not good enough for an accurate determination of the bases, the samples were re-amplified and sequenced. The determined sequences were run on the Blast program of NCBI for comparing the sequence homology. The base composition and the mutations of gene sequences were compared using the Mega 6 software. The unaligned bases at the end of the sequence were deleted. The genetic distance was calculated based on the Kimura-2-parameter (K2P) model[8]. Phylogenetic trees of *COI* and *Cyt-b* gene sequences were constructed using the neighbor-joining (NJ) method, and the NJ trees were analyzed using 1,000 bootstraps to determine the confidence level of each branch[9].

A total of 27 *Spermophilus* samples were evaluated using DNA barcoding technology, and the gene sequences of *COI* and *Cyt-b* were obtained from all these 27 samples.

The genetic distance of the *COI* gene sequence obtained from the *Spermophilus* samples in the plague area of Dingbian County and from *S. alashanicus* samples in Ningxia was found to be ≤ 0.5%. When compared with *S. dauricus* samples collected from Inner Mongolia, the genetic distance was found to be between 7.9% and 9.3%. The genetic distance of the *Cyt-b* gene sequence obtained from the *Spermophilus* samples in the plague area of Dingbian County and from *S. alashanicus* samples in Ningxia was found to be ≤ 2.2%. When compared with *S. dauricus* samples collected from Inner Mongolia, the genetic distance was found to be between 8.9% and 11.3%.

NJ trees were constructed using the Mega 6 software (Figure 1A: *COI* gene, Figure 1B: *Cyt-b* gene). The samples form two distinct branches with high support in each NJ tree. One branch included the *Spermophilus* samples collected from Inner Mongolia, and the other branch included the samples collected from the plague foci of Shaanxi and Ningxia.

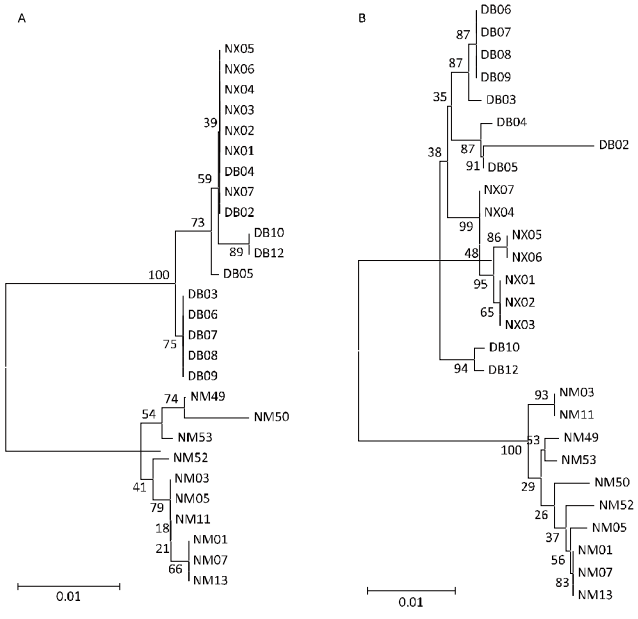

Figure 1. Phylogenetic tree analysis based on gene sequences of *COI* (A) and *Cyt-b* (B).

Hebert et al.[3] analyzed the *COI* gene sequence of the same genus from GenBank and reported that the degree of intraspecific difference of the COI gene sequence was < 2% and the average interspecific genetic distance was 11.3%.

By analyzing the genetic distance and NJ phylogenetic trees, we found that the *Spermophilus* collected from the plague area of Shaanxi and the *S.dauricus* collected from Inner Mongolia were different species, whereas *S. alashanicus* collected from Ningxia were the same species.

Five live *Spermophilus* species collected from the plague area of Dingbian County were investigated using chromosome karyotype analysis[10]. The chromosomes obtained from a total of 218 cells isolated from these five *Spermophilus* species were observed under the microscope with an oil-immersion lens (Supplementary Table S3 available in www.besjournal.com) and counted according to their morphology and type. Results showed that the chromosome number 2*n* = 38 accounted for 84.4% of all cells, whereas 2*n* = 37 accounted for 5.96% of all cells. However, the karyotype 2*n* = 36 accounted for 9.63% of all cells. These results indicate that the *Spermophilus* species collected from the plague area of Shaanxi must be classified as *S. alashanicus*.

Taken together, based on the morphological characteristics, chromosome karyotype, and the results of DNA barcoding technology, the *Spermophilus* species collected from the plague area of Dingbian County is categorized as *S. alashanicus*.

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