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Microsatellite and Skull Morphology Analysis of *Tupaia belangeri chinensis* in Yunnan Province

Yue Ren[1], Ting Jia[2], Hao Zhang[1], Zhengkun Wang[1] and Wanlong Zhu[1]*

[1]*Key Laboratory of Ecological Adaptive Evolution and Conservation on Animals Plants in Southwest Mountain Ecosystem of Yunnan Province Higher Institutes College, School of Life Sciences, Yunnan Normal University, Kunming, 650500, China*
[2]*Yunnan College of Business Management, Kunming, 650106, China*

ABSTRACT

Chinese tree shrew (*Tupaia belangeri chinensis*) is an experimental animal with a close affinity to primates. A lot of genetic marker selections and physiological adaptation studies of *T. belangeri chinensis* have been reported in detail. But genetic diversity and skull adaptation within *T. belangeri chinensis* populations from different habitats remain largely unknown or are controversial. In the present study focuing on *T. belangeri chinensis* that from Mengla, Puer, Jingdong, Kunmimng, Chuxiong, Dali, Pianma and Lijiang. Using microsatellite approaches, we observed considerable genetic variation and high heterozygosity in *T. belangeri chinensis* populations in different Yunnan province, and Chuxiong population and Lijiang population had relative low genetic diversity. Moreover, skull morphology changed for adapting to changing environment in *T. belangeri chinensis*. Finally, MengLa population had remarkable differences with the other populations both in genetic diversity and skull morphology. Our study provides genetic diversity and skull morphology knowledge of *T. belangeri chinensis*, their adaptation of genetic level and skull offers new insight into their classification and adaptation.

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Authors' Contribution
WLZ and ZKW conceived the study and participated in design and coordination. YR carried out the studies of microsatellite and skull, and drafted the manuscript. TJ and HZ made the skull specimens.

Key words
***Tupaia belangeri chinensis*, Genetic diversity, Microsatellite, Skull morphology, Fst**

INTRODUCTION

Genetic diversity protection, ecosystem diversity protection as well as species diversity protection were listed as three priority contents of global biodiversity conservation by International Union for Conservation of Nature (IUCN). Genetic diversity has been generally regarded as genetic variation between different populations and within population, and has been studied mainly from the phenotype, chromosome, protein, and DNA (Ellegren and Galtier, 2016). Genetic diversity both helps understand the population genetic structure and polymorphism, and provides valuable baseline information for species classification, utilization and protection, and the environment factors play an important role in species genetic diversity (Scheiner, 2003). Species genetic diversity in different environment with different altitude, temperature, latitude and longitude has been reported in detail, including in a variety of flora and fauna species (Slatking, 1987; Huang et al., 2005). Microsatellite, a kind of universal molecular marker, has been wildly used to explore the genetic variation, evolution and origin of mammals (Ellegren, 2004; Scibner and Pearce, 2000).

Environment factors effect can directly reflect in mammalian body traits and skull morphology (Caumul and Polly, 2005; Klingenberg, 2010). It is easy and quick to distinguish species and even subspecies based on the animals phenotypic polymorphism. For small mammal, skull morphology is an important phenotypic trait (Mu *et al*., 2015) that change for adapting to changing environment (Gao *et al*., 2017).

Tupaia belangeri chinensis (Tupaiidae, Scandentia) has a wide distribution cover a large variety of environments, ranging from South Asia, Southeast Asia and Southwest China (Wang, 1987; Roberts *et al*., 2011). Due to its unique characteristics, such as small body size, high encephalization quotient, short reproductive cycle or life span, *T. belangeri chinensis* has been proposed to be alternative experimental animal to primates in biomedical research (Yu *et al*., 2016; Lu *et al*., 2016; Pryce and Fuchs, 2017; Gawne *et al*., 2017). Previous studies have shown that there were significant differences in the phenotypic morphological characteristics of *T. belangeri chinensis*

* Corresponding author: zwl_8307@163.com
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from different environments (Wu *et al*., 2013; Gao *et al*., 2017), and there were high diversity and microsatellite polymorphism within population from one region (He *et al*., 2009; Chen *et al*., 2011; Liu *et al*., 1989; Li *et al*., 2012), knowledge of classification and adaptation within *T. belangeri chinensis* populations is limited because of the lack of the comparison studies between multiple populations. The primary objective of this paper is to resolve this issue by combining microsatellite as well as skull morphology to explore the genetic variation and skull morphology of *T. belangeri chinensis* among the different populations in Yunnan province.

MATERIALS AND METHODS

Experimental animals

Adult healthy male tree shrews (*T. belangeri chinensis*) used in the present study were captured from MengLa (ML, n=7), PuEr (PE, n=26), JingDong (JD, n=6), KunMing (KM, n=12), ChuXiong (CX, n=7), DaLi (DL, n=15), PianMa (PM, n=6), LiJiang (LJ, n=15) from January, 2016 to January, 2017. All animals were captured using standard rat cages. Detailed sampling site information is given in Table I. Animals caught in wild were immediately sacrificed by anesthesia, and their livers were immediately dissected and frozen in liquid nitrogen. Samples were stored in dry ice and transported to the laboratory of Yunnan Normal University and stored at -80ºC refrigerator until assayed. All procedures were licensed under and approved by Animal Care and Use Committee of School of Life Science, Yunnan Normal University. This study was approved by the Committee (13-0901-011). Total genomic DNA was extracted from the tissue samples of the animals using the phenol/chloroform method. DNA samples were detected by using agar-gel electrophoresis and stored at -20ºC until used (Zhu *et al*., 2014).

The PCR amplification of microsatellite DNA

Eight pairs microsatellite DNA primers with easy amplification and high polymorphism of *T. belangeri chinensis* were screened from the 27 pairs of microsatellite primers which have been reported (Srikwan *et al*., 2002; Olson *et al*., 2004, 2005; Munshi-souyh and Wilkinson, 2006), and the detailed primer information is shown in the Table II. Eight loci were coamplified in a single reaction (15µl reaction volume) which consisted of 1.5µl10×PCR buffer, 2.5mM dNTPmixture, 2.5U Taq polymerase, 10pM of the reverse primers and 10pM of the forward primers, 10ng/µl DNA template and 2.5mM Mg^{2+}. The normal cycle consisted of denaturation at 94°C for 8 min followed by 30 cycles each of 94°C for 1 min, 50~58°C for 1 min, and 72°C for 1 min, and final excision at 72°C for 6 min. 3µl of the PCR product was detected by using 2% agarose gel electrophoresis. And performed by capillary electrophoresis. Further, 1ul of the PCR product was mixed with 0.8µl of LIZ 500 internal standard and 8.0 µl of HI-DI formamide (Applied Biosystems), and the mixture was incubated at 95°C for 5min. The PCR product was detected using capillary electrophoresis by using ABl3730XL (Applied Biosystems). Finally, microsatellite fragments were read by using GeneMarkerV2.2.0 software (Soft Genetics LLC, State College, PA) (Yang *et al*., 2016). We deleted the microsatellite loci with less than 4 loci (Barker, 1994).

Table I. Sampling sites of *Tupaia belangeri chinensis*.

Population	Code	Latitude	Longitude	Climate	Altitude (m)	Annual precipitation (mm)	Average annual temperature (ºC)	Sample size
Mengla	ML	N21°28′48″	E101°33′56″	Tropical monsoon climate	646	1540	21	7
Puer	PE	N22°47′9″	E100°58′20″	Subtropical monsoon climate	1320	1100-1780	15-20	26
Jingdong	JD	N24°26′44″	E100°50′53″	Subtropical monsoon climate	2450	1086	18.3	6
Kunming	KM	N24°52′7″	E100°50′53″	Subtropical monsoon climate	1895	1011	18.5	12
Chuxiong	CX	N25°06′0″	E101°01′48″	Subtropical and subhumid plateau monsoon	1857	838	14.6	7
Dali	DL	N25°41′26″	E100°12′41″	Low latitude plateau monsoon climate	1976	1056	15	15
Pianma	PM	N26°0′48″	E98°37′35″	Tropical monsoon climate of the Indian Ocean	1900	1200	14-16	6
Lijiang	LJ	N26°52′21″	E100°13′19″	Subtropical humid climate	2418	1000	12-20	15

Table II. Characteristics of microsatellite loci amplified from eight *Tupaia belangeri chinensis* geographic populations.

Primer	Primer sequence 5' →3'	Locus repeat motif	Tm (°C)	Size	Fis	Fit	Fst
TBC1	F: AGGAGGTCCAAGCAATGAGA; R: AATAATCCAGCCACCAAGAA	$(CA)_{19}$	48	376-384	-0.34	-0.17	0.13
TBC3	F: TGGCAGGATTTCCTTCATTC; R: TCATTGCACGAGAATTTCCA	$(TG)_3TATGC(GT)_5$	44	162	-0.18	-0.08	0.09
TBC5	F: CACTTGCCATTAGTCTTTGA; R: TTGGGAGAATTAGGTTTGAG	$(AC)_{10}GCAG(AC)^4$	48	147-172	-0.35	-0.19	0.12
TBC8	F: TCTGCTTTATGTCTGTATCTTT; R: CTGTGTCTCTCCGCAATGAG	$(TC)_{11}AGTCA(CT)_5$	51.5	240	0.16	0.40	0.28
TG4	F: TGAAAACTGGCAATTCATATGC; R: CAATCCTTTTTCGTTAGTTTTGTG	$(CA)_9TA(CA)_2$	57.5	150-152	0.05	0.25	0.22
TG19	F: ACCCCTCCCTAAAGGAACT; R: CGCCCTATAGAAACCTCTCC	$(CA)_7TAAA(CA)_8$	54	173-177	-	-	-
TG22	F: GTGAGTGCACTTGCCCTGTA; R: TCCTGAACCTGGTGGCTAAC	$(CA)_8A(CA)_{10}$	54.5	149-182	-0.15	0.04	0.16
JS22	F: CAATGTCCTGGTGGTTATGG; R: GAAGTGGTCACTCTGCAATCC	$(GT)_3GC(GT)_{14}$	55.2	139-201	-0.26	-0.12	0.11
Mean					-0.15	0.02	0.16

The detection of skull geometric morphology

We used method of using larva of Tenebrio molitor to manufacture skull specimen of small mammals (Gao *et al*., 2017). The damaged skull specimens were not performed following analysis. Finally, a total 81 complete, adult and healthy male individual skulls were examined in the morphological study, and skull morphological data were obtained by using landmark methods to digitally dispose each skull sample. The processes mainly including image acquisition, image digitization, de-morphing. First, digital SONY Camera (DSC-Y110), which was 10 cm from the skull, was used to obtain pictures of the dorsal cranium, lateral cranium, ventral cranium and lateral mandible of the skull. Secondly, the pictures were digitally disposed by using TPSDIG2 software (Rohlf, 1990). According to the method of marking the skull of beasts (Cardini and Higgins, 2004), we dotted and marked the pictures, and selected homologous points including 16 points in dorsal cranium, 18 points in lateral cranium, 22 points in ventral cranium, and 12 points in lateral mandible to obtain the coordinate data (Fig. 1). The left structure of skull samples were marked to avoid data repetition, and all markers were made by one person for repeating six time, and the average coordinate data were used in the following statistical analysis. Moreover, generalized procrustes analysis (GPA) was used to remove the non-morphological variations.

Data analyses

First, we converted data format by using CONVERT software (version 1.31) (Liu *et al*., 2018). Secondly, POPGENE software (version 1.32) (Yeh and Yang, 1999) was used to calculate genetic diversity indexes including the number of alleles (Na), effective number of allele (Ne), observed heterozygosity (Ho), expected heterozygosity (He), F-statistics (Fis, Fit, Fst), and polymorphism information content (PIC). To determine whether the pattern of differentiation among *T. belangeri chinensis* populations supported a hypothesis of isolation-by-distance, Mantel test of matrix correspondence (Mantel, 1967) as implemented in Ecodist package in R (version 3.6.3). This approach tested whether genetic distances between populations increased with greater geographic distances between their sites of origin. Point-to-point geographic distances were measured from topographic maps of the study area. Genetic distances were calculated as mean pairwise Fst between populations (Nei and Li, 1979).

Moreover, Hardy-Weinberg equilibrium (HWE) test of populations was performed by using GENEPOP software (version 4.2) (Raymond and Rousset, 1995). UPGMA cluster method was used to evaluate the reliabilities of construction of phylogenetic trees based on the microsatellite data from eight *T. belangeri chinensis* populations in Yunnan Province. Finally, factorial correspondence analysis (FCA) and the population structure were performed using Genetix software (version 4.05) (Pritchard *et al*., 2000).

Morphologika (version 2v2.5) (Zhang *et al*., 2019) was used to analyze skull lice spline. Moreover, SPSS software (version 22.0) (Jia *et al*., 2009) was used to

analyze the skull multidimensional scaling and preform principal component analysis (PCA).

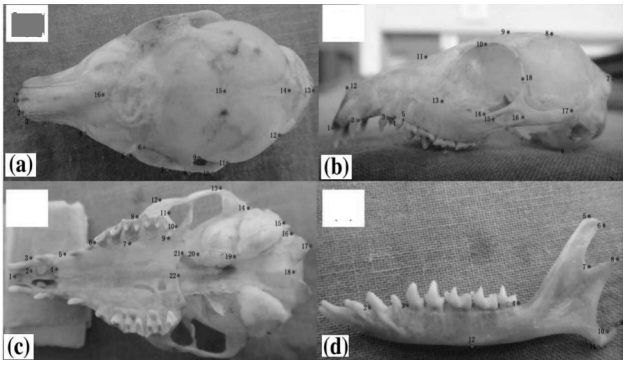

Fig. 1. *T. belangeri chinensis* landmark configurations of dorsal cranium (a), ventral cranium (b), lateral cranium (c) and lateral mandible (d).

RESULTS

Genetic diversity of T. belangeri chinensis

Partial results of capillary electrophoresis were showed in Figure 2. Detection results of TBC1, TBC3, TBC8, TG4 and TG19 loci showed that TBC3 and TBC8 were homozygous microsatellite with 162 and 240bp size of fragments respectively, and TBC1, TG4 and TG19 were heterozygous microsatellite, and the fragment sizes of them were 376/384, 150/152 and 173/177, respectively. Total 84 alleles at eight microsatellite loci were detected, and the microsatellite loci with less than 4 loci were deleted, and finally total 82 alleles at seven microsatellite loci were used in the following analysis.

Indeed, high Na (mean: 4.04, min: 1, max: 9), Ho (mean: 0.58, min: 0.08, max: 0.96) and He (mean: 0.65, min: 0.08, max: 0.93) were observed for each of the microsatellite markers (Table III) in this study, which indicating that there was high genetic diversity among *T. belangeri chinensis* populations in Yunnan province. Moreover, PM population and PE population had the highest PIC value (0.68), followed by ML population (0.62), JD population (0.58), DL population (0.53), KM population (0.52), LJ population (0.48), and CX population (0.47). Hardy-Weinberg equilibrium test was further performed, and the result showed that most of the microsatellite loci were conform to the Hardy-Weinberg equilibrium ($P_{HWE} > 0.05$), but few microsatellite loci were not as $P_{HWE} < 0.05$.

Differentiation of T. belangeri chinensis *populations*

F-statistics results were shown in Table II. Five Fis counts of loci were less than zero, indicating that there are many heterozygotes within population, and the absolute value ranged from 0.05 to 0.35. Four Fit counts of loci were less than zero, and the absolute value ranged from 0.02 to 0.40. Pairwise Fst value ranged from 0.09 to 0.28 with average 0.16. Pairwise Fst that visualized genetic differentiation between the populations are shown in Table IV. Pairwise Fst ranged from 0.02 to 0.33 (average, 0.12). Mantel test between geographic distance and pairwise Fst was showed in Figure 3. This result reflected that there was no correlation between geographic distance and pairwise Fst and indicated that geographic distance may not effect *T. belangeri chinensis* distribution and diffusion (*mantel r* = 0.047, *P* = 0.812).

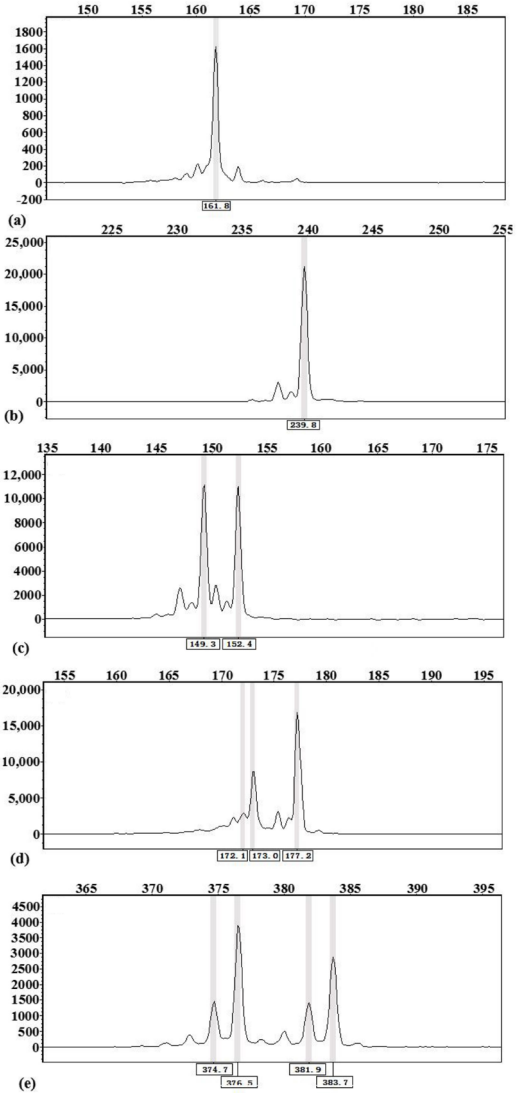

Fig. 2. The result of genotypic alleles of TBC3 locus (a), TBC8 locus (b), TBC1 locus (c), TG4 locus (d) and TG19 locus (e).

Table III. Genetic diversities and Hardy-Weinberg equilibrium test of eight microsatellite loci in the eight *Tupaia belangeri chinensis* geographic populations.

Population	Loci	Na	Ho	He	PIC	P_{HWE}
ML	TBC3	5.00	0.43	0.51	0.45	0.21
	TBC5	4.00	0.52	0.82	0.70	0.01
	TBC8	5.00	0.40	0.76	0.64	0.05
	TG4	6.00	0.33	0.89	0.79	0.00
	TG22	1.00	0.64	0.76	0.64	0.81
	JS22	5.00	0.67	0.68	0.56	0.91
	TBC1	4.00	0.54	0.69	0.55	0.26
	Mean	4.29	0.47	0.73	0.62	-
PE	TBC3	3.00	0.23	0.21	0.19	0.96
	TBC5	8.00	0.96	0.83	0.79	0.08
	TBC8	6.00	0.81	0.75	0.69	0.51
	TG4	4.00	0.67	0.70	0.63	0.03
	TG22	1.00	0.90	0.87	0.83	0.65
	JS22	8.00	0.69	0.78	0.83	0.01
	TBC1	8.00	0.78	0.84	0.80	0.13
	Mean	5.43	0.71	0.71	0.68	-
JD	TBC3	3.00	0.29	0.38	0.33	0.11
	TBC5	5.00	0.67	0.78	0.68	0.70
	TBC8	4.00	0.38	0.52	0.44	0.60
	TG4	3.00	0.33	0.59	0.46	0.30
	TG22	2.00	0.74	0.89	0.80	0.17
	JS22	7.00	0.75	0.67	0.59	0.07
	TBC1	5.00	0.73	0.84	0.74	0.98
	Mean	4.14	0.53	0.67	0.58	-
KM	TBC3	2.00	0.80	0.51	0.36	0.05
	TBC5	4.00	0.80	0.60	0.51	0.70
	TBC8	1.00	0.00	0.00	-	-
	TG4	3.00	0.30	0.28	0.25	0.98
	TG22	2.00	0.60	0.75	0.67	0.65
	JS22	4.00	0.69	0.75	0.66	0.14
	TBC1	4.00	0.72	0.90	0.64	0.00
	Mean	2.86	0.54	0.54	0.52	-
CX	TBC3	2.00	0.71	0.49	0.35	0.20
	TBC5	3.00	0.86	0.69	0.57	0.03
	TBC8	3.00	0.29	0.27	0.24	0.99
	TG4	3.00	0.64	0.65	0.52	0.11
	TG22	2.00	0.65	0.69	0.57	0.30
	JS22	3.00	0.65	0.65	0.53	0.11
	TBC1	3.00	0.66	0.65	0.52	0.11
	Mean	2.71	0.64	0.58	0.47	-
DL	TBC3	3.00	0.50	0.44	0.36	0.94
	TBC5	3.00	0.66	0.68	0.55	0.17
	TBC8	3.00	0.17	0.32	0.27	0.01
	TG4	5.00	0.50	0.67	0.58	0.34
	TG22	2.00	0.79	0.86	0.60	0.53
	JS22	5.00	0.65	0.71	0.60	0.54
	TBC1	4.00	0.83	0.85	0.75	0.65
	Mean	3.57	0.55	0.65	0.53	-
PM	TBC3	3.00	0.33	0.44	0.36	0.17
	TBC5	5.00	0.76	0.82	0.71	0.80
	TBC8	6.00	0.67	0.88	0.78	0.08
	TG4	5.00	0.67	0.67	0.58	0.85
	TG22	2.00	0.50	0.93	0.79	0.07
	JS22	6.00	0.67	0.91	0.81	0.10
	TBC1	7.00	0.78	0.86	0.76	0.58
	Mean	4.86	0.60	0.79	0.68	-
LJ	TBC3	3.00	0.25	0.24	0.21	0.98
	TBC5	6.00	0.75	0.59	0.54	1.00
	TBC8	2.00	0.08	0.08	0.08	1.00
	TG4	4.00	0.75	0.66	0.56	0.89
	TG22	2.00	0.75	0.57	0.49	0.96
	JS22	5.00	0.86	0.86	0.80	0.24
	TBC1	9.00	0.75	0.74	0.68	0.81
	Mean	4.43	0.60	0.53	0.48	-

Groups: ML, Mengla population; PE, Puer population; JD, Jingdong population; KM, Kunming population; CX, Chuxiong population; DL, Dali population; LJ, Lijiang population.

Table IV. Pairwise Fst between *Tupaia belangeri chinensis* populations.

	ML	PE	JD	KM	CX	DL	PM
ML	-						
PE	0.04	-					
JD	0.10	0.06	-				
KM	0.33	0.21	0.14	-			
CX	0.28	0.17	0.09	0.02	-		
DL	0.15	0.11	0.06	0.13	0.12	-	
PM	0.03	0.04	0.04	0.19	0.12	0.09	-
LJ	0.22	0.16	0.07	0.20	0.14	0.02	0.15

For details of the groups, see Table III.

Genetic structure of T. belangeri chinensis *populations*

UPGMA cluster method and FCA analysis were further used to evaluate the construction of cluster analysis tree and perform population structure (Fig. 4). The results showed that ML population and PM population respectively became a separate cluster. Moreover, the other

six populations branch off in pairs, including JD population and PE population, DL population and LJ population, CX population and KM population.

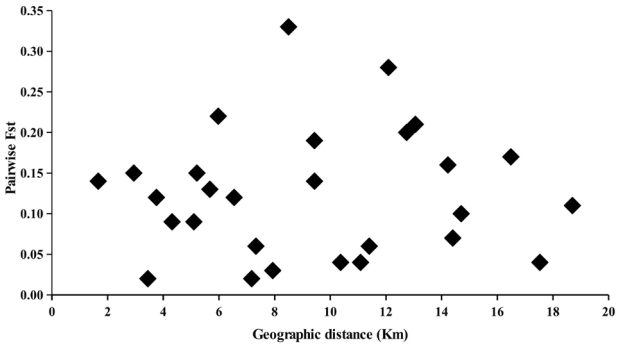

Fig. 3. Correlation analysis of geographic distance and pairwise Fst. Data were analyzed by Mantel test, *mantel r* = 0.047, *P* = 0.812.

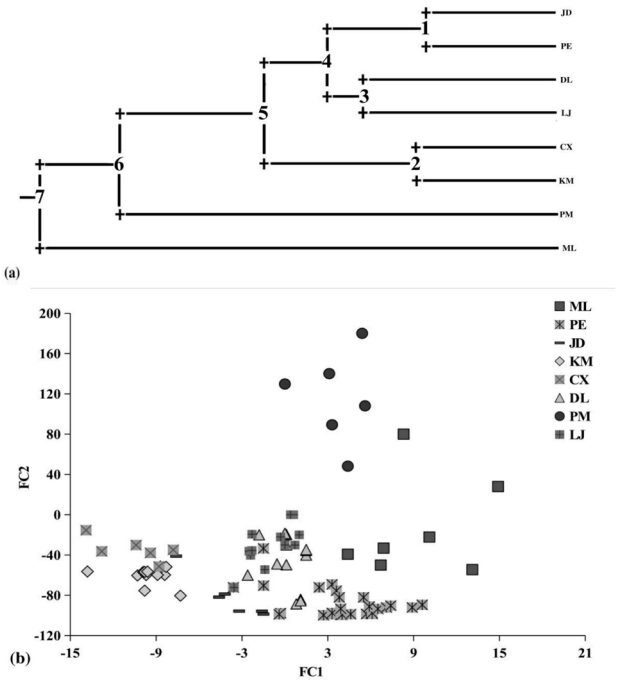

Fig. 4. Population structure. (a) Clustering analysis of haplotype diversity of microsatellites of *Tupaia belangeri chinensis* by UPGMA. (b) Scatter plot of Factorial components 1 versus 2 (FC1 versus FC2) for the eight populations. Groups: ML, Mengla population; PE, Puer population; JD, Jingdong population; KM, Kunming population; CX, Chuxiong population; DL, Dali population; LJ, Lijiang population.

Variations of skull morphology

The PCA result of dorsal cranium, lateral cranium, ventral cranium and lateral mandible were summarized in Table V. In the dorsal cranium, the eigenvalues for the first three principal components were 0.00963, 0.00213 and 0.00140, respectively, and accounted for 89.15% of the total variance. The first and second principal components were mixed together (Fig. 5A). In the lateral cranium, the eigenvalues for the first three principal components were 0.00120, 0.00107, 0.00057, respectively, and accounted for 73.23% of the total variance. The first two principal components were mixed together, except for the ML population, which had very little overlap that could be due to intraspecific individual variations (Fig. 5B). In the ventral cranium, the eigenvalues for the first three principal components were 0.00195, 0.00154, 0.00038, respectively, and accounted for 92.37% of the total variance. The first two principal components of JD and ML populations mixed together, and the other populations were mixed together (Fig. 5C). In the lateral mandible, the eigenvalues for the first three principal components were 0.00063, 0.00040, 0.00011, respectively, and accounted for 76.72% of the total variance. The first two principal components were mixed together, except for the KM population, which had very little overlap with the other population. Moreover, PE population were mixed with all of the other populations.

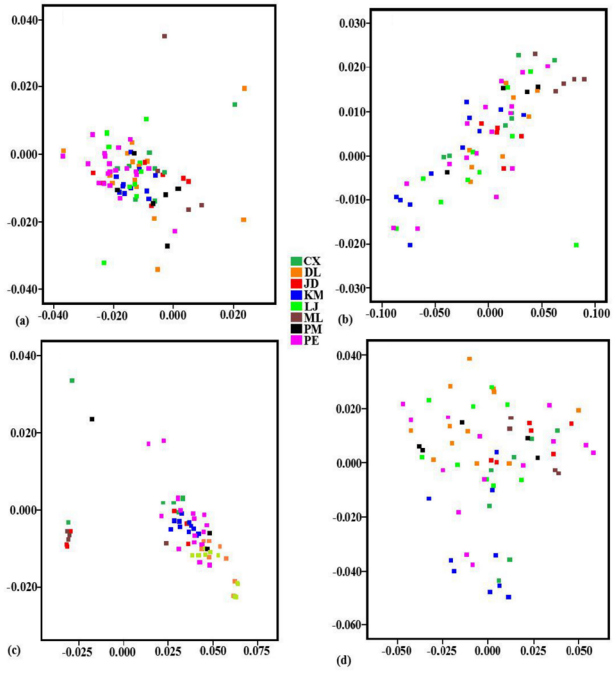

Fig. 5. Plots of principal component factors 1 and 2 for dorsal cranium (a), ventral cranium (b), lateral cranium (c) and lateral mandible (d) data of *T. belangeri chinensis*.

We combined the data of dorsal cranium, lateral

cranium, ventral cranium and lateral mandible of animals from CX, JD, DL, KM, LJ, ML, PM, and PE populations, and used multidimensional scaling analysis analyze the combination data. The result showed that there were differences between eight populations, and there was great difference between ML population and the other seven populations (Fig. 6).

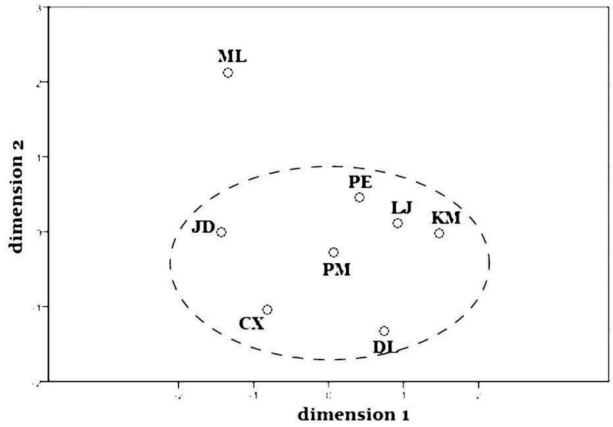

Fig. 6. Multidimensional scaling diagram for combinnation data of skull form eight geography populations in *T. belangeri chinensis.* Groups: ML, Mengla population; PE, Puer population; JD, Jingdong population; KM, Kunming population; CX, Chuxiong population; DL, Dali population; LJ, Lijiang population.

DISCUSSION

Heterozygosity is a measure of genetic variation within the population, and its value reflects the uniformity of individuals within the population. A high value indicates high genetic variation within the population, while a low value indicates low genetic variation within the population, mainly including Ho and He. Ho refers to the proportion of heterozygous individuals in the population. In the case of the same Na, the higher the proportion of heterozygous individuals in the population, the richer the genetic diversity of the population. He is the heterozygosity calculated on the premise that each gene locus conforms to HWE. Moreover, the average He can be used to measure the genetic variation of population. The higher the average of He level is, the richer the genetic diversity of the population becomes (Botstein *et al*., 1980). When He range from 0.5 to 0.7, the population has high genetic diversity and low inbreeding number which belong to the characteristics of a closed colony. When He is lower than 0.5, the genetic diversity is low and the inbreeding number is high; When He is greater than 0.7, the genetic heterozygosity is high and the individual differences of the population are great (Liu *et al*., 2018), neither of these cases belong to the characteristics of closed colony. In the present study, the high genetic diversity among *T. belangeri chinensis* populations in Yunnan province is consistent with the other studies (Liu and Yao, 2013; Zhu *et al*., 2014). The most He were higher than Ho, indicating that there may be heterozygosity among populations. The average He level of JD population, CX population, KM population, DL population and LJ population ranged from 0.5 to 0.7 reflecting a high genetic diversity which are corresponding to the characteristics of enclosed group (Li *et al*., 2011; Liu *et al*., 2018). Increased genetic diversity of a population means it has increased environmental adaptability (Ellegren and Galiter, 2016). While the He level of ML population, PE population and PM population revealed that the four populations did not corresponding to the characteristics of closed colony (Li *et al*., 2011; Liu *et al*., 2018).

Table V. Principal component, variance explained and cumulative variance explained of dorsal cranium, ventral cranium, lateral cranium and lateral mandible.

Skull/ Principal component	Eigenval-ue	Variance explained (%)	Cumulative variance explained (%)
Dorsal cranium			
PC1	0.00963	65.14	65.14
PC2	0.00213	14.41	79.54
PC3	0.00140	9.50	89.04
Ventral cranium			
PC1	0.00120	81.26	81.26
PC2	0.00107	7.23	88.48
PC3	0.00057	3.88	92.37
Lateral cranium			
PC1	0.00195	36.85	36.85
PC2	0.00154	29.11	65.96
PC3	0.00038	7.27	73.23
Lateral mandible			
PC1	0.00063	42.33	42.33
PC2	0.00040	26.71	69.03
PC3	0.00011	7.68	76.72

We further compared genetic diversity between different *Tupaia* species which showed in Table VI. In the present study, the average He was 0.64 which is lower than *Tupaia longipes* (average 0.69) (Brunke *et al*., 2020), *Tupaia belangeri* in Guangxi (average as 0.70) (Tang *et al*., 2016; Yang *et al*., 2016), but is higher than *T. belangeri yaoshanensis* (average as 0.48) (Su *et al*., 2017), *T.*

belangeri chinensis in laboratory (average as 0.52) (Zhang *et al*., 2015) and *T. belangeri chinensis* born in laboratory including F1, F2, F3 and F4 of *T. belangeri chinensis* (average as 0.62, 0.61, 0.60 and 0.60, respectively) (Yang *et al*., 2010; Liu *et al*., 2018), and is similar with *Tupaia glis* (average as 0.65) (Srikwan *et al*., 2002), *Tupaia* spp (average as 0.66) (Munshi-South *et al*., 2006), *T. belangeri chinensis* in Yunnan (average as 0.62) (Liu and Yao, 2013).

Table VI. Genetic diversity comparison between different *Tupaia* species.

Species	Ho	He	PIC	Reference
Tupaia glis	0.83	0.65	-	Srikwan *et al*., 2002
Tupaia spp.	0.67	0.66	-	Munshi-South *et al*., 2006
Tupaia longipes	0.78	0.69	-	Brunke *et al*., 2020
Tupaia belangeri yaoshanensis	0.40	0.48	0.44	Su *et al*., 2017
T. belangeri in Guangxi	0.48	0.70	0.73	Tang *et al*., 2016
T. belangeri in Guangxi	0.62	0.70	0.64	Yang *et al*., 2016
T. b. chinensis in laboratory	0.61	0.52	-	Zhang *et al*., 2015
T. b. chinensis born in laboratory	-	0.62	0.52	Yang *et al*., 2010
T. b. chinensis in Yunnan	0.57	0.62	0.58	Liu *et al*., 2012
T. belangeri F1	0.50	0.62	0.56	Liu *et al*., 2018
T. belangeri F2	0.56	0.61	0.54	Liu *et al*., 2018
T. belangeri F3	0.53	0.60	0.53	Liu *et al*., 2018
T. belangeri F4	0.48	0.60	0.47	Liu *et al*., 2018
T. b. chinensis in Mengla	0.47	0.73	0.62	Present study
T. b. chinensis in Puer	0.71	0.71	0.68	Present study
T. b. chinensis in Jingdong	0.53	0.67	0.58	Present study
T. b. chinensis in Kunming	0.54	0.48	0.52	Present study
T. b. chinensis in Chuxiong	0.64	0.58	0.47	Present study
T. b. chinensis in Dali	0.55	0.65	0.53	Present study
T. b. chinensis in Pianma	0.60	0.79	0.68	Present study
T. b. chinensis in Lijiang	0.60	0.53	0.48	Present study

The heterozygosity and PIC of microsatellites are optimal parameters for measuring genetic variations within population, and the high value reflect the high genetic diversity of population (Rousset, 1997). Botstein *et al*. (1980) suggested that population has high genetic polymorphism when PIC value is more than 0.5, and has moderate genetic polymorphism when PIC range from 0.25 to 0.5, and has low genetic polymorphism when PIC value is less than 0.25. Our data showed that CX population and LJ population had moderate genetic polymorphism, and the other populations had high genetic polymorphism.

In the present study, most of the microsatellite loci conformed to the Hardy-Weinberg equilibrium, but few microsatellite loci were not. It may be caused by the small sample size, however, in the study of genetic structure of wild specie populations, there are general phenomena of deviation from the Hardy-Weinberg equilibrium caused by invalid allele or inbreeding among populations (Lade *et al*., 1996).

The F-statistics results indicated that there are more heterozygotes in *T. belangeri chinensis* populations, and this may caused by different degrees of inbreeding, indicating that it is necessary to strengthen the selection to breed excellent varieties in the breeding process of *T. belangeri chinensis* in the future (Yang *et al*., 2016). The low pairwise Fst may reflected that there were high gene flow between *T. belangeri chinensis* populations in Yunnan province and genetic variation mainly comes from within the population. Because of geographical differences, the same species populations from different habitat exhibit different levels of genetic diversity (Ellegren and Galtier, 2016). In this study, the selected populations mainly belong to mountainous areas category, and the result showed that geographic distance may not effect *T. belangeri chinensis* distribution, so there are other factors to influence the genetic diversity among different populations, such as longitude, latitude, temperature and food recourse (Zhu *et al*., 2014).

The UPGMA cluster and FCA analysis indicate that *T. belangeri chinensis* populations in Yunnan province had obvious genetic structure, and they could be divided into five branches, which first divided ML population and PM population as a separate cluster. Moreover, the other six populations branch off in pairs, including JD population and PE population, DL population and LJ population, CX population and KM population. This is consistent with the previous result of D-loop gene sequence of *T. belangeri chinensis* in Yunnan province in our group (Zhu *et al*., 2014), and it may be caused by the geographical barrier of the Nu Jiang and Wuliang mountain (Zhu *et al*., 2014).

In general, species can survive from changing climates in three ways: migration, plasticity, and adaptive evolution (Williams *et al*., 2008; Chen *et al*., 2018). Animals can change phenotypic traits for adapting to the changing environment with different altitude, vegetation and climate (Caumul and Polly, 2005). Our geometric morphological analysis of skull revealed that there was difference in skull in different *T. belangeri chinensis* populations in Yunnan province, which consistent with the results shown in previous studies (Jia *et al*., 2009;

Zhu *et al*., 2013; Gao *et al*., 2017). Interestingly, the ML population had more difference with the other populations by using multidimensional scaling analysis, while there were no difference between the other populations. These differences may be related to the habitat and/or environment. For example, ML population living in low altitude area with tropical monsoon climate, and the food (in terms of quality and quantity) is sufficient, so that environmental stress is small. As a result, there are difference between ML population with the other populations.

CONCLUSION

In conclusion, we characterized a set of eight polymorphic microsatellite markers and skull morphology for the *T. belangeri chinensis* in Yunnan province. Our data confirm the *T. belangeri chinensis* had a considerably high heterozygosity and genetic diversity, and CX population and LJ population have relatively lower genetic diversity and have characteristics of enclosed group. Moreover, skull morphology change for adapting to changing geographical environment in *T. belangeri chinensis*. Finally, ML population has remarkable differences with other population both in genetic diversity and skull morphology. We hope that these results will provide essential help for clarifying the classification and relationships of *T. belangeri chinensis* from different regions and advancing the inbreeding project of the *T. belangeri chinensis*.

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Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Barker, J.S.F., 1994. A global protocol for determining genetic distance among domestic livestock breeds. *Proc. 5th World Cong. Genet. Appl. Livest. Prod*., **21**: 501–508.

Botstein, D., White, R.L., Skolnick, M., and Davis, R.W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet*., **32**: 314–331.

Brunke, J., Russo, I.M., Orozco-Terwengel, P., Zimmermann, E., Bruford, I.W., Goossens, B., and Radespiel, U., 2002. Dispersal and genetic structure in a tropical small mammal, the bornean tree shrew (*Tupaia longipes*), in a fragmented landscape along the Kinabatangan river, sabah, malaysia. *BMC Genet*., **21**: 43. https://doi.org/10.1186/s12863-020-00849-z

Cardini, A., and Higgins P., 2004. Patterns of morphological evolution in Marmota (Rodentia, Sciuridae): geometric morphometrics of the cranium in the context of marmot phylogeny, ecology and conservation. *Biol. J. Linn. Soc*., **82**: 385–407. https://doi.org/10.1111/j.1095-8312.2004.00367.x

Caumul, R., and Polly, Y.P.D., 2005. Phylogenetic and environmental components of morphological variation: Skull, mandible, and molar shape in marmots (Marmota, Rodentia). *Evolution*, **59**: 2460–2472. https://doi.org/10.1111/j.0014-3820.2005.tb00955.x

Chen, C., Wang, H., Liu, Z., Chen, X., Tang, J., Meng, F., and Shi, W., 2018. Population genomics provide insights into the evolution and adaptation of the Eastern honey bee (*Apis cerana*). *Mol. Biol. Evol.*, **9**: 2260–2271. https://doi.org/10.1093/molbev/msy130

Chen, S.Y., Xu, L., Lü, L.B., and Yao, Y.G., 2011. Genetic diversity and matrilineal structure in Chinese tree shrews inhabiting Kunming, China. *Zool. Res*., **32**: 17–23.

Ellegren, H., 2004. Microsatellites: Simple sequences with complex evolution. *Nat. Rev. Genet*., **5**: 435–445. https://doi.org/10.1038/nrg1348

Ellegren, H., and Galtier, N., 2016. Determinants of genetic diversity. *Nat. Rev. Genet*., **17**: 422–433. https://doi.org/10.1038/nrg.2016.58

Gao, W.R., Zhu, W.L., Fu, J.H., Yang, T., and Wang, Z.K., 2017. Morphometric variation of tree shrews (*Tupaia belangeri*) from different regions. *Anim. Biol*., **67**: 177–189. https://doi.org/10.1163/15707563-00002527

Gawne, T.J., Siegwart, J.T., Ward, A.H., and Norton, T.T., 2017. The wavelength composition and temporal modulation of ambient lighting strongly affect refractive development in young tree shrews. *Exp. Eye Res*., **155**: 75–84. https://doi.org/10.1016/j.exer.2016.12.004

He, B.L., Shen, P.Q., Chen, L.L., Jiao, J.L., Liu, R.W., Li.B., Zhen, P.Q., and Li, M.L., 2009.

Polymorphism microsatellites in tree shrews (*Tupaia belangeri chinensis*). *Acta Labrary Anim. Sci. Sin*., **17**: 143–145.

Huang, Z., Liu, N., Zhou, T., and Ju, B., 2005. Effects of environmental factors on the population genetic structure in chukar partridge (*Alectoris chukar*). *J. Arid Environ*., **62**: 427–434. https://doi.org/10.1016/j.jaridenv.2005.01.011

Jia, T., Lin, A.Q., Wang, R., Zhu, W.L., Xiao, C.H., Liu, C.Y., Meng, L.H., Lian, X., Wang, Z.K., 2009. Pilot study of *Tupaia belangeri* from Yunnan Province based on morphometrics of the skulls and mandibles *Acta Theriol. Sin*., **29**: 259–268.

Klingenberg, C.P., 2010. Evolution and development of shape: Integrating quantitative approaches. *Nat. Rev. Genet*., **11**: 623–635. https://doi.org/10.1038/nrg2829

Lade, J.A., Murray, N.D., and Marks, C.A., Robinson, N.A., 1996. Microsatellite differentiation between Phillip Island and mainland Australian populations of the red fox *Vulpes vulpes. Mol. Ecol.*, **5**: 81–87. https://doi.org/10.1111/j.1365-294X.1996.tb00293.x

Li, R.X., Xu, W., Wang, Z., Liang, B., Wu, J.R., and Zeng, R., 2012. Proteomic characteristics of the liver and skeletal muscle in the Chinese tree shrew (*Tupaia belangeri chinensis*). *Prot. Cell*., **3**: 691–700. https://doi.org/10.1007/s13238-012-2039-0

Li, W., Jiang, Q.H., Du, X.Y., Shang, S.C., and Chen, Z.W., 2011. Establishment of genetic standards of closed colony gerbil. *Lab. Anim. Sci.*, **28**: 31–36.

Liu, C.X., Li, N., Tong, P.F., Wang, W.G., Lu, C.X., and Han, Y.Y., 2018. Analysis of microsatellite genetic characteristics in of closed colony of tree shrews. *Lab. Anim. Comp. Med*., **38**: 1–9.

Liu, R.Q., Shi, L.M., and Chen, Y.Z., 1989. Comparative studies on chromosoes of 3 subspecies of *Tupaia belangeri. Zool. Res*., **10**: 195–200.

Liu, X.H., and Yao, Y.G., 2013. Characterization of 12 polymorphic microsatellite markers in the Chinese tree shrew (*Tupaia belangeri chinensis*). *Zool. Res*., **34**: E62–E68. https://doi.org/10.3724/SP.J.1141.2013.E02E62

Lu, J.S., Yue, F., Liu, X.Q., Chen, T., and Zhou, M., 2016. Characterization of the anterior cingulate cortex in adult tree shrew. *Mol. Pain*., **12**: 1744806916684515. https://doi.org/10.1177/1744806916684515

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. *Cancer Res*., **27**: 209–220.

Mu, Y., Yang, T., Ma, Z.Q., Zhang, H., Zhu, W.L., and Wang, Z.K., 2015. Genetic diversity of mitochondrial cytochrome b and control region in *Eothenomys miletus* of Jianchuan, Yunnan Province. *J. Biol*., 187: 34–38.

Munshi-South, J., and Wilkinson, G.S., 2006. Isolation and characterization of polymorphic microsatellite loci in Bornean treeshrews (*Tupaia* spp.). *Mol. Ecol. Notes*, **6**: 698–699. https://doi.org/10.1111/j.1471-8286.2006.01314.x

Nei, M., and Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. natl. Acad. Sci. USA*., **76**: 5269–5273. https://doi.org/10.1073/pnas.76.10.5269

Olson, L., Sargis, E., and Martin, R., 2005. Intraordinal phylogenetics of treeshrews (Mammalia: Scandentia) based on evidence from the mitochondrial 12S rRNA gene. *Mol. Phyl. Evol*., **35**: 656–673. https://doi.org/10.1016/j.ympev.2005.01.005

Olson, L.E., Sargis, E.J., and Martin, R.D., 2004. Phylogenetic relationships among treeshrews (Scandentia): A review and critique of the morphological evidence. *J. Mamm. Evol*., **11**: 49–71. https://doi.org/10.1023/B:JOMM.0000029145.28207.6d

Pritchard, J.K., Stephens, M., and Donnelly, P., 2000. Inference of population structure using multilocus genotype data. *Genetics*, **155**: 945–959. https://doi.org/10.1093/genetics/155.2.945

Pryce, C.R., and Fuchs, E., 2007. Chronic psychosocial stressors in adulthood: Studies in mice, rats and tree shrews. *Neurobiol. Stress*, **6**: 94–103. https://doi.org/10.1016/j.ynstr.2016.10.001

Raymond, M., and Rousset, F., 1995. GENEPOP (version1.2): Population genetics software for exact tests and ecumenicism. *J. Hered.*, **86**: 248–249. https://doi.org/10.1093/oxfordjournals.jhered.a111573

Roberts, T.E., Lanier, H.C., Sargis, E.J., and Olson, L.E., 2011. Molecular phylogeny of treeshrews (Mammalia: Scandentia) and the timescale of diversification in Southeast Asia. *Mol. Phyl. Evol*., **60**: 358–372. https://doi.org/10.1016/j.ympev.2011.04.021

Rohlf, F.J., 1990. *Tpspline: A program to compare two shapes using a thinplate spline, department of ecology and evolution*. State University of New York at Stony Brook, New York, USA, 1990.

Rousset, F., 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, **145**: 1219–1228. https://doi.org/10.1093/genetics/145.4.1219

Scheiner, S.M., 2003. Genetics and evolution of phenotypic plasticity. *Annu. Rev. Ecol. Evol. Syst*., **24**: 35–68. https://doi.org/10.1146/annurev.es.24.110193.000343

Scibner, K.T., and Pearce, J.M., 2000. Microsatellites: Evolutionary and methodological background and empirical applications at individual, population, and phylogenetic levels. *Mol. Ecol.*, **11**: 235–273.

Slatking, M., 1987. Gene flow and the geographic structure of natural populations. *Science*, **236**: 787–792. https://doi.org/10.1126/science.3576198

Srikwan, S., Hufford, K., Eggert, L., and Woodruff, D.S., 2002. Variable microsatellite markers for genotyping tree shrews, *Tupaia*, and their potential use in genetic studies of fragmented populations. *Science Asia*, **28**: 93–97. https://doi.org/10.2306/scienceasia1513-1874.2002.28.093

Su, A.L., Lan, X.W., Huang, M.B., Nong, W., Li, Q.Q., and Leng, J., 2017. Microsatellite analysis of genetic diversity in the *Tupaia belangeri yaoshanensis. Biomed. Rep*., **7**: 349–352. https://doi.org/10.3892/br.2017.969

Tang, Y.P., Cao, Y., Yang, X.T., Yang, C., Li, Y., and Zhou, L.L., 2016. Comparison of the genetic diversity between Guangxi and Yunnan tree shrew populations (*Tupaia belangeri chinensis*). *Acta Lab. Anim. Sci. Sin*., 24: 572–578.

Wang, Y.X., 1987. Taxonomic research on burma-Chinese tree shrew, *Tupaia belangeri* (Wagner), from southern China. *Zool. Res*., **8**: 214–226.

Williams, S.E., Shoo, L.P., Isaac, J.L., Hoffmann, A.A., Langham, G., 2008. Towards an integrated framework for assessing the vulnerability of species to climate change. *PLoS Biol.*, **6**: e325. https://doi.org/10.1371/journal.pbio.0060325

Wu, X., Chang, Q., Zhang, Y., Zou, X., Chen, L., Zhang, L., Lv, L., and Liang, B., 2013. Relationships between body weight, fasting blood glucose concentration, sex and age in tree shrews (*Tupaia belangeri chinensis*). *J. Anim. Physiol. An. N*., **97**: 1179–1188. https://doi.org/10.1111/jpn.12036

Yang, F., He, B.L., He, Y.S., LI, Q., and Jiao, J.L., 2010. Elementary study on five biochemistry sites and eleven microsatellites markers sites of tree shrews (*Tupaia belangeri chinensis*). *J. Kunming med. Univ*., **31**: 8–11.

Yang, X.D., Cao, J., Yang, C., Li, Y., Zhou, L.L., Luo, W., Wang, G.Y., and Li, K.Z., 2016. The genetic polymorphism analysis in tree shrew (*T. belangeri chinensis*) based on flourescent microsatellite. *China Anim. Husb. Vet. Med*., **43**: 182–190.

Yeh, F.C., and Yang, R., 1999. *Microsoft window-based freeware for population genetic analysis (POPGENE Ver. 1.31).* University of Alberta, Alberta, 1999.

Yu, W.H., Yang, C.C., Bi, Y.H., Long, F.Y., Li, Y.L., Wang, J., and Huang, F., 2016. Characterization of hepatitis E virus infection in tree shrew (*Tupaia belangeri chinensis*). *BMC Infect. Dis*., **16**: 80. https://doi.org/10.1186/s12879-016-1418-1

Zhang, H.J., Zhang, H., Qin, X.X., Wang, Z.K., and Zhu, W.L., 2019. Geometric morphometric analysis of skull dimensions of *Eothenomys miletus* from five areas of Hengduan mountains, Yunnan. *Chin. J. Wildl*., **40**: 51–61. https://doi.org/10.1038/s41598-019-51493-2

Zhang, Y., Li, X.F., Li, Z.Y., Tong, Z.Y., Tong, P.F., Chen, L.X., Yin, B.W., and Dai, J.J., 2015. Isolation and chatracterization of microsatellite markers in *Tupaia belangeri chinensis. Chin. J. comp. Med*., **25**: 36–41.

Zhu, W.L., Jia, T., Cai, J.H., and Wang, Z.K., 2014. Study on genetic diversity of mitochondrial cytochrome b gene and D-loop gene in *Tupaia belangeri* from Yunnan Province. *J. Biol*., **31**: 11–14.

Zhu, W.L., Jia, T., Huang, C.M., and Wang, Z.K., 2013. Morphometrics investigation of the skulls, mandibles and molar in *Tupaia belangeri* from Yunnan, Guizhou Guangxi. *Acta Ecol. Sin*., **33**: 1721–1730. https://doi.org/10.5846/stxb201112141910