Genetic diversity and population structure of a Sichuan sika deer (*Cervus sichuanicus*) population in Tiebu Nature Reserve based on microsatellite variation

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Abstract: *Cervus sichuanicus* is a species of sika deer (*Cervus nippon* Group). To date, research has mainly focused on quantity surveying and behavior studies, with genetic information on this species currently deficient. To provide scientific evidence to assist in the protection of this species, we collected Sichuan sika deer fecal samples from the Sichuan Tiebu Nature Reserve (TNR) and extracted DNA from those samples. Microsatellite loci of bovine were used for PCR amplification. After GeneScan, the genotype data were used to analyze the genetic diversity and population structure of the Sichuan sika deer in TNR. Results showed that the average expected heterozygosity of the Sichuan sika deer population in TNR was 0.562, equivalent to the average expected heterozygosity of endangered animals, such as *Procapra przewalskii*. Furthermore, 8 of 9 microsatellite loci significantly deviated from the Hardy-Weinberg equilibrium and two groups existed within the Sichuan sika deer TNR population. This genetic structure may be caused by a group of Manchurian sika deer (*Cervus hortulorum*) released in TNR.

Keywords: Sichuan sika deer; Microsatellite; Genetic diversity; Population structure

The Sichuan sika deer (*Cervus sichuanicus*) (Guo et al, 1978) is a species of the *Cervus nippon* group (Groves and Grubb, 2011). It is an endemic species in China and is listed as a class Ⅰ endangered species, as well as an endangered species by the International Union for Conservation of Nature and Natural Resources (IUCN). Currently, the Sichuan sika deer is distributed in three unconnected areas located in northwest Sichuan Province and southwest Gansu Province. Compared to other species in China, Sichuan has the largest sika deer population in the wild, consisting of about 850 individuals (Guo, 2000).

In recent years, most research has focused on macroscopic aspects such as social behavior (Guo et al, 1991), distribution and quantity surveying (Guo, 2000), activity rhythms (Liu et al, 2004), and food habits (Guo, 2001). At the genetic level, Wu et al (2008) chose 16 microsatellite loci to analyze the genetic diversity of Sika deer in China, including 24 samples of Sichuan sika deer. Lü et al (2006) and Liu et al (2003) studied the genetic relationship of sika deer using mitochondrial DNA, which also had a small number (16) of Sichuan sika deer samples. These studies focused on the differences among species and genetic structure of sika deer in China. Due to the limited samples, however, the above data do not reflect genetic information in one species or population. Moreover, using traditional capture methods makes it difficult to obtain abundant samples to study genetic diversity and population structure within Sichuan sika deer populations.

The Tiebu Nature Reserve (TNR) in the Zoige administrative district of northwest Sichuan has the largest sika deer population. According to monitoring data from TNR, there were about 1 000 Sichuan sika deer

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in the reserve in 2012. TNR has been chosen as the main area to study Sichuan sika deer due to large population size and better protection situation (Li, 2010; Ning et al, 2008; Qi et al, 2010). To date, however, no genetic information studies on TNR sika deer have been conducted and the genetic diversity and possible population substructures remain unknown. Previous studies have shown that the geographical distribution pattern of the population is related to geographical isolation (Boyce et al, 1999; Gavin et al, 1999; Scandura et al, 1998). However, it is unclear whether the rivers, roads, villages, and crop protection fences across the reserve affect gene flow or generate genetic structures within the sika deer population. Therefore, we hypothesized that geographic and artificial factors may affect the gene flow of sika deer, and thus established seven sampling areas in TNR to obtain sika deer fecal samples. Overall, we collected five hides, one tissue sample, and 149 fecal samples. After extracting DNA from those samples, nine Bovinae microsatellite loci were amplified and the products were used for gene scanning. We then used the genotype data to analyze genetic diversity and population structure. The results of this study provide a scientific basis for the protection of the Sichuan sika deer.

MATERIALS AND METHODS

Study area and sampling collection

Fecal samples were collected in seven areas in TNR (E102°46′–103°14′, N33°58′–34°16′, about 206 922 km^2), as shown in Figure 1. To avoid cross-contamination when sampling, we only collected one pellet from each fecal heap. We collected five hides, one tissue sample, and 149 fecal samples. The hide samples were collected from dead individuals in the wild. The only tissue sample came from an individual that had been killed by poachers. All samples were stored in 95% ethanol.

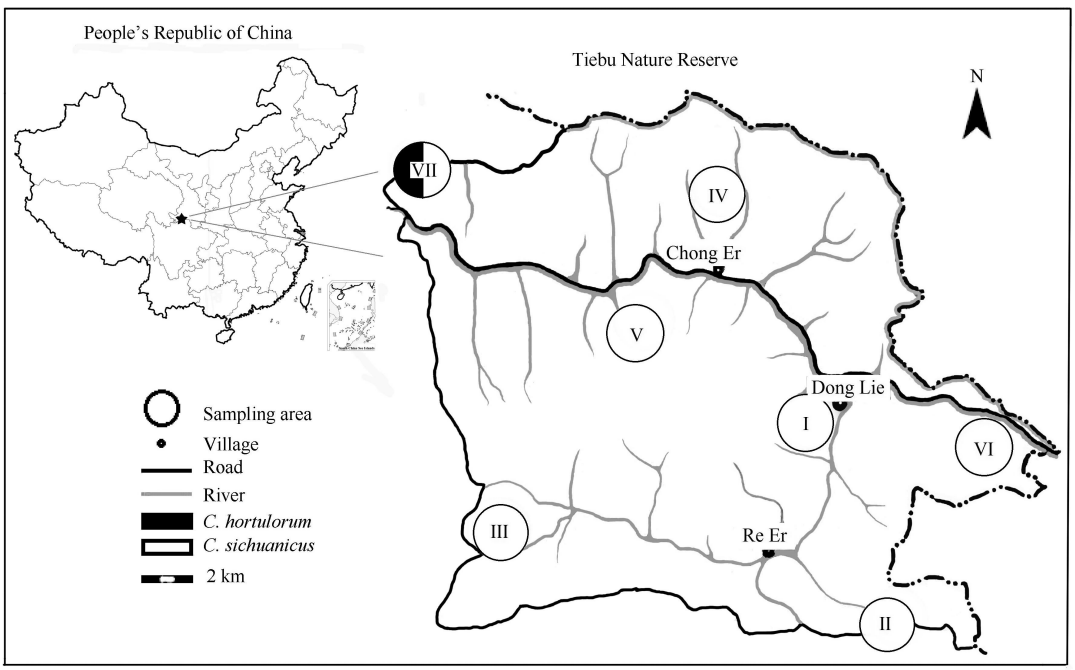

Figure 1 Distribution of sampling areas in Tiebu Nature Reserve

DNA extraction and amplification

DNA was extracted using an E.Z.N.A HP Tissue DNA Maxi Kit and a Stool DNA kit (OMEGA) according to the manufacturer's protocols. The former primers of nine Bovinae microsatellite loci (IDVGA29, BM4107, RT1, TGLA53, RM188, RT13, BM6506, BL42, CSSM019, ETH225, and CERVID14) were modified fluorescent groups. The PCR mixture contained 1.5 μL genomic DNA (100 ng/μL), 1 μL of each primer (10 pmol/μL), 0.5 μL BSA (20 mg/μL), and 7.5 μL of Premix Ex Taq (Takara), with deionized water used to make the sample volume up to 15 μL. The PCR amplification was carried out using an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s,

annealing for 30 s, primer extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The products were preserved at 4 °C. One tissue sample was used as positive control and water as negative control to ensure that PCR was performed correctly and to avoid contamination. The PCR products were sent to Shanghai MAP Biotechnology Co., Ltd (Shanghai, China) for GeneScan.

Table 1 Information on the nine microsatellite bovine loci used in this research

Locus	Repeat motif	Primer sequence	Size range (bp)	Annealing temperature (℃)	Sources of primers
IDVGA29	(AC)n	CCC ACA AGG TTA TCT ATC TCC AG CCA AGA AGG TCC AAA GCA TCC AC	137−157	60	Konfortov et al (1996)
BM4107	(AC)n (TC)n (TG)n	AGC CCC TGC TAT TGT GTG AG ATA GGC TTT GCA TTG TTC AGG	162−172	60	Bishop et al (1994)
RT1	(GT)n	TGC CTT CTT TCA TCC AAC AA CAT CTT CCC ATC CTC TTT AC	222−240	55	Wilson & White (1998)
TGLA53	(AC)n	CAG CAG ACA GCT GCA AGA GTT AGC CTT TCA GAA ATA GTT TGC ATT CAT GCA G	176−216	50	Hoffmann et al (2004)
RM188	(AC)n	GGG TTC ACA AAG AGC TGG AC GCA CTA TTG GGC TGG TGA TT	141−151	50-52	Barendse et al (1994)
RT13	(GT)n	GCC CAG TGT TAG GAA AGA AG CAT CCC AGA ACA GGA GTG AG	293−324	60	Bishop et al (1994)
BM6506	(AC)n	GCA CGT GGT AAA GAG ATG GC AGC AAC TTG AGC ATG GCA C	196−212	59−63	Moore et al (1994)
BL42	(AC)n	CAA GGT CAA GTC CAA ATG CC GCA TTT TTG TGT TAA TTT CAT GC	246−258	58−60	Bishop et al (1994)
CSSM019	(AC)n	TTG TCA GCA ACT TCT TGT ATC TTT TGT TTT AAG CCA CCC AAT TAT TTG	138−156	52−58	Wilson & White (1998)
ETH225	(AC)n	GAT CAC CTT GCC ACT ATT TCC T ACA TGA CAG CCA GCT GCT ACT	140−193	60	Steffen et al (1993)
CERVID14	(AC)n	TCT CTT GCG TCT CCT GCA TTG AC AAT GGC ACC CAC TCC AGT ATT CTT C	214−231	61−65	De Woody et al (1995)

Species identification and population assessment

We amplified all samples using two pairs of primers of the mitochondrial control region and compared the sequences in GenBank to ensure the samples in our research were from sika deer. We used GeneMapper v. 4 (Applied Biosystems) to obtain the microsatellite fragment sizes and identified the heterozygotes and homozygotes. We did two repeats if the genotypes of the two repetitive PCRs were different; for example, if there was a heterozygote in one repeat but a homozygote in the other repeat. If there was a heterozygote in three of four repeats, we considered this site to be a heterozygous locus; the same for a homozygote locus. The probability of identity (*PI*) and the *PI* between siblings were calculated in Gimlet 1.3.3 (Valière, 2002; Liu & Yao, 2013) using genotype data of the nine microsatellite loci. We used the following principle when using Mstools (Park, 2001) to assess the population number: we allowed one genetic mismatch at one allele for one locus to be considered as two samples belonging to the same individual (Bellemain et al, 2005). To detect the validity of the above principle, we randomly selected 50 fecal samples (accounting for a third of the total number) and amplified the nine loci twice. At the same time, we amplified another two loci (ETH225 and CERVID14) of the selected 50 samples. We tested whether the influence of population number may be caused by the repeat amplification and increasing loci.

Genetic diversity and population structure

We computed the expected heterozygosity (H_E), observed heterozygosity (H_O), and polymorphism information content (*PIC*) using Mstools. Arlequin 3.1 (Excoffier et al, 2005) and Fstat 2.9.3.2 (Goudet, 2001) were

used to test the Hardy-Weinberg equilibrium and *Fis* separately. The fixation index (F_{ST}) (Weir & Cockerham, 1984) among sampling areas was tested in Arlequin 3.1, using 1 000 random iterations to calculate confidence intervals (*CI*). Software structure 2.3 (Pritchard et al, 2000) was used to estimate the potential populations (*K*). The admixture model was used and run three times using 10 replications of *K* ranging from 1 to 7. For those runs the following conditions were adopted: a burn-in period at 10 000 following 10 000 000 replicates of the MCMC. The ΔK calculated as per Evanno et al (2005) was used to estimate optimal *K*. The formulas were: $\Delta K=m|L(K+1)-2L(K)-L(K-1)|/s[L(K)]$. We referred to the structure output $\ln P(D)$ as $L(K)$. *m* was the mean value and *s* was the standard deviation. Software CLUMPP Windows.1.1.2 (Jakobsson & Rosenberg, 2007) and destruct (Rosenberg, 2004) were used to visualize the individual coefficients of membership in the subpopulation calculated by structure 2.3.

RESULTS

Species identification and population assessment

Comparing the obtained sequences of mitochondrial hypervariable region I from our research to the corresponding sequence in GenBank, all samples were obtained from sika deer. Thereinto, 143 of 155 samples belonged to the haplotypes of Sichuan sika deer and the remainder belonged to the haplotypes of Manchurian sika deer (*Cervus hortulorum*).

Most of the Sichuan sika deer samples could be amplified at all the nine microsatellite loci, few samples (11) had a loss at one locus. Analyzing the united *PI* values for nine loci, we found that the discrimination power of nine loci was close to 100% (Figure 2). According to the rule of Bellemain, we assessed 76 sika deer individuals from 143 face samples. After the second amplification of the randomly selected samples, the population number did not change. The additional two loci (ETH225 and CERVID14) increased the population by one individual, accounting for 3% of the total number. The distribution of the 76 individuals in the samplings areas is shown in Table 3.

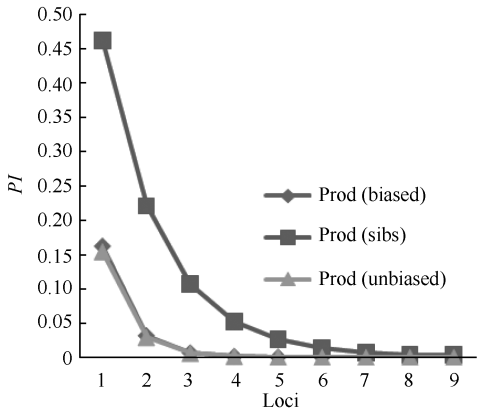

Figure 2 Multi-loci *PI* (biased and unbiased) and *PI* (sibs) for sika deer in increasing order of single-locus values

Genetic diversity and population structure

In the Sichuan sika deer population in TNR, the mean number of alleles / locus was 6.56 and the H_E was 0.562. The *PIC* values were higher than or close to 0.5, except for RT13 (0.129). All detected loci deviated from the Hardy-Weinberg equilibrium significantly (Figure 2) and the *P* value was 0. In addition to the locus RT13, the *Fis* values of the remaining eight loci were negative. The values of H_E ranged from 0.4292 to 0.6865 in the seven sampling areas and all H_O values were higher than those of H_E (Table 3). The F_{ST} among the seven sampling areas ranged from –0.04781 to 0.05243. The highest F_{ST} (0.05243) appeared between Area Ⅳ and Ⅵ but the lowest (–0.04781) appeared between Area Ⅰ and Ⅲ (Table 4).

Table 2 Microsatellite diversity indices of Sichuan sika deer in Tiebu Nature Reserve

Locus	No. of alleles	H_E	H_O	*PIC*	*Fis*
IDVGA29	5	0.587	0.722	0.516	–0.230
BM4107	7	0.560	0.654	0.495	–0.169
RT1	6	0.633	0.951	0.558	–0.506
CSSM019	6	0.635	0.926	0.560	–0.462
TGLA53	8	0.643	0.899	0.571	–0.401
BL42	9	0.607	0.913	0.523	–0.509
RM188	6	0.656	0.738	0.607	–0.125
BM6506	8	0.604	0.974	0.519	–0.620
RT13	4	0.134	0.026	0.129	0.810
Mean	6.56	0.562	0.756	0.496	–0.347

Table 3 Indices of genetic diversity of Sichuan sika deer in different sampling areas

Sample area	Sample size	Loci typed	H_E	H_O
Ⅰ	10	9	0.4292	0.7160
Ⅱ	13	9	0.5499	0.8034
Ⅲ	7	9	0.4831	0.7778
Ⅳ	8	9	0.6804	0.7284
Ⅴ	20	9	0.6865	0.7444
Ⅵ	11	9	0.4350	0.7063
Ⅶ	7	9	0.5368	0.8571

Table 4 *F*-statistics (F_{ST}, below diagonal) and significant differences among different areas

Sample area	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ	Ⅶ
Ⅰ		+	–	+	+	–	+
Ⅱ	–0.00493		–	–	–	+	–
Ⅲ	–0.04781	–0.03753		–	–	–	–
Ⅳ	0.03553	–0.0052	–0.00225		–	+	–
Ⅴ	0.05177	0.00789	0.01646	–0.01778		+	–
Ⅵ	–0.02823	–0.00142	–0.0334	0.03287	0.05243		+
Ⅶ	0.01389	–0.02825	–0.03837	–0.01869	0.01487	–0.00278	

+ denotes significant difference, – in contrast (95% confidence interval).

Population structure

Structure analysis showed that when K=2, the ΔK exhibited an obvious apex (Figure 3). The F_{ST} between subpopulations was 0.535. Individual coefficients of membership in subpopulations (Figure 4) showed that when K=2, the genetic amount of cluster 1 (Yellow) was larger and distributed in all sampling areas. In contrast, the genetic amount of cluster 2 (Blue) was less than cluster 1, and had a relatively smaller distribution area. Most of the blue genotype was distributed in Areas Ⅳ and Ⅴ.

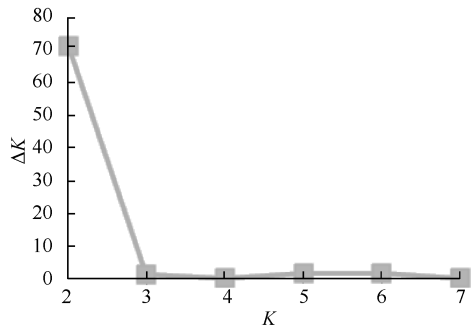

Figure 3 Value of ΔK as a function of K based on 10 runs

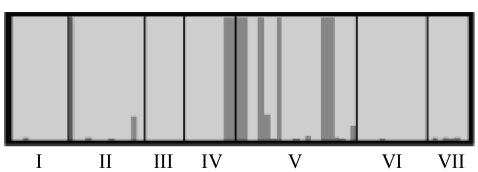

Figure 4 Individual coefficients of membership in each sampling area

DISCUSSION

Population assessment

In recent years, non-invasive sampling methods have been widely used in genetic studies of endangered animals (He et al, 2010; Wang et al, 2012). Because of the endangered status and fugacious feature of sika deer, non-invasive sampling methods (obtaining fecal samples) have become an important means of population assessment and individual identification. In this study, the stability of repeat amplification of nine microsatellite loci had no effect on assessing the population number, that is, our genotype data were reliable. After adding two loci, there was a 3% error between previous and post assessment results, which is much higher than 2% (Bellemain et al, 2005). Bellemain et al (2005) attributed such errors in population assessment to pseudogenes, pollution and missing data.

Genetic diversity and differentiation

Genetic diversity helps populations adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. The population will continue for more generations because of the success of these individuals (Khater et al, 2011). In our research, the H_E of Sichuan sika deer

population was 0.562, close to the mean H_E (0.559) of sika deer in China, but much higher than the mean H_E for Sichuan sika deer (0.477) and slightly lower than the mean H_E for Manchurian sika deer (0.584) and south China sika deer (Jiangxi, 0.585; Zhejiang, 0.589) (Wu et al, 2008). Compared to other Ungulata, such as red deer (*Cervus elaphus*) (H_E=0.78, Hajji et al, 2007; H_E=0.804, Pérez-Espona et al, 2008; H_E=0.62−0.85, Zachos et al, 2007), forest musk deer (*Moschus berezovskii*) (H_E=0.8−0.9, Zhou et al, 2005), and Mongolian wild ass (*Equus hemionus*) (H_E=0.83, Kaczensky et al, 2011), Sichuan sika deer had a relatively lower H_E and was equivalent to Przewalski's Gazelle (*Procapra przewalskii*) (H_E=0.552, Yang & Jiang, 2011) and milu (*Elphurus davidianus*) (H_E=0.46−0.54, Zeng et al, 2007). The H_Es values were different in each sampling area. Area Ⅰ had the lowest H_E (0.4292), while Area V had the highest (0.6865). Polymorphism information content (*PIC*) is a measure of variation between microsatellite loci. It is generally considered that a *PIC* value greater than 0.5 is for highly polymorphic loci, a *PIC* value of less than 0.5 and greater than 0.25 is for moderately polymorphic loci, and a *PIC* of less than 0.25 is for low polymorphic loci (Niu et al, 2001). In this study, the *PIC* values of six of the nine microsatellite loci were higher than 0.5, indicating highly polymorphic loci, while two were moderately polymorphic loci and one was a low polymorphic locus. Heterozygosity and *PIC* information indicated Sichuan sika deer in TNR had abundant genetic diversity.

Hartl & Clark (1997) considered that population deviation from the Hardy Weinberg equilibrium was mainly due to small populations, not random mating, gene mutation, migration or other factors. The present study showed that nine microsatellite loci of the Sichuan sika deer population in TNR deviated significantly from the Hardy Weinberg equilibrium. The Sichuan sika deer population numbers in TNR grew steadily from 650 in 2000 (Guo, 2000) to 1000 in 2012 (monitoring data from TNR). As a result, the deviation from the Hardy Weinberg equilibrium is unlikely attributed to a reduction in the population. The coefficient of genetic differentiation (F_{ST}), on the other hand, is an important indicator of genetic differentiation among subpopulations. According to Wright (1978), F_{ST}s values between 0−0.5 suggest no differentiation between subgroups. The F_{ST}s values in our seven sampling areas were all less than 0.5, ranging from 0.04781 to 0.05243, and showing negative value in some regions. This indicated that the genetic variation among the seven sampling areas mainly came from inside the sampling area, and the geographical barriers and human factors do not affect gene flow in Sichuan sika deer in TNR. In addition, due to the relatively closed geographical position of TNR, very few little sika deer were distributed in the periphery of the protected areas (Guo, 2000). Gene flow through immigration and emigration could not significantly affect the Sichuan sika deer populations to deviate from the Hardy Weinberg equilibrium.

Microsatellite polymorphism studies of sika deer in China found that 16 microsatellite loci significantly deviated from the Hardy Weinberg equilibrium, but H_Es were higher than H_Os (Wu et al, 2008). In the present study, H_Os were significantly higher than the H_Es. The *Fis* values of eight microsatellite loci were negative, showing heterozygosis excess. When a population experiences a reduction in its effective size, it generally develops heterozygosity excess at selectively neutral loci (Cornuet & Luikart, 1996). Xu et al (2009) used microsatellites to detect the population structure of Himalayan marmots and found heterozygosity excess due to historic population decline. Wu et al (2008) tested bottlenecks in four Chinese sika deer species and found that Sichuan sika deer had not experienced a population bottleneck in recent history. Therefore, the heterozygosity excess of Sichuan sika deer in TNR could not be caused by population reduction.

Population structure

A population composed of individuals from two different source populations will tend to have an excess of heterozygotes (Milkman, 1975). In testing population structure in the present study, we found that when K=2, ΔK exhibited a significant apex, indicating that two subpopulations existed within the Sichuan sika deer population. Considering that the Sichuan sika deer population had heterozygote excess and two subpopulations, we speculated that the sika deer in TNR might have two different sources populations. According to Wu (2004) and TNR staff, a captive-bred Manchurian sika deer group was bred and released in TNR in the late 1960s. The mitochondrial data showed that haplotypes of Manchurian sika deer (unpublished data) do exist. Previous research has shown that introduced species often hybridize with local sibling species (Avise et al, 1974; Gutiérrez, 1993; Perry & Goudet, 2001) and

panmictic admixtures can develop rapidly over a short period of time (Echelle & Connor, 1989; Echelle, 1987; 1997). Hybridization abounds in Cervidae, and introduced sika deer have been shown to hybridize with native red deer (Senn & Pemberton, 2009). Therefore, there is a strong possibility that hybridization occurred between the two sika deer species in our research. The released Manchurian sika deer explained the existence of subpopulations in the Sichuan sika deer population in TNR. We speculate that the Manchurian sika deer genotype is the minority – the blue parts in the individual coefficients of membership, which were mainly distributed in Areas Ⅳ and Ⅴ– because the number of introduced Manchurian sika deer is not clear and of the 155 sika deer fecal samples examined, only 12 belonged to the haplotypes of Manchurian sika deer. The Sichuan sika deer population were in larger quantities than that of Manchurian sika deer. So, the yellow parts in Figure 4 indicate the genotypes of Sichuan sika deer.

In the past, researchers have defined the Sichuan sika deer and Manchurian sika deer as two subspecies of sika deer. Groves & Grubb (2011) compared antecedent research on the morphology and molecular data of sika deer and suggested the Sichuan and Manchurian sika deer were two independent species in the *Cervus nippon* group. If hybridization and introgression occurred, it may lead to a local genetic extinction of a population (Avise et al, 1997; Templeton, 1986). From the perspective of conservation biology, how genes flow between two species mixed by human influence is a focus for future research.

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