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Genetic diversity among Chinese sika deer (*Cervus nippon*) populations and relationships between Chinese and Japanese sika deer

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Abstract Sika deer (*Cervus nippon*) is a cervid endemic to mainland and insular Asia and endangered. We analyzed variation in the mitochondrial DNA (mtDNA) control region for four subspecies to understand the genetic diversity, population structure and evolutionary history in China. 335 bp were sequenced and eight haplotypes were identified based on 25 variable sites among the populations. Sika deer in China showed lower genetic diversity, suggesting a small effective population size due to habitat fragmentation, a low number of founder individuals, or the narrow breeding program. AMOVA analysis indicated that there was significant genetic subdivision among the four populations, but no correlation between the genetic and geographic distance. Phylogenetic analyses also revealed that Chinese sika deer may be divided into three genetic clades, but the genetic structure among Chinese populations was inconsistent with subspecies designations and present geographic distribution. Including the sequence data of Japanese sika deer, the results indicated that Chinese populations were more closely related to Southern Japanese populations than to the Northern Japanese one, and the Taiwan population was closer to populations of Northeastern China and Sichuan than to those of Southern China.

Keywords: ***Cervus nippon*****, mtDNA, tandem repeat, genetic diversity, population structure, phylogenetic relationship.**

Sika deer, *Cervus nippon*, was historically widespread throughout northeastern Asia, from the Ussuri region to Vietnam, including the Korean peninsula, mainland China and Taiwan, and the Japanese archipelago, and up to 13 subspecies have been described[1]. The fossil record indicates that the sika deer originated during the late Pliocene or early Pleistocene and their ancestors were distributed across most of China during the Pleistocene[2]. The species has been extirpated from much of its historical range due largely to hunting and habitat degradation, and is now only found in small fragmented populations on the Asian mainland and in Japan[2,3]. This species currently has Class I Protected Status in China, and is on CITES Appendix I.

Historically, six subspecies were described based on morphological data and their distribution in China: *C. n. sichuanicus* in Sichuan and Gansu Provinces; *C. n. kopschi* in the southern of China including Anhui, Jiangxi, Zhejiang, Hubei, Hunan, Fujian, Guangdong and Guangxi provinces; *C. n. hortulorum* in northeast China; *C. n. grassianus* in Shanxi Province; *C. n. mandarinus* in Hebei Province; *C. n. taiouanus* in Taiwan[2]. Of these six subspecies, *C. n. grassianus* and *C. n. mandarinus* are extinct in the wild[4]. *C. n. hortulorum* had been considered to be on the edge of extinction in the wild until a 300-individual stable population was rediscovered along the border between China and Russia[5]. The Taiwanese subspecies, *C. n. taiouanus,* was extremely abundant on the southwestern coastal plain, but declined rapidly and was extinct in the wild by the late 1960s because of heavy hunting and conversion of its habitat[6]. However, a captive breeding program for sika deer was also established in the Kenting area in 1984, and some wild sika deer populations have been founded from captive stock in Kenting National park and Green Island with about 210 and 200—250 individuals present, respectively[6]. The remaining two subspecies, *C. n. sichuanicus* are distributed in a few small isolated locations in Sichuan and Gansu Provinces, and *C. n. kopschi*, is limited to some small fragmental ranges in Anhui, Jiangxi and Zhejiang provinces[4].

Several molecular studies on Japanese sika deer have been also conducted recently. Molecular analyses indicate that the Japanese sika deer is separated into two main lineages[7,8], with northern distributions encompassing Hokkaido and Northern Honshu and southern distribution Honshu and Kyushu. The two lineages form a contact zone in mid-Honshu in the region of

Hyogo. The distribution of genetic variation in Japanese sika deer has several other interesting features. Deer populations in Hokkaido have at least six maternal lineages and appear to have recolonized the island after a historical bottleneck[3]. Elsewhere, Tamate *et al.*[9] suggested the Kinkazan Island deer preserve extensive genetic variation despite its small population size and isolation. Goodman *et al.*[8] observed extreme levels of differentiation and significant differences in diversity among populations across the whole of Japan resulting from recent drift. In his study, although habitat fragment size was not significantly associated with genetic diversity, there was a significant correlation between habitat fragment size and effective population size.

Although many studies conducted on sika deer in China to date have concentrated mainly on their morphological, ethological, ecological variation, as well as management and conservation, there is no study concerning the genetic status of Chinese sika deer at present. Because China is the primary distribution of sika deer and evolutionary centre of deer[10], and the above-mentioned molecular studies did not include any non-Japanese sika deer populations, especially Chinese sika deer. In this study, we used mtDNA control region sequences to characterize the genetic diversity and structure among Chinese sika deer, and to explore the evolutionary history and the phylogenetic relationships among the populations from China and Japan through the genetic analysis of our data and published Japanese sika deer data[3,7].

1 Materials and methods

Forty-five samples (blood, muscle, hair, bone, or skin) were collected from four populations in China, including 10 bone and 6 skin samples of *C. n. sichuanicus* in Sichuan population (SC), 5 skin and 1 muscle samples of *C. n. kopschi* in Southern China population (SHC) from the wild, 17 blood samples of *C. n. hortulorum* in Northeastern China population (NEC), and 6 hair samples of indigenous *C. n. taiouanus* in Taiwan population (TW) (Green Island) sampled by Dr. Pei Jia-Chyi (Fig. 1). In addition, one haplotype in Table 1, CN6, was cited from Nagata *et al.*[7], which came from *C. n. kopschi* in SHC. 171 mtDNA CR sequences of Japanese sika deer[3,7], which came from Southern Japanese population (SJ) and Northern Japanese population (NJ), were added to this study to explore the re-

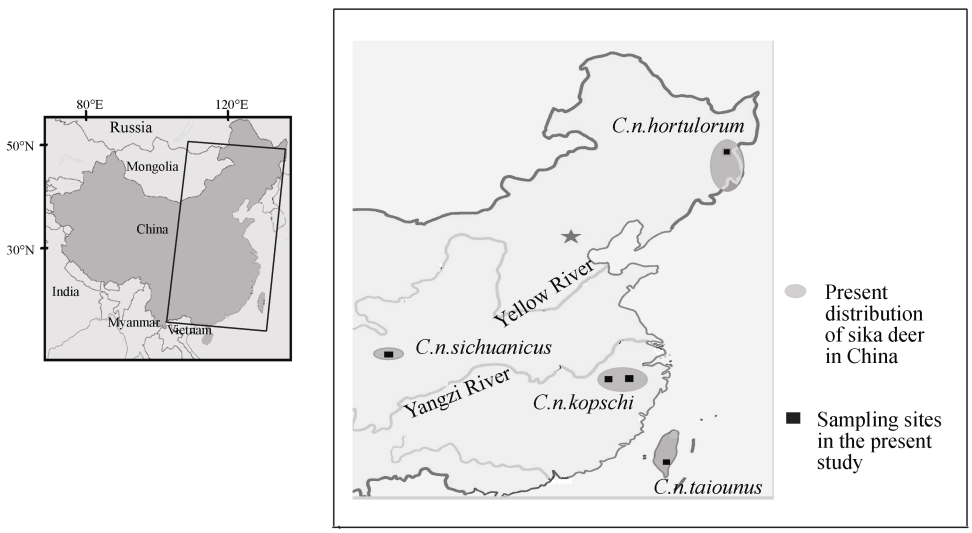

Fig. 1. Present distribution of the sika deer (*Cervus nippon*) in China, and the sampling sites used for this study.

Table 1 Subspecies, tissue types, number of samples (*N*), location and haplotype in this study

Subspecies	Tissue	N	Location	Haplotypes
C. n. hortulorum	blood	17	Jilin Province	CN1, CN2, CN3, CN4
C. n. sichuanicus	skin, bone	16	Sichuan Province	CN4
C. n. kopschi	muscle, skin	6	Anhui and Jiangxi Provinces	CN5
		1	Anhui Province	CN6
C. n. taiouanus	hair	6	Taiwan Province	CN7, CN8

lationships between the Chinese sika deer and Japanese sika deer.

Genomic DNA from blood and muscle was extracted following standard protocols[11]. Genomic DNA from hair was isolated using Chelex 100 (Bio-Rad) and the 20 μL resultant supernatant was used for PCR. DNA from skin samples was extracted following[12]. DNA extraction from bone samples was also based on Rao *et al.*[12] after removing calcium with 0.5 mol/L EDTA (pH8.0).

Deer-specific primers (LD5 5′-AAGCCATAACCCCACTATCAA-3′ and HD8 5′-TGGACGTAATGCGCTATGTA-3′) were used for polymerase chain reaction (PCR) amplification and sequencing[3]. Double-stranded DNA was amplified in a total reaction volume of 30 μL, which included 5—25 ng of genomic DNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2.0 mmol/L Mg^{2+}, 200 μL each dNTP, 10 pmol of each primer, and 1.0 U *Taq* DNA polymerase (Promega). The PCR was conducted by pre-denaturing at 94℃ for 5 min, then following by 35 amplification cycles of 94℃ for 30 s, 54℃ for 30 s, and 72℃ for 50 s with a final 7 min extension step at 72℃ using a PTC-200 DNA thermocycler (MJ Research Inc.). PCR products were purified using the Spin Column-PCR Purification Kit (Shanghai Huaxun Biology and Technology Ltd.) and sequenced using a Prism™ BigDye Terminator Ready Reaction kit (Applied Biosystem Inc.) and run on an *ABI-PRISM*™ 310 Genetic Analyzer (Applied Biosystem Inc.). Each PCR product was sequenced in both directions.

Forward and reverse sequences for each sample were aligned using *Clustal* X and rechecked by eye. The sequences for each haplotype are available in GenBank (AF465415—AF465419, AY764391, and AY764392).

Genetic diversity among all populations was estimated using haplotype (h) and nucleotide diversities (π) using DnaSP 3.0[13]. ARLEQUIN 2.0[14] was used for analysis of molecular variance (AMOVA) to test for differentiation among populations. Differentiation was quantified using Φ_{ST} with 1000 random permutations. We assessed the correlation between genetic distance and geographic distance by applying a Mantel's permutation test[15]. Also, the genetic distances between Chinese populations, within and among Chinese populations, SJ and NJ were estimated using the Kimura 2-parameter model in MEGA2.0[16].

In order to explore the relationships among all haplotypes from Chinese and Japanese sika deer populations, phylogenetic trees between all haplotypes were reconstructed in PAUP* 4.0b4a[17] using maximum likelihood (ML) and parsimony (MP) methods. The red deer (*Cervus elaphus*) was used as an outgroup. The Tamura-Nei model (TrN) with gamma (G) distribution was applied as it was selected as best-fit model for maximum-likelihood analysis by Modeltest 3.06[18]. In phylogenetic analyses, all gaps were regarded as missing sites, and bootstrap values were derived from 100 and 1000 replicates in ML and MP analysis, respectively. Because low sequence diversity was expected, a statistical parsimony network was constructed with MINSPNET[19], since haplotype networks may more effectively portray the relationships among closely related populations[20].

2 Results

2.1 Control region sequence variation and genetic diversity

Sequence data were collected for 335 bp of the mtDNA control region from 45 sika deer individuals from Chinese populations (including Sichuan population, Southern China population, Northeastern China population and Taiwan population). There were 25 variable sites including 20 transitions, 5 insertions or deletions, and no transversion. Eight haplotypes (CN1—CN8) were identified among four Chinese populations (haplotype CN6 cited from Nagata *et al.*[7] (Table 1). Of all haplotypes, CN4 was the most frequent occurring in two populations: Sichuan and Northeastern China populations. Four haplotypes (CN1—CN4) were found in 17 individuals from the Northeastern China population. CN5 and CN6 appeared only in the Southern China population, and CN7 and CN8 were only observed in Taiwan population. The Sichuan population had a single haplotype CN4 that was shared with Northeastern China population.

After combining our data with 171 mtDNA control region sequences from Japanese sika deer[3,7], the final alignment was 470 bp in length due to tandemly repeated elements (see below). Among the 216 sequences, there were 189 variable sites including 63 transitions, one transversion and 114 insertions or deletions. Among 470 bp of sika deer control region sequence, there were seven repeat units, where their length was 37—40 bp, from 133th to 404th site reported by Nagata *et al.*[7]. Of these, unit 1—4 were shared by all Chinese and Japanese populations; unit 5—7 were not found in

Chinese populations; units 5－7 or 6－7 were not observed in Southern Japanese populations; unit 7 was found only in some populations of Northern Japanese, and only unit 1 was present in red deer. To prevent the large repeats in the control region from biasing the results, the repeat sequences of unit 5－7 were excluded from the data set before analyzing phylogenetic relationships among Chinese and Japanese populations. Consequently, twenty-five haplotypes were identified with six in mainland China (CN1－CN6), two in Taiwan Island of China (CN7 and CN8), eleven in Northern Japan (N1－N11) and eight in Southern Japan (S1－S8).

Haplotype diversity (h) ranged from 0 (Sichuan population) to 0.684 (Northeastern population) among all Chinese populations (Table 2). The sequence differences among eight haplotypes were 0.28%－4.85% (excluding insertions and deletions). Nucleotide diversity (π) among all mainland China samples was 2.136% but varied widely between populations, ranging from 0 in Sichuan population to 1.95% in Northeastern population (Table 2). When all populations within mainland China were regarded as one population, and compared with the Taiwan population and Southern (8 haplotypes) and Northern (11 haplotypes) populations of Japan, the haplotype diversity in the Southern Japan was the highest and that in Taiwan was the lowest (Table 2).

2.2 Genetic structures of Chinese sika deer and phylogenetic relationships between Chinese and Japanese sika deer

From an AMOVA analysis in Arlequin 2.0, the proportion of genetic diversity attributable to variation within populations and among Chinese populations was 25.6% and 74.4%, respectively (Table 3). Also, there are high average Φ_{ST} values [Φ_{ST} = 0.744 (0.694－1), p<0.05]. Thus, our data demonstrated the strong population subdivision at the level of mtDNA among Chinese sika deer populations. Matrices of geographic and genetic distances were not significantly correlated (g= –0.4875, p>0.05).

The MP and ML trees based on control region sequences among all sika deer haplotypes (including Japanese haplotypes) (Fig. 2) supported the results obtained from the genetic variance analysis and were also identical to the minispan network (MSN) (Fig. 3). Haplotypes from China, Southern Japan and Northern Japan each form monophylectic clades which were supported by bootstrapping, indicating that strong phylogeographic structure is present among Chinese and Japanese populations. Figs. 2 and 3 show that all Chinese populations, Southern Japanese populations and Northern Japanese populations form three different

Table 2 Haplotype distribution and frequency, haplotype diversity (h±SD), and nucleotide diversity (π) of sika deer, *Cervus nippon*, within 4 population in China, and Southern and Northern population of Japan

Population	MtDNA haplotype								$h\pm$SD	π
	CN1	CN2	CN3	CN4	CN5	CN6	CN7	CN8		
Northeast China	3	3	9	2					0.684±0.096	0.0195
Sichuan				16					0	0
Southern China					6	1			0.286±0.196	0.00173
Taiwan							2	4	0.533±0.172	0.0016
Total	3	3	9	18	6	1	2	4		
Mainland of China									0.731±0.051	0.02136
Taiwan									0.533±0.172	0.0016
Southern Japan									0.916±0.06	0.0146
Northern Japan									0.623±0.03	0.0035

Table 3 Φ_{st} values between populations and percent of variation within and among populations in Chinese sika deer[a)]

	NEC	SC	SHC	TW
Northeast China (NEC)				
Sichuan (SC)	0.694*			
Southern China (SHC)	0.718*	0.875*		
Taiwan (TW)	0.711*	1.000*	0.971*	
Percent of variation among populations		74.4		
Percent of variation within populations		25.6		
Φ_{ST} among populations		0.744		

a) *, p<0.05.

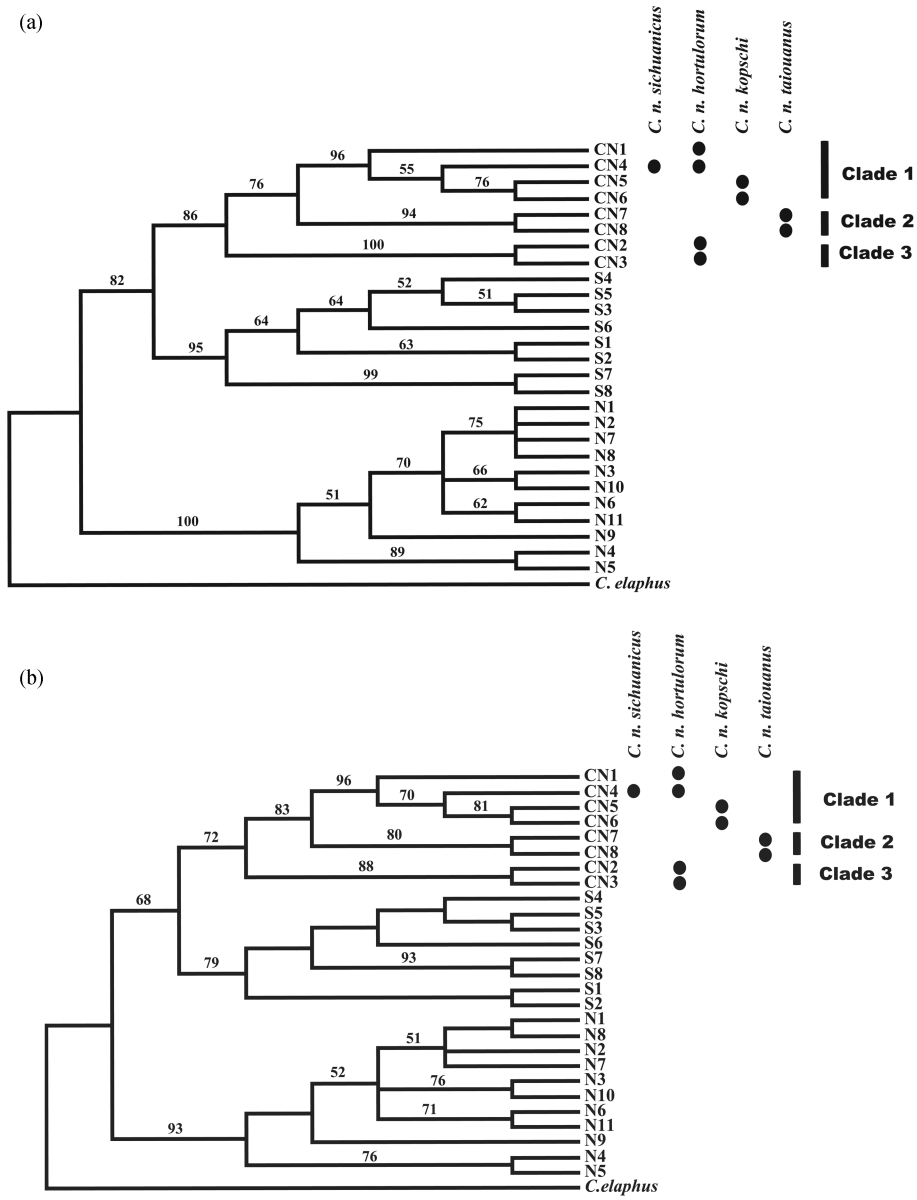

Fig. 2. Phylogenetic trees of the sika deer based on the control region 470 bp sequence. (a) ML tree was constructed based on the TrN+*G* model and rooted from *Cervus elaphus* (–In*L*=1215.33966), and the values above branches are bootstrap percentages (100 replications); (b) MP tree was constructed using exhaust search and bootstrap values were obtained from 1000 replications (Tree length = 98, CI = 0.6735).

lineages. However, the phylogeographic structure among haplotypes of the Chinese populations was not related to geographic distribution or subspecies designation (Fig. 2). The results indicate three clades among Chinese haplotypes: 4 haplotypes in Clade 1 from Northeastern China, Sichuan and Southern China populations, 2 haplotypes in Clade 2 from the Taiwan population, and 2 haplotypes in Clade 3 from the Northeastern China population. The number of substitutions separating Chinese populations and Southern Japan populations, and Chinese populations and Northern Japan populations is 19 and 25, respectively, greater than that within populations. For the Chinese populations, the number of substitutions between Clade 3 and the other two Clades was 14, which was more than that between Clade 1 and Clade 2, and within each clade (2—4, 0 and 2 within Clade1, Clade 2 and Clade 3, respectively) in the Chinese populations (Fig. 3). Thus, our results suggested a close relationship between Chinese populations and the Southern Japanese popula-

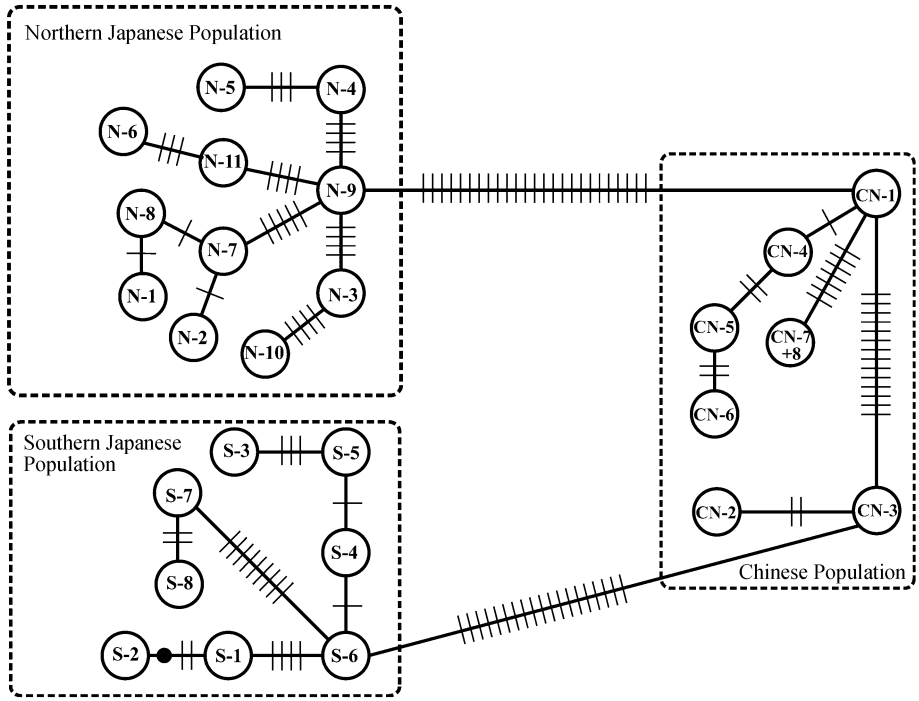

Fig. 3. Minimum spanning network for haplotypes. Haplotype names correspond to the names in Table 1. Nucleotide transitions and transversions are indicated by dashes and dots, respectively.

tions, and that the Taiwan population was closer to populations of Northeastern China and Sichuan than those of Southern China (Fig. 2).

3 Discussion

3.1 Tandemly repeated sequences and genetic diversity of sika deer in China

Previous studies have shown the existence of tandemly repeated sequences in the control region of several artiodactyl species (such as Japanese sika deer[3,7,21], Red deer[22] and other mammals[23]. For sika, Nagata *et al.*[3,7] reported up to seven tandemly repeated sequences of 37—40 bp with the number of repeat units differing among Japanese sika deer populations. However, Cook *et al.*[21] reported that samples of *C. n. nippon* had only three and not seven tandem repeats, although seven tandem repeat was found in one sample which may mistake its name[24]. Randi *et al.*[24] thought that the number of tandem repeats was up to six within Japanese sika deer. Comparing our data with Japanese sika deer in the present study, we found a maximum of 7 and 4 repeat units (37—40 bp) was present within Japanese sika deer and Chinese sika deer, respectively. Of all repeat units, units 1—4 were shared by all Chinese and Japanese populations, and units 5—7 were missing in Chinese populations and some populations of Southern Japan. Also, we suggest that unit 1 of all units may reflect the ancestral form with other units being derived from this unit by replication slippage[7,21,24], because the this unit also presented in outgroup (*Cervus elaphus*). The reasons that our result was different from those of other studies might result from the following factors: few sequences from Chinese sika deer samples in above-mentioned studies when aligning, or partial sequences for alignment, or sequences from mislabeled samples or hybrid deer[25].

Our estimates of genetic diversity values (Table 2) of Chinese sika deer were lower than those estimated for the mtDNA control region of many ungulates, such as kob (4.6%)[26], but similar to those of other deer, e.g. wapiti (1.07%)[27], 1.46% and 0.35% for Southern Japan and Northern Japan sika deer, respectively[3,7], and eld's deer[28]. When the three populations in mainland China were regarded as one large population and compared with the Taiwan population and the two Japanese populations, the highest genetic and haplotype diversity within populations were found in mainland China and Southern Japan, while the lowest variation was detected in the northern Japanese population. This limited variation may be attributed to a bottleneck[29] or a small ef-

fective population size relative to current census size[30]. For Japanese sika deer, Nagata *et al.*[3] and Goodman *et al*.[8] concluded that low genetic and haplotype diversity of northern Japanese population resulted from a severe historical bottleneck. China is regarded as the evolutionary center of deer and fossils of sika deer have been found in many sites[10]. However, the distribution and numbers sharply have decreased since the 19th century[2] which may have led to the loss of genetic diversity due to bottleneck, small effective population size, or genetic drift. Then, what is primary factor resulting in the low genetic diversity of Chinese sika deer? For the Sichuan population, h and π were 0 based on 17 samples. Guo[31] reported that *C. n. sichuanicus* were distributed over most of Sichuan Province, but that small populations existed in only three isolated regions at present. These samples were bone from died individuals before population isolation[1)]. Thus, we suggest that the low genetic diversity of this population might be the result of a small effective population size due to habitat fragmentation[30]. As to *C. n. kopschi*, it was also reported that the present population is very small in fragmented regions[32], however, only 6 samples may not be sufficient to describe the erosion of genetic diversity. Thus, more samples are needed for further analysis. Our results for the Taiwan population were in accord with those of Cook *et al.*[21]. Taiwan sika deer has evolved in isolation for about 10000－15000 years since the flooding of the last landbridge between the island and mainland China, which separated it from other sika deer populations[33], and it has been extinct in the wild since the late 1960s[6]. The present wild Taiwan population was founded from the captive herd[34]. Accordingly, the low number of founder individuals, bottlenecks and isolation might have led to the low genetic diversity[21]. For the Northeastern population, it is not surprising that h and π are higher than those of other populations because the captive populations were from more wild population lineages in the past[2)].

3.2 Population structure among Chinese sika deer populations

The AMOVA indicated that there was significant genetic subdivision among the four Chinese populations (average $\Phi_{ST} = 0.744$, $p<0.01$), and 74.4% of the total variation was also found among populations (Table 3). However, phylogenetic analysis revealed that the genetic structure among Chinese populations was inconsistent with subspecies designations and present geographic distributions (Fig. 2). Although the distances among these populations are more than 1000 km, there was no significant correlation between genetic and geographical distances by a Mantel test ($p>0.05$)[15]. Accordingly, the genetic structure of the Chinese sika deer might not result from isolation by distance. Of the eight haplotypes, CN4 was shared by NEC and SC, and all NEC haplotypes were in clades1 and 3 (Fig. 2), which suggested that either hybridization or secondary contact has occurred between NEC and SC or they were so recently derived that there was incomplete lineage sorting. Fossil records indicate that the ancestor of modern sika deer was mainly distributed in NEC and expanded westwards and to the south and north after the uplift of Qinhai-Tibetan plateau[2]. This scenario is supported by phylogenetic analysis, which indicates that NEC haplotypes branch basally from the Chinese populations.

3.3 Relationship between Chinese and Japanese sika deer

Generally, it is thought that sika deer originated in northeast Asia during the late Pliocene or early Pleistocene after diverging from an ancestor shared with *Cervus elaphus*, and the insular populations of Japan were the result of colonization from the mainland during one or more sea level lows[21]. In the present study, the data showed that there were three lineages among Chinese populations, Southern Japanese populations and Northern Japanese populations (Figs. 2 and 3), which agrees with the results[7,21] that Japanese populations were descendants from at least two lineages. As depicted in Figs. 2 and 3, Chinese and Southern Japanese populations appear more closely related than the Chinese and Northern Japanese populations. Based on the supposed evolutionary processes of tandem repeats[7,21] and the different numbers of repeats observed, we also suggest that Chinese and Southern Japanese population are more closely related than Chinese and Northern Japanese populations. The evolutionary center of deer and the earliest sika fossils are in China[10,21] and the Japanese sika deer might come from Asia continent[7,21], but whether the Japanese sika came from Chinese sika is

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not clear yet. More samples from Siberia, the Korean peninsula and Vietnam must be added to these data for further analysis.

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