

ORIGINAL ARTICLE

Classification and phylogeny of sika deer (*Cervus nippon*) subspecies based on the mitochondrial control region DNA sequence using an extended sample setHengxing Ba^{1,2}, Fuhe Yang^{1,2}, Xiumei Xing^{1,2}, and Chunyi Li^{1,2}¹Institute of Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences, Jilin, People's Republic of China and²State Key Laboratory for Molecular Biology of Special Economical Animals, Chinese Academy of Agricultural Sciences, Jilin, People's Republic of China

Abstract

To further refine the classification and phylogeny of sika deer subspecies, the well-annotated sequences of the complete mitochondrial DNA (mtDNA) control region of 13 sika deer subspecies from GenBank were downloaded, aligned and analyzed in this study. By reconstructing the phylogenetic tree with an extended sample set, the results revealed a split between Northern and Southern Mainland Asia/Taiwan lineages, and moreover, two subspecies, *C.n.mantchuricus* and *C.n.hortulorum*, were existed in Northern Mainland Asia. Unexpectedly, Dybowski's sika deer that was thought to originate from Northern Mainland Asia joins the Southern Mainland Asia/Taiwan lineage. The genetic divergences were ranged from 2.1% to 4.7% between Dybowski's sika deer and all the other established subspecies at the mtDNA sequence level, which suggests that the maternal lineage of uncertain sika subspecies in Europe had been maintained until today. This study also provides a better understanding for the classification, phylogeny and phylogeographic history of sika deer subspecies.

Keywords

Cervus nippon, control region, mitochondrial DNA, phylogeny, phylogeographic history, sika deer

History

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Introduction

Sika deer (*Cervus nippon*) has been classified in the order of family Cervidae, subfamily Cervinae and genus *Cervus*. The fossil records indicate that the sika deer originated during early Pleistocene after diverging from an ancestor shared with wapiti (Guo & Zheng, 2000; Sheng & Ohtaishi, 1993). The modern range of sika deer was widespread throughout Northeast Mainland Asia, from the Ussuri region of Siberia to North Vietnam including the Korean peninsula, Mainland China/Taiwan and the Japanese archipelago (Sheng & Ohtaishi, 1993; Whitehead, 1993). Due to the consequence of human activities, sika deer subspecies, such as *C.n.mandarinus* and *C.n.grassianus*, have become extinct in the last two centuries, while other populations of subspecies are now only found associated with small, fragmented and isolated vegetation (Groves, 2006).

Currently, many questions about the classification and phylogeny of sika deer are still open, although several relevant studies have been conducted. Whereas three deep split lineages, including the Mainland Asia/Taiwan, Northern Japan and Southern Japan lineage (Kuwayama & Ozawa, 2000; Lu et al., 2006; Nagata et al., 1995, 1999; Tamate & Tsuchiya, 1995; Tamate et al., 1998), are widely accepted, the subdivision at the subspecies level is still problematic and incompatible among the previous studies (Cook et al., 1999; Lu et al., 2006; Randi et al., 2001).

Moreover, recently up to 16 sika subspecies have been described based on their native range (Table 1; Figure 1) combined with morphological characteristics including body size, color pattern, bell and color of the antler velvet (Groves, 2006). The work from Groves (2006) suggested that two subspecies, *C.n.mantchuricus* and *C.n.hortulorum*, used to exist in Northern Mainland Asia. In contrast, some previous studies proposed that only one subspecies (*C.n.hortulorum*) was existed in the Northern Mainland Asia (Guo & Zheng, 2000; Lu et al., 2006; Sheng & Ohtaishi, 1993). Although *C.n.mantchuricus* was elevated to subspecies ranking by Groves (2006), to date classification of this subspecies has never been verified by modern molecular techniques.

In addition, due to demand of the hunting activities in the last two centuries, sika deer was introduced into most of the European countries from their distant native range (Bartoš, 2009). Based on recent mitochondrial DNA (mtDNA) analyses, these introduced individuals include three widely accepted sika lineages: Northern Japan, Southern Japan and Mainland Asia (Barančková et al., 2012). Among these exotic animals, Dybowski's sika deer, named by Dybowski, a polish biologist, was thought to be originated from Northern Mainland Asia, including Northern China, Korea and Russian Far East (Beolens et al., 2009). It is generally believed that Dybowski's sika deer is the same subspecies to *C.n.mantchuricus* or *C.n.hortulorum* (Cook et al., 1999; Groves, 2006; Krojerova-Prokesova et al., 2013; Randi et al., 2001). Most importantly, these sika deer subspecies, especially those that were lived in Mainland Asia, have seldom been tested all together due to lack of DNA samples in the previous studies. The extent of genetic divergence among them

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Table 1. Subspecies of sika deer.

Nr.	Subspecies	Common name	Native range
1	<i>C.n.hortulorum</i>	Ussurisika deer	Russian Far East, north-eastern Heilongjiang and the northern part of North Korea
2	<i>C.n.mantchuricus</i>		Korea, south-western Heilongjiang, Jilin and northern Liaoning
3	<i>C.n.grassianus</i> ^a		Shanxi
4	<i>C.n.mandarinus</i> ^a		Hebei and Shandong
5	<i>C.n.sichuanicus</i>		Sichuan and Gansu
6	<i>C.n.kopschi</i>	South China sika deer	Anhui, Jiangsu and Jiangxi
7	<i>C.n.taiovanus</i>	Formosan sika deer	Taiwan
8	<i>C.n.pseudaxis</i>		Northern Vietnam
9	<i>C.n.yesoensis</i>	Hokkaido sika deer	Hokkaido
10	<i>C.n.centralis</i> ^b		Northern and central Honshu
11	<i>C.n.nippon</i>		Southern Honshu, Shikoku and Kyushu
12	<i>C.n.mageshimae</i>		Mageshima and Tanegashima
13	<i>C.n.yakushimae</i>	Yakushimasika deer	Yakushima
14	<i>C.n.keramae</i>		Kerama
15	<i>C.n.pulchellus</i>		Tsushima islands
16	<i>C.n.infelix</i>		Goto islands

^aConsidered extinct (Sheng & Ohtaishi, 1993).
^bAn early name for *C.n.centralis* was named *C.n.aplodontus* (Groves, 2006).

Figure 1. The present distribution of sika deer (*Cervus nippon*) subspecies in mainland Asia, Taiwan and Japan. The ellipse shadows on map refer to the natural range of sika deer subspecies. The ellipse of dashes indicated the corresponding sika deer subspecies that have become extinct. The dark dots represent the geographic location of fossils of the ancestor of modern sika deer (*Cervus grayi*, Zdansky, 1925), which are noted by Guo & Zheng (2000). The black diamond legend between the natural range of *C.n.hortulorum* and that of *C.n.mantchuricus* represents the location of the wild sika deer population found in the mid-1980s (Ma, 1986).



at the molecular level is largely unknown. The well-annotated mtDNA sequences of sika deer subspecies have recently become available in the public databases. This has allowed us to investigate these subspecies and to reconstruct the phylogeny of sika deer subspecies at the molecular level with an extended sample set of these resources.

Genetic analysis of mtDNA can provide reliable information of the intraspecific variation because the mitochondrial genome is featured with more rapid evolution, maternal inheritance and non-recombination (Brown et al., 1979; Larson, 1995). The main aim of this study was to reconstruct a mtDNA phylogeny with a focus on the classification and phylogenetic relationships of sika

Table 2. The complete mtDNA control region sequences analyzed in this study.

Nr.	Subspecies in GenBank	Accession No.	Origin and reference
1	<i>C.n.kopschi</i>	JN389444	Yang et al. (2012)
2	<i>C.n.sichuanicus</i>	JN389443	Yang et al. (2012)
3	<i>C.n.hortulorum</i>	NC_013834	Zha et al. (unpublished)
4	<i>C.n.mantchuricus</i>	JQ624420	Chung et al. (unpublished)
5	<i>C.n.taioanus</i>	NC_008462	Chen et al. (unpublished)
6	<i>C.n.pseudaxis</i>	AF291881	Randi et al. (2001)
7	<i>C.n.yakushimae</i>	NC_007179	Wada et al. (2007)
8	<i>C.n.mageshimae</i>	AB279718	Yamada et al. (unpublished)
9	<i>C.n.nippon</i> ^a	AB279714	Yamada et al. (unpublished)
10	<i>C.n.nippon</i> ^a	AB279724	Yamada et al. (unpublished)
11	<i>C.n.yesoensis</i>	NC_006973	Wada et al. (2007)
12	<i>C.n.centralis</i>	NC_006993	Wada et al. (2007)
13	Dybowski's sika deer	AF291880	Randi et al. (2001)
14	<i>Cervus canadensis</i> ^b	GQ304772	Tu et al. (unpublished)

^aTwo *C.n.nippon* samples were used in this study because there are the different number of tandem copy repeats within their mtDNA control region sequences, and were named *C.n.nippon* 1 (AB279714) and *C.n.nippon* 2 (AB279724), respectively.

^b*Cervus canadensis* represents Chinese Wapiti as outgroup.

deer subspecies in the Mainland Asia/Taiwan region and to clarify the phylogenetic position of Dybowski's sika deer. In addition, we have also discussed the migration history of this species in reference to its Pleistocene fossil records, molecular date and its native range.

Materials and methods

Sequences of the complete mtDNA control region from 13 sika deer subspecies were retrieved from GenBank (2012.09), and all the sequences were well-annotated at the level of the subspecies unambiguously (Table 2). These sika deer sequences include five individuals in Mainland Asia, one in Taiwan, six in Japan and one introduced to Europe (Dybowski's sika deer). As well, one Chinese wapiti (*Cervus canadensis*) individual was used as phylogenetic outgroup. The two extinct sika deer subspecies in the Mainland Asia, *C.n.grassianus* and *C.n.mandarinus*, were not included because their DNA samples are no longer available. The mtDNA control region sequences of three subsepecies, *C.n.keramae*, *C.n.pulchellus* and *C.n.infelix*, were also unavailable or unannotated in the present public databases. But in the previous study, these subspecies were joined to the Southern Japan lineage by constructing the phylogenetic tree of their cytochrome b sequences (Nagata et al., 1999).

The mtDNA control region sequences were aligned using the ClustalX v1.83 (Larkin et al., 2007), and the sequence alignments were manually edited using GenDoc (Nicholas et al., 1997). As the variable tandem repeats of 38 to 40 bp in the control region sequence could be formed by parallel duplication events, these variable repeat copies were excluded except for those original repeats (Randi et al., 2001). All the sites with indels were also excluded in the sequence alignments. The estimates of genetic divergence and measurements of variability were performed using MEGA v5.0 (Tamura et al., 2011).

Four methods of phylogenetic analyses were employed to investigate the evolutionary relationships: neighbour-joining (NJ) as implemented in MEGA v5.0 (Tamura et al., 2011), maximum parsimony (MP), maximum-likelihood (ML) using PAUP*4.0b10 (Swofford, 2002), and Bayesian inference (BI) of phylogeny as implemented in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001). For the NJ analyses, the genetic divergences were calculated through Tamura-Nei (TrN) model (Tamura & Nei,

1993) with the uniform rates among sites (estimated gamma, $\alpha=0$). For the MP analyses, we excluded the constant and uninformative sites, weighted all characters and character transformations equally, and used 10 replicates of random addition of terminal sequences and tree bisection-reconnection (TBR) branch swapping option. According to Akaike information criterion (Akaike, 1974) implemented in MrModeltest v2.3 (Nylander, 2004), the best-fitting model chosen for ML and BI was Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) with equal rates for all the sites and an allowance for invariant sites (0.8381). Heuristic ML searches were performed with 10 replicates of random sequence addition and TBR branch swapping. BI tree was constructed with two independent Markov chain Monte Carlo (MCMC) analyses. Samples from the posterior were drawn every 1000 generations over a total of 10 million generations per MCMC run. The first 20% burn-in was discarded and the resulting trees for each replicate were combined. Node support of the phylogenetic tree was assessed by means of 1000 bootstrap replicates (Felsenstein, 1985).

Likelihood tests of alternative phylogenetic trees were also performed, and *p* values for each topology were calculated with CONSEL (Shimodaira & Hasegawa, 2001). Assuming a constant molecular clock, the time of divergence (*t*) was calculated as $t = K/2\mu$, where μ is the rate of nucleotide substitution and *K* is the proportion of nucleotide differences between two sequences (Polzahn & Strobeck, 2002). The divergence rate of 1.11%–1.31% per million years (Myr) was used for the control region of the deer mtDNA (Randi et al., 2001).

Results

Sequence alignment of the complete control region (923 nt) from 13 individuals of sika deer samples was conducted only using the original tandem unit. There were 111 variable sites, including 93 transitions (83.8%), five transversion (4.5%) and 13 insertions or deletions (11.7%). The number of singleton variable sites and parsimony informative sites (two variants) were 37 and 61, respectively. The transition/transversion bias was estimated to be 18.6. In order to provide further evidence for the classification of the two existing subspecies: *C.n.hortulorum* and *C.n.mantchuricus* (Groves, 2006), we calculated the genetic divergence between them. The value of pair-wise genetic divergences was ranged from 0.4% to 5.3% (average $3.4\pm0.4\%$) among all the tested samples. The value of genetic divergences between *C.n.hortulorum* and *C.n.mantchuricus* subspecies was 1.0%. This genetic divergence value was higher than that (0.4%) between *C.n.sichuanicus* and *C.n.kopschi* subspecies, and equal to that (1.0%) between *C.n.yakushimae* and *C.n.mageshimae*, indicating that *C.n.hortulorum* and *C.n.mantchuricus*, which were classified based on morphology, would belong two different subspecies. The level of genetic divergence (1.0%) between *C.n.nippon* 1 and *C.n.nippon* 2 (for the meaning of nippon 1 and nippon 2, refer to Table 2) samples was also identical to that between *C.n.hortulorum* and *C.n.mantchuricus*. In addition, the range of genetic divergence between Dybowski's sika deer and the other sika deer samples was between 2.1% and 4.8%.

The mean value of genetic divergence inter-geographical regions (Table 3) was from 2.5% to 4.6%; and within each geographical region, the mean value was relatively small and ranged from 1.0% to 1.7%. Thus, our results demonstrate that the strong subdivision within sika deer species in genetic divergence is in accordance with their geographical regions.

All four methods of phylogenetic analyses yielded an essentially identical tree topology (Figure 2A). Therefore, our acquired sequence data are robust. According to the branching patterns

Table 3. Pair-wise mean genetic divergence (below diagonal) and standard error (above diagonal) and mean divergence within group (diagonal).

	Southern Mainland ^a	Northern Mainland	Southern Japan	Northern Japan
Southern Mainland ^a	1.7% ± 0.3%	0.4%	0.5%	0.6%
Northern Mainland	2.5%	1.0% ± 0.3%	0.6%	0.6%
Southern Japan	3.9%	3.7%	1.5% ± 0.3%	0.6%
Northern Japan	4.6%	4.4%	4.4%	1.4% ± 0.4%

^aIncluding Taiwan.

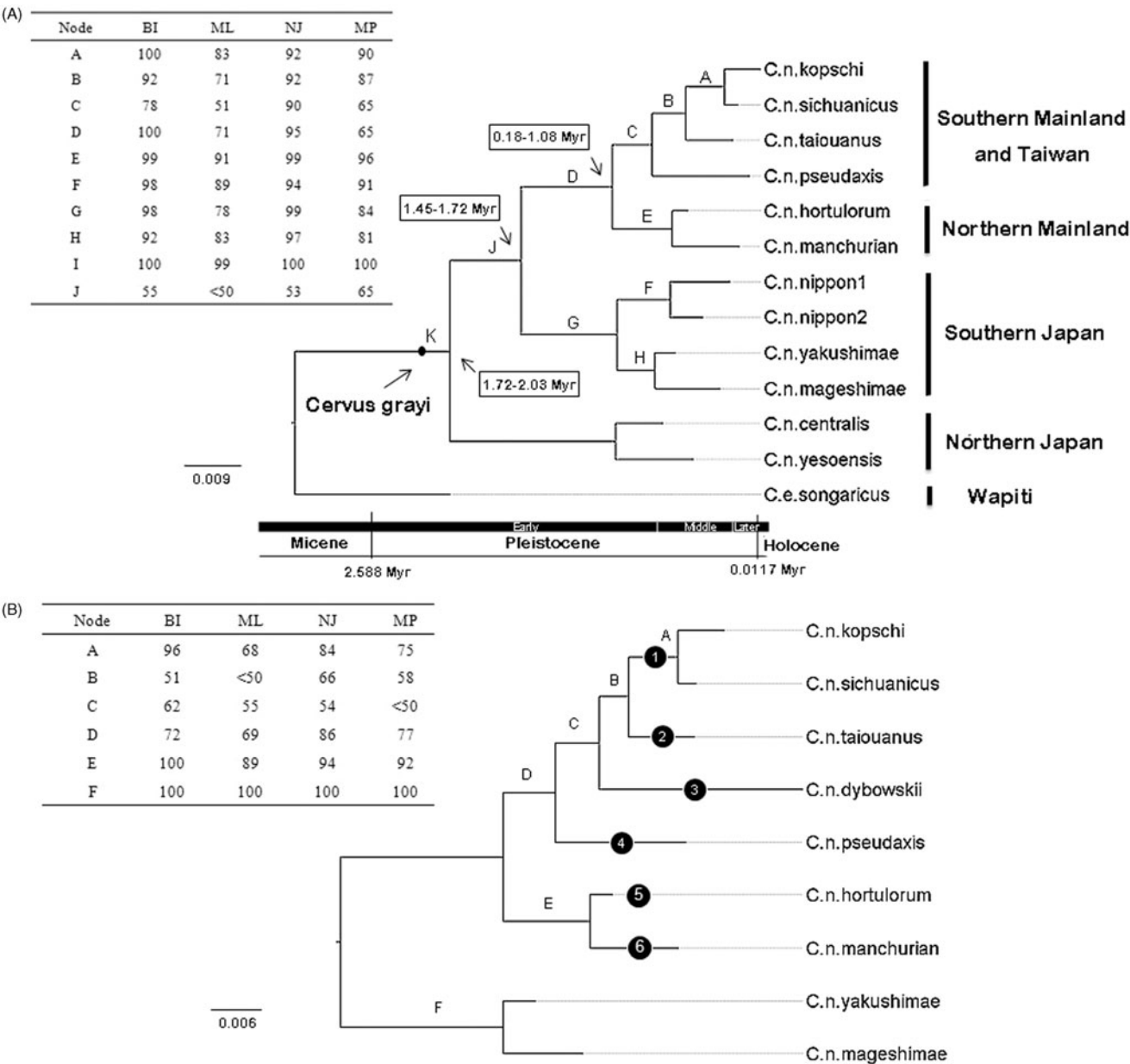


Figure 2. Bayesian inference tree shows the relationships among sika deer subspecies inferred from mtDNA control region sequence. The model of site substitution was the HKY model for ML and BI analyses, while the TN93 model was used for NJ analyses. Four phylogenetic trees (ML, NJ, MP and BI trees) showed the identical topology. Support values for nodes, detailed according to analytical approach are listed on the left. The Geologic Time Scale with major relevant epochs is shown below the tree. (A) Wapiti as outgroup. (B) Dybowski's sika deer joining and southern Japan sika deer as outgroup. See Table 4 about the numbers 1 to 6 in circles.

of the identical phylogenetic tree, we propose the following three points.

- (1) Four divergent lineages, Southern and Northern Mainland Asia/Taiwan, Southern and Northern Japan, were supported by the strong bootstrap value (refer to Figure 2A). More importantly, a split (genetic divergence was 2.5%)

- between Southern and Northern Mainland Asia/Taiwan lineage was also observed in the present data (Figure 2A, node D).
- (2) Within Southern Mainland Asia/Taiwan lineage, *C.n.kopschi* had the sister relationship with *C.n.sichuanicus*, *C.n.taiouanus* and *C.n.pseudaxis* were two basal clades.

Table 4. Results of likelihood-based tests of alternative topologies.

Rank	Tree topology (hypotheses) ^a	ln <i>l</i>	Δln <i>l</i>	AU probability	KH probability	SH probability
1	(((1,2),3),4),(5,6))	-1797.75	Optimal	0.682	0.574	0.940
2	(((1,2),(3,4)),(5,6))	-1798.83	1.1	0.724	0.426	0.843
3	(((1,3),2),4),(5,6))	-1799.52	1.8	0.482	0.342	0.820
4	(((1,(2,3)),4),(5,6))	-1799.80	2.1	0.452	0.323	0.807
5	(((1,2),4),3),(5,6))	-1800.11	2.4	0.491	0.343	0.784
6	(((1,2),4),(5,6),3))	-1805.97	8.2	0.239	0.180	0.439
7	(((1,2),4),(3,6),5))	-1824.35	26.6	0.039 ^b	0.042 ^b	0.048 ^b
8	(((1,2),4),(3,5),6))	-1827.44	29.7	0.014 ^b	0.027 ^b	0.030 ^b

^aThe number in brackets corresponding to the numbers 1 to 6 in circles in Figure 2(B).

^bSignificant at $p < 0.05$.

- (3) Mainland Asia/Taiwan lineage was more closely related to Southern Japan lineage (Figure 2A), but the relationship was only weakly supported by bootstrap value (Figure 2A, node J). The results of Likelihood-based tests ($p > 0.05$) were not significantly different from the alternative tree topologies of the arrangement of three deep split lineages including Mainland Asia/Taiwan, Southern and Northern Japan lineages.

To resolve the phylogenetic position of Dybowski's sika deer, a phylogenetic tree was constructed using four analysis methods, and likewise an identical tree topology was generated (Figure 2B). The results showed that Dybowski's sika deer joined the Southern Mainland/Taiwan lineage, but the relationships between Dybowski's sika deer and other subspecies were very weak. In order to determine whether alternative phylogenetic trees were statistically different to the established optimal tree, the likelihood-based tests were performed. The corresponding p values are shown in Table 4. When Dybowski's sika deer was allocated to Southern Mainland Asia/Taiwan clades, all alternative trees were not significantly different from the optimal tree. In contrast, if Dybowski's sika deer was allocated to Northern Mainland Asia clades, these alternative trees would be significantly different from the optimal tree. These results suggest that Dybowski's sika deer should be allocated to Southern Mainland Asia/Taiwan lineage although its exact position within the phylogenetic tree is not clear.

Discussion

As genetic divergence estimates may be best applied for the classification of a subspecies status, such as *Cervus elaphus* subspecies (Polziehn & Strobeck, 2002), caribou subspecies (Duck, 1998) and other mammals (Mikko & Andersson, 1995), the genetic divergence (1.0%) between *C.n.hortulorum* and *C.n.mantchuricus* supports that sika deer in Northern Mainland Asia have maintained two distinct genetic backgrounds for tens of thousands of years. In addition, historical records of wild animals indicated that two populations (differing in morphology) of wild sika deer were once found in the mid-1980s in Wangqing County of Jilin Province, China (Ma, 1986). One population of wild sika deer had larger body size, deep russet pelage, significant ranked spots and black back line. Another type had smaller body size, fawn pelage and scattered spots without black back line. Wangqing County just locates in the overlap region of the native ranges of the two subspecies, *C.n.hortulorum* and *C.n.mantchuricus* (Figure 1). Importantly, the descriptions of morphological characteristics of these two sika deer populations coincide with those of sika subspecies, *C.n.hortulorum* and *C.n.mantchuricus*, reported by Groves (2006).

Sika deer that are used for velvet production are generally thought to be the descendant of the subspecies that was

lived in Northern Mainland Asia (Sheng & Ohtaishi, 1993). Molecular phylogenetic techniques can be used to provide correct information of classification of sika deer subspecies and their native geographical origin. The information would in turn help for the domestication and management of deer population for velvet production.

It is generally accepted that the natural range for Dybowski's sika deer was in Northern Mainland Asia (Beolens et al., 2009). Unexpectedly, the phylogenetic analysis results showed that Dybowski's sika deer was closer to the Southern Mainland Asia/Taiwan lineage rather than to the Northern Mainland Asia lineage (Figure 2B). Therefore, Dybowski's sika deer would be of the Southern Mainland Asia/Taiwan lineage origin. Alternatively, the sample (AF291880, Randi et al., 2001) we used for this analysis could have hybridized with other sika subspecies after it was introduced into Europe. The hybridization status among the sika deer subspecies in the Czech Republic was also verified by another study (Krojerova-Prokesova et al., 2013). Our analysis showed that genetic divergence between Dybowski's sika deer and all other established samples is from 2.1% to 4.7% at the mtDNA sequence level, which suggests that the maternal genetic background of Dybowski's sika deer is more divergent than that of the established subspecies mentioned in the present study. It is also suggested that Dybowski's sika deer, thus far uncertain subspecies, should belong to *C.n.grassianus* or *C.n.mandarinus* because their native ranges are closer to Northern Mainland Asia (Figure 1). Sika deer subspecies with mixed genetic background could also have been introduced into Europe, and all of these deer were designated Dybowski's sika deer. As no relevant information is available for the two extinct subspecies, *C.n.grassianus* and *C.n.mandarinus*, it would be difficult to confirm the identity of this unconfirmed subspecies. Overall, our findings indicate that the distinct genetic background from the confirmed sika subspecies in Mainland Asia/Taiwan and Japan has been maintained within the European sika deer population until today.

The occurrence of the distinct clades in sika deer phylogenetic tree implies some important factors that formed the geographical distribution patterns in Mainland Asia/Taiwan and Japanese Islands. To elucidate the possible events that caused the observed patterns, it is important to determine the age of the split. Based on the divergence rate of 1.11%–1.31% per million years (Myr) for the deer mtDNA control region (Randi et al., 2001), the molecular date for sika deer lineages was estimated.

The estimated molecular dates of main split lineages have been mapped to the corresponding nodes of the phylogenetic tree (Figure 2A, node K, J and D). The deer, named *Cervus grayi*, Zdansky, 1925 is the first appearance of the direct ancestor of sika deer during the Early Pleistocene (Guo & Zheng, 2000). The geographical district of *Cervus grayi* fossils have also been mapped to Mainland Asia (Figure 1) (Guo & Zheng, 2000). Based on the available information, it was suggested that sika deer evolved from an ancestor in Northeast Mainland Asia. Their range

then expanded southwardly and eastwardly along two paths: one path passed the Korean peninsula and migrated to Japanese islands across land bridges at least twice (Ohshima, 1990) between the southern part of the Japanese islands and the Korean Peninsula during Early-Middle Pleistocene; the other path moved down the east coast of the Mainland, eventually into Taiwan and Vietnam. This scenario is also supported by some previous studies (Cook et al., 1999; Lu et al., 2006; Nagata et al., 1999).

The complex climatic oscillations during the Pleistocene in Mainland Asia are thought to be crucial in shaping phylogeographic patterns of many species (Mahmut et al., 2002; Qu et al., 2005), and the glacial history of a region plays a substantial role in shaping intraspecific variation (Hewitt, 1999). Due to frequent glaciations during the Pleistocene, we thought that the present geographical distribution of modern sika deer subspecies stemmed directly from Pleistocene glacial cycles. In addition, the chilly climate formation with the uplift of the Qinghai-Tibet Plateau during Late Pleistocene (Guo & Zheng, 2000) caused the disappearance of the forest-scrub-meadow environment in many places, which further led to the expansion of sika deer subspecies in Mainland Asia/Taiwan.

The main straits between Japanese islands would have formed during Mid-Later Pleistocene (Ohshima, 1990). The biogeographic boundaries, such as strait formation, confined sika deer populations to each island. Such isolation had stopped gene exchange and accumulated genetic variability among the Japanese sika deer subspecies, and subsequently, modern Japanese subspecies could have been shaped rapidly during Middle-Later Pleistocene.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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