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The origin and genetic variability of the Czech sika deer population

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Abstract Sika deer (*Cervus nippon*), native to Asia, formed two well-established free-living populations in the Czech Republic over the last century and continue to spread. Sika are also maintained in a large number of enclosures; these continue to introduce new individuals from the places of its origin as well as from other European countries. Despite extensive research into the morphology and ethology of the Czech sika deer, conducted over the last three decades, no study using genetic methods has been done. This study aimed to determine the genetic variability and the geographic origin of the Czech sika deer population. Two mitochondrial markers, the cytochrome *b* and the control region were analyzed in this study. Analysis of the two markers confirmed that the founder individuals of the Czech population originated from both native island (Japanese Islands) and native mainland (Far East Russia) populations. Results showed that the genetic variability of the Czech sika deer population is lower than the variability of the native Japanese population, but higher than that of the sampled part of the native Russian population. Also, the genetic variability was found to be higher within the samples from enclosures.

Keywords *Cervus nippon* · Cytochrome *b* gene · Control region · Introduced species

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Introduction

The sika deer (*Cervus nippon* Temminck 1838) belongs to the subfamily Cervinae. It originated on the mainland of southeastern Asia and subsequently spread to Taiwan and the Japanese Archipelago (McCullough 2009a). Over the last two centuries, sika deer have been introduced into many parts of the world and they have become a stable element of the fauna in various European countries (Bartoš 2009; McCullough et al. 2009).

Sika originally spread from Japan through the southern Ussuri, Korea, Manchuria, and Eastern China to Vietnam. However, sika in their native range have greatly suffered due to habitat changes (Yuasa et al. 2007) and uncontrolled hunting, which led to the extinction of local wild populations in Vietnam, Taiwan, and South Korea (McCullough 2009a, b). Based on morphological characters, sika in the world were originally classified into 13 subspecies (Whitehead 1993). Six of these subspecies are found in Japan (Ohtaishi 1986): *Cervus nippon yesoensis* (Hokkaido Island), *C. n. centralis* (Honshu and Tsushima Island), *C. n. nippon* (Kyushu Island, Shikoku Island, and Goto Island), *C. n. mageshimae* (Mageshima and Tanegashima Island), *C. n. yakushimae* (Yakushima and Kuchinoerabu Island), and *C. n. keramae* (Ryukyu Island). Small, free-living populations of sika can still be found in mainland Asia and Taiwan (McCullough 2009b). The historical range of Russian sika (*C. n. hortulorum*) was primarily in Primorsky Krai extending to the borders of China (Aramilev 2009). Up until the early 19th

century, five morphological subspecies were abundant throughout mainland China (Sheng and Ohtaishi 1993). At present, wild sika are extinct over most of their original mainland range, with only three subspecies, *C. n. hortulorum*, *C. n. sichuanicus*, and *C. n. kopschi*, surviving in northeastern, southern, and western China (Guo and Zheng 2000). Wild sika are extinct in South Korea (Wo and Smith 1999), and there is only a small amount of information on their current distribution in North Korea (McCullough 2009b). In Vietnam, only captive populations survive, with a rather low, but not dangerously low, genetic diversity, suggesting a bottleneck (Thévenon et al. 2004). The last free-living Taiwan sika (*Cervus nippon taiouanus*) was killed in 1969, and its current population exists thanks to the release of animals bred in captivity (McCullough 2009a).

However, the classification based on morphology is not completely supported by genetic studies. Several studies reported the existence of two mitochondrial lineages in Japan the northern and southern Japanese groups (Nagata et al. 1995, 1999; Tamate and Tsuchiya 1995; Tamate et al. 1998), which does not reflect the morphological classification. The divergence of these lineages probably occurred before the colonization of Japan from China (Kawamura 2009). Furthermore, analyses showed that the southern Japanese lineage is more closely related to the mainland subspecies *C. n. hortulorum* than to the northern Japanese lineage (Nagata 2009). The three surviving Chinese subspecies appear to be valid based on DNA evidence; however, due to the lack of DNA samples, the status of the two extinct subspecies (*C. n. grassianus* and *C. n. mandarinus*) cannot be verified (Wilson 2000). Also, DNA studies confirmed the status of Vietnamese *C. n. pseudaxis* as a separate subspecies (Wilson 2000).

The introduction of sika to Europe began about 150 years ago. Altogether, sika were introduced to 35 different European countries at the end of the 19th century and the beginning of the 20th century (Bartoš 2009). Outside Asia, free-ranging populations are established in Australasia (New Zealand); North America (Kentucky, Maryland, North Carolina, Texas, Virginia); Europe (Austria, Czech Republic, Denmark, France, Germany Ireland, Poland, United Kingdom). Most of these introductions date from the last years of the 19th century (1890s) through to 1930s, although some have continued until more recently. The most authoritative reviews of distribution, history of introductions, and current status will be found in McCullough (2009b), Feldhamer and Demarais (2009), Banwell (2009), Bartoš (2009), and Swanson and Putman (2009) (see also Eick (1995a, b) for Continental Europe and Ratcliffe (1987) for United Kingdom).

In the Czech Republic, sika were first released into the Bor enclosure near Poděbrady in 1891 (Kokeš 1970). The geographical origin and subspecies status of most of the introduced individuals can no longer be ascertained as the archives of the main supplier, the Hagenbeck Company from Hamburg (Eick 1995a, b), were destroyed (Bartoš 2009). The animals reportedly originated from Japan, eastern China, Korea, and Russia. Later, animals from England and Austria were also imported (Kokeš 1970). Up until World War II the sika were kept in enclosures; however, after the war some of these enclosures were destroyed or the animals were released on purpose, and the sika escaped (Vavruněk and Wolf 1977). These escaped sika were the basis of the West Bohemian and Moravian (Bouzovsko) populations (Doležal 1960; Babička et al. 1977). Since then, sika have colonized a large area of the Czech Republic (Vavruněk and Wolf 1977). Currently, there are two well-established populations inhabiting both the West Bohemia and the Bouzovsko area, and their populations are reported to be spreading—hunters repeatedly report the presence of sika males in previously sika-free localities. The recommendations for management of this exotic species are strictly set. However, due to very imprecise methods for the estimation of deer densities combined with the unwillingness of hunters to stop sika spreading, the situation in reality is greatly different. At present, the population size of sika in the Czech Republic, as provided by hunters for the year 2009, is 9,031 individuals (http://eagri.cz/public/web/file/67788/sumar_2009.pdf). However, real population numbers are much higher than the figures estimated and reported on a yearly basis.

Sika may have considerable ecological impacts due to their potential to cause significant damage to forestry, agriculture, and habitat structure (Chadwick et al. 1996; Abernethy 1998; Putman and Moore 1998; Putman 2000; Kelly 2002; Lowe 1994). There is also the additional concern of hybridization between introduced sika and native deer species. Sika subspecies are not only able to hybridize intra-specifically, they can interbreed with other deer species, such as red deer, hog deer, and axis (Bartoš et al. 1981). Hybridization occurs in regions of natural contact of sika with another deer species, for example the Ussuri River on the Russia–China border (Heptner et al. 1961), and sika have also been intentionally crossbred with other deer species for research purposes (e.g., Harrington 1973, 1982) or to improve the quality of sika trophies (Eick 1995b). This fact has been known for a long time and the first hybrids in Europe were recorded in 1884 in Ireland (Powerscourt 1884). Despite this, the possibility of interspecific hybridization has been ignored and the introduced animals have often been kept together with other deer species. Hybridization of sika has been recorded in Great Britain (Lowe and Gardiner 1975; Abernethy 1994; Goodman et al. 1999; Diaz et al. 2006; Pemberton et al. 2006; Pérez-Espona et al. 2009; Senn and Pemberton 2009; Swanson and Putman 2009), Germany (Herzog 1987; Gehle and Herzog 1998), Ireland (Harrington 1973; McDevitt et al. 2009), and in the Czech Republic (Bartoš and Žirovnický 1981; Bartoš and Vítek 1993) by morphological and karyological analyses.

One of the first applications of molecular phylogenetic approaches was to simultaneously determine both the phylogenetic and the spatial relationships between different mtDNA haplotypes or sequences. Avise (2000)

termed the joint use of phylogenetic techniques and geographic distributions phylogeography. The rapid mutation rate of the mitochondrial genome, the basis for phylogeographic research, makes it particularly suitable for studies of intraspecific variation (Avise 1994). Two regions of mitochondrial DNA, the cytochrome *b* gene and the control region, have also been extensively used in studies of various deer species (e.g., sika—Nagata et al. 1995; Tamate and Tsuchiya 1992, 1995; red deer—Polziehn and Strobeck 2002; Ludt et al. 2004; Eld's deer—Balakrishnan et al. 2003). The genetic variability and the phylogenetic and geographic relationships of both native (e.g., Nagata et al. 1995; Tamate and Tsuchiya 1995) and introduced (Cook et al. 1999; Pitra et al. 2005) sika populations have been extensively studied.

Even though over the last three decades several studies researched the ecology (Heroldová 1990), ethology (e.g., Bartoš and Žirovnický 1981, 1982; Bartoš et al. 1998), and morphology (Bartoš and Žirovnický 1981; Bartoš and Vítek 1993) of the Czech sika population, until now there have been no studies of the genetic variability and origin of this population. The aim of this study was to obtain the first genetic data about the Czech sika population by investigating (1) the genetic variability of the sika population using mitochondrial DNA markers for the cytochrome *b* gene and the control region and (2) the origins of Czech sika and their phylogeographic relationships with native Asian populations.

Materials and methods

Tissue samples (muscles, ear pieces, skin) for the analyses were collected from introduced Czech (236 samples), native Japanese (102 samples), and Russian (111 samples) populations, and stored in 96 % ethanol. In the Czech Republic, the samples were collected from free-living populations by hunters during the hunting seasons and by J. Lamka during veterinary examinations of the individuals kept in enclosures during the years 2007–2010 (Table 1). The Russian samples from Primorsky Krai were collected by hunters, mostly from cadavers found during winter in the years 2007–2010, while the Japanese samples primarily came from meat samples sent to Obihiro University of Agriculture and Veterinary Medicine in the years 2009–2010 (Table 1). All of the samples collected were tested for genetic polymorphisms in the cytochrome *b* (cyt *b*) gene and in the control region (CR). A further nine sequences for the cyt *b* gene and nine sequences for the CR, representing haplotypes of various sika subspecies from Asia, were downloaded from GenBank database (see the Supplementary material for the accession numbers).

The differences between introduced Czech and native Asian populations were analyzed as well as the differences between the free-living populations and populations from enclosures in the Czech Republic. The decision to analyze the differences between free and kept populations was based on the fact that the population of sika in enclosures continues to get "fresh genes"—all introductions are to enclosures and not to the wild—and that the population in enclosures includes also subspecies from mainland Asia, which is not present in the free-living population.

Table 1 Tissue samples collected in introduced and native populations

Country	Locality	Sample size
Czech Republic	Bílá Lhota	1
	Bouzovsko	20
	Bydlo	1
	Častolovice	9
	Český les	2
	Doupov	47
	Horšovský Týn	23
	Hrotovice	3
	Lány	49
	Lidmovice	15
	Manětínsko	21
	Měřín	4
	Opočno	8
	Pastviny	2
	Podrážnice	10
	Rovensko	2
	Rouchovany	2
	Uherčice	4
	Úsobí	1
	Veselsko	1
	Vidlice	2
	Vysočina	5
	Private enclosures	4
Russia	Lazovsky District	97
	Terneysky District	14
Japan	Ehime	1
	Hokkaido	34
	Chiba	18
	Kochi	7
	Kyoto	8
	Nagasaki	10
	Osaka	12
	Yamaguchi	12

Table 2 The cyt *b* haplotype and nucleotide diversity of free-living sika deer and sika deer in enclosures in the Czech Republic

	N	N_H	H_D	P_i (JC)	k	S
Enclosures	144	6	0.666	0.02025	22.599	72
Free-living	95	4	0.461	0.01498	16.682	42

N number of samples, N_H number of haplotypes, H_D haplotype diversity, P_i (JC) nucleotide diversity Jukes–Cantor-corrected, k average number of nucleotide differences, S number of polymorphic sites

DNA extraction, PCR, and sequencing

The DNA was isolated using a Qiagen DNeasy Blood & Tissue Kit (Hilden, Germany) and a Genomed Jetquick Tissue DNA Spin Kit (Löhne, Germany), following the manufacturer's instructions. The PCR amplification of cyt *b* was carried out using the primers L14724 and H15915 (Irwin et al. 1991), and the amplification of the

CR using primers L15926 and H00651 (Kocher et al. 1989). The PCR protocol for cyt *b* was as follows: (1) 94 °C, 3 min; (2) 35 × (94 °C, 40 s; 50 °C, 40 s; 65 °C, 90 s); (3) 65 °C, 5 min, and for CR: (1) 94 °C, 3 min; (2) 35 × (94 °C, 30 s; 53 °C, 30 s; 65 °C, 1 min); (3) 65 °C, 5 min. The amplified DNA fragments were visualized by electrophoresis on a 2 % agarose gel stained with ethidium bromide or GoldView™. The DNA sequencing was performed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Complete sequences of the cyt *b* and the CR were edited and aligned using Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA).

Genetic diversity analysis and statistics

The Akaike information criterion (AIC; Akaike 1974), implemented in the MrModeltest 2.3 program (Nylander 2004), was used to select the best-fitting nucleotide substitution models for PAUP*4 (Swofford 2003) and MrBayes for both the cyt *b* and the CR datasets. Phylogenetic reconstructions were performed using maximum-likelihood criterion (ML; Felsenstein 1981) implemented in PHYML software (Guindon and Gascuel 2003). The robustness of the trees was assessed by bootstrap re-sampling (1,000 random replications for both NJ and ML analyses; Felsenstein 1985). Bayesian estimation (BE) of phylogeny (Yang and Rannala 1997) was performed in the program MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed with five chains run for 1,000,000 iterations, using estimated model parameters as the starting values. Bayesian posterior probabilities were obtained from the 50 % majority rule consensus of trees sampled every 20 generations, discarding the trees obtained before the chains a reached stationary distribution. The cyt *b* gene sequence of *Odocoileus virginianus* and CR sequence of *Capreolus pygargus* were used as outgroups to root the phylogenetic trees (see the Supplementary material).

A median-joining network (MJ; Bandelt et al. 1999) was used as another way of visualizing relationships between the haplotypes. The MJ network was constructed using freely accessible Network software (http://www.fluxus-engineering.com).

The estimation of genetic diversity was conducted in DnaSP v5 (Librado and Rozas 2009). Genetic diversity indices consisted of haplotype diversity (H_D), nucleotide diversity (P_i; Jukes-Cantor-corrected) and number of polymorphic sites (S).

Results

A total of 449 samples were collected from 33 localities (Table 1) and analyzed. The analyses yielded 449 complete cyt *b* sequences, 1140 bp, and 447 CR sequences, 975–1143 bp. All of the haplotype sequences were deposited in GenBank (accession numbers for cyt *b*: JF893469–JF893492, for CR: JF893497–893538).

Sequence divergence of the *cyt b* region

A total of 24 distinct cytochrome *b* haplotypes was identified among the 449 analyzed samples. The haplotype diversity of the whole data set was $H_D = 0.861$. The most frequent haplotype J67D was found in 22.94 % of the samples, and three other haplotypes (J1N, J4N, J35D) were found in more than 10 % of the samples each. While none of the Japanese haplotypes were found in the Czech sika population, both Russian haplotypes were also shared by the Czech sika. The number of haplotypes found on the Czech localities varied from one to four (Fig. 1). The most frequent Czech haplotype was J1N, which was found in 30.38 % of the samples; haplotypes J35D and J4N were present in more than 20 % of the samples. The number and distribution of haplotypes at localities sampled in Japan was similar to that in the Czech Republic and varied from one to five haplotypes (Fig. 2a). Only three of these haplotypes reached more than 10 % representation in the data set, with the most frequent being J132NJ (33.33 %). The haplotype variability in the native population in Russia was very low with only two cyt *b* haplotypes found at both sampled localities (Fig. 2b). More than 80 % of the Russian samples belonged to haplotype J67D.

Out of the 1140 bp in the cyt *b* sequences, up to 78 were variable. The base composition in the sequences was 31 % of A, 28 % of C, 13 % of G and 28 % of T. The highest number of haplotypes and the highest haplotype diversity were found in Japanese samples, whereas the Russian population was the least diversified (see the Supplementary material).

Using Akaike information criterion, the best-fitting model chosen for maximum likelihood (ML) and Bayesian estimation (BE) was a general time reversible model (GTR + I + G; Tavaré 1986), with the gamma shape parameter $\Gamma = 0.6963$ and the proportion of invariable sites $I = 0.486$. Both ML and BE methods, yielded phylogenetic trees with an almost identical topology. The topology of the cyt *b* trees indicated the presence of three haplotype groups: *nippon1*, *nippon2*, and *hortul* (Fig. 3a), with the highest diversity found in the haplogroup *nippon1*. The divergence of the three haplogroups is well supported by both used methods (ML = 83; BE = 0.96) (Fig. 3a). The k2p-distance between the two *nippon* groups was estimated to be between 2.9 and 3.5 %, and the p-k2p-distances between *nippon1*/*hortul* and *nippon2*/*hortul* were 2.9–3.7 % and 3.3–4.2 %, respectively (see the Supplementary material). The haplogroups *nippon1* and *nippon2* contained haplotypes found in both Czech and Japanese sika populations. The most frequent haplotype within *nippon1* was J4N (35.14 %), within *nippon2* it was haplotype J1N (70.59 %), and within the group *hortul* two of three haplogroups were present in more than 40 % of

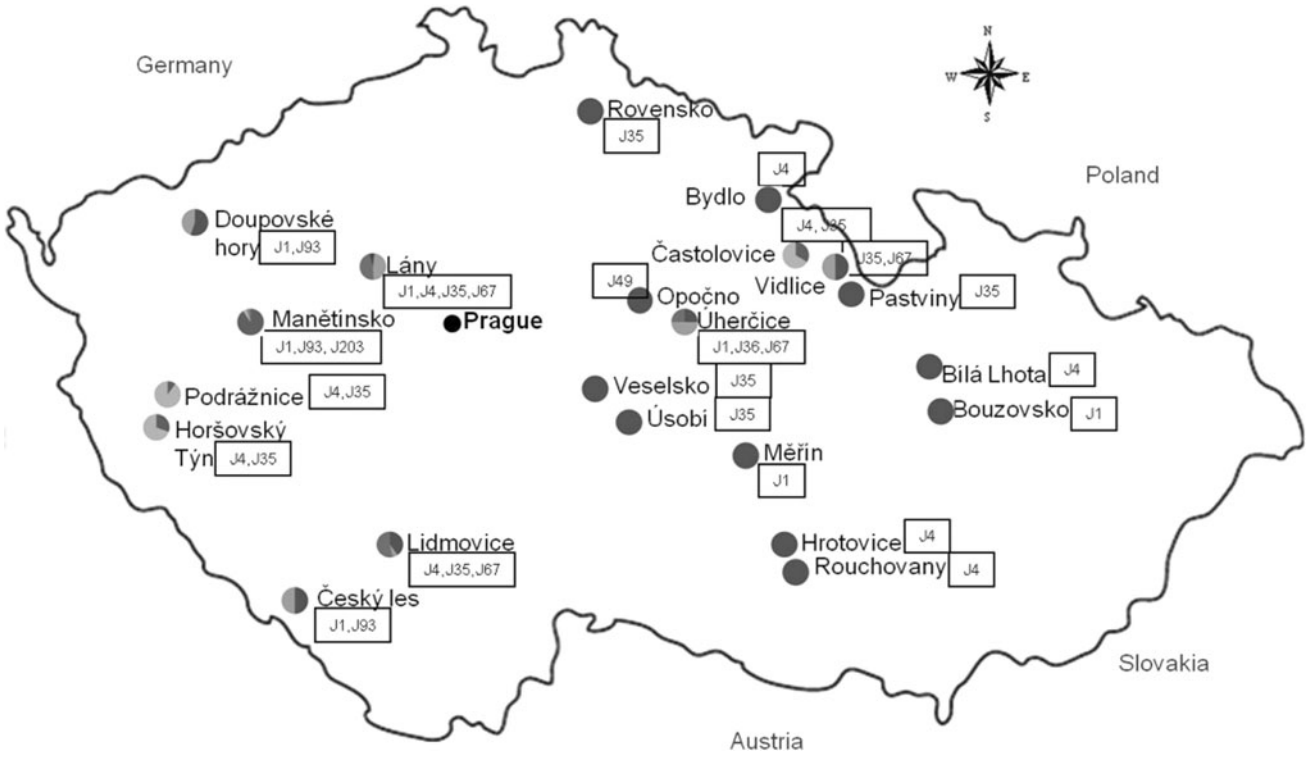

Fig. 1 Distribution and variability of cyt *b* haplotypes at the localities sampled in the Czech Republic

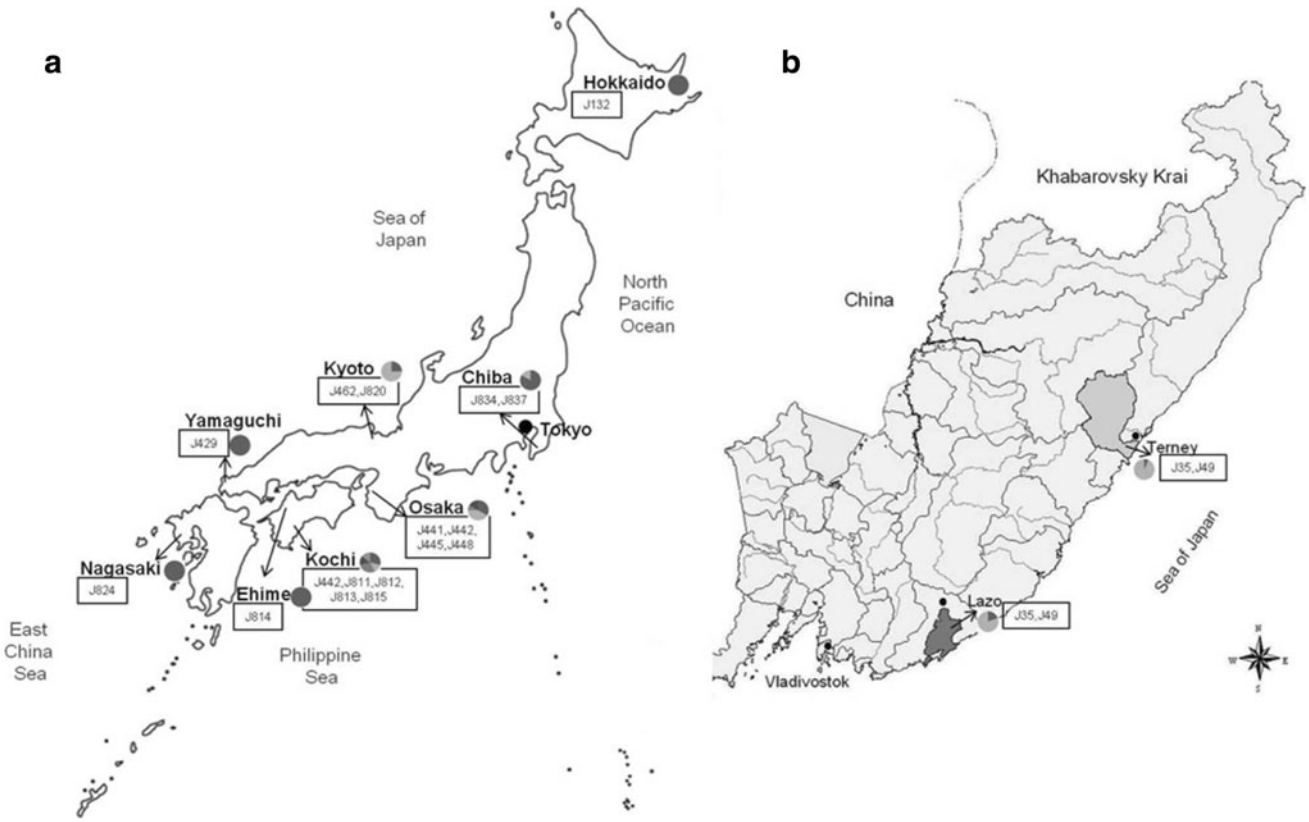

Fig. 2 Distribution and variability of cyt *b* haplotypes at the localities sampled in **a** Japan and **b** Russia

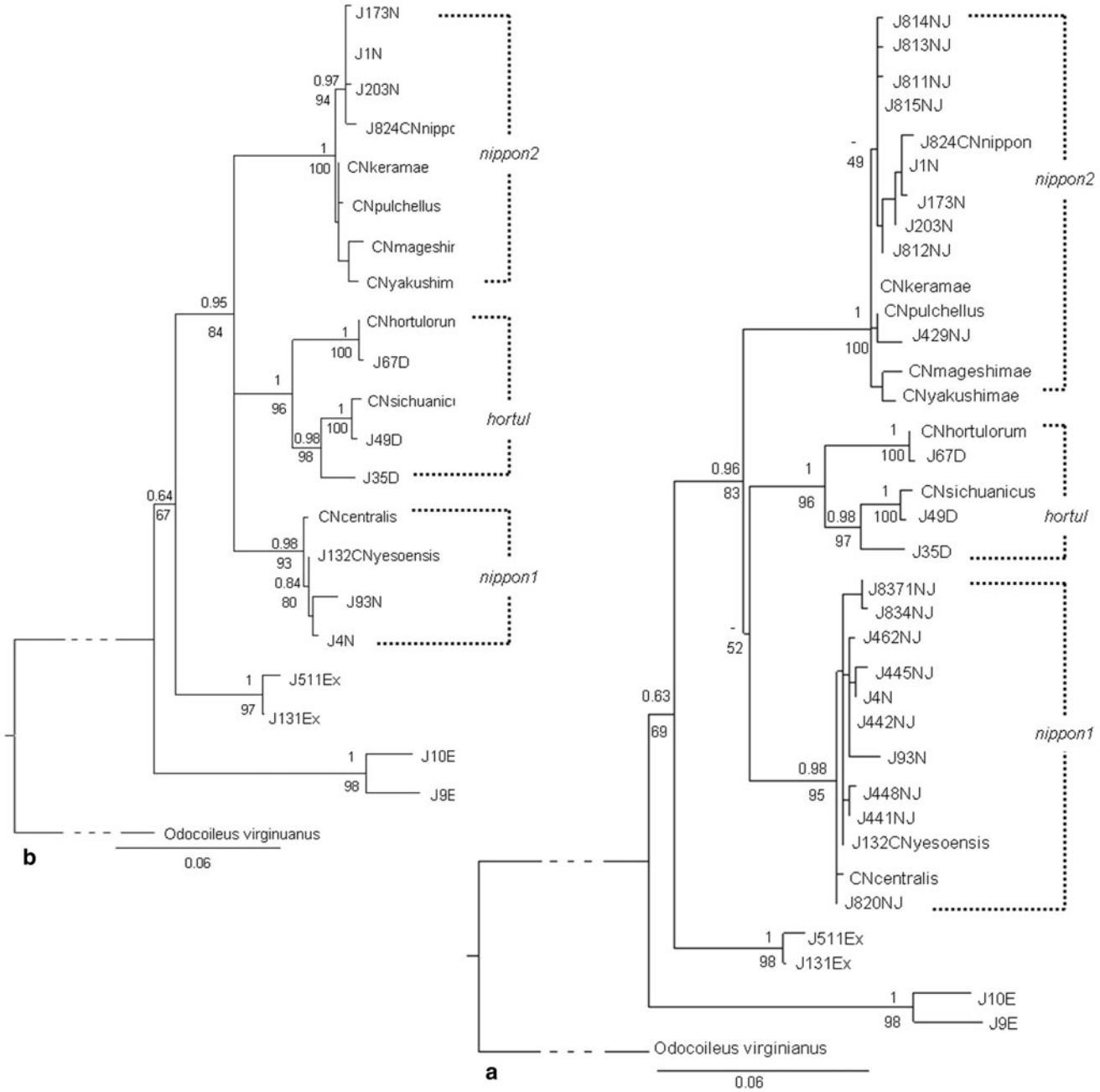

Fig. 3 Maximum likelihood trees for cytochrome *b* sequences. Bootstrap support of ML analysis is indicated *below the branches*, posterior probabilities of BE *above the branches*; **a** whole sample set, **b** Czech samples

the samples analyzed (D35 = 43.37 %; J67D = 52.55 %). The haplotypes from the *nippon1* group belong to the morphological subspecies with larger bodies, found in northern Japan (*C. n. yesoensis*, *C. n. centralis*), while the haplogroup *nippon2* includes the subspecies with smaller bodies that are found in southern Japan (*C. n. mageshimae*, *C. n. nippon*, *C. n. keramae*, *C. n. pulchellus*). The haplogroup *hortul* includes haplotypes found in the samples from Russian and Czech specimens. These haplotypes belong to the mainland subspecies *C. n. hortulorum*. The largest average number of nucleotide differences was found between the haplogroups *nippon1* and *hortul* (see the Supplementary material). The most distinctive difference found in the topology of two cyt *b* phylogenetic trees was found in the position of haplogroup *nippon1*.

A haplotype network (Fig. 4) constructed using the full dataset revealed the same structure as the phylogenetic trees reconstructed by ML and BE methods. Each of the three main haplogroups was diverged from the neighboring haplogroups by at least ten mutation steps and contained one to two common haplotypes (Fig. 4).

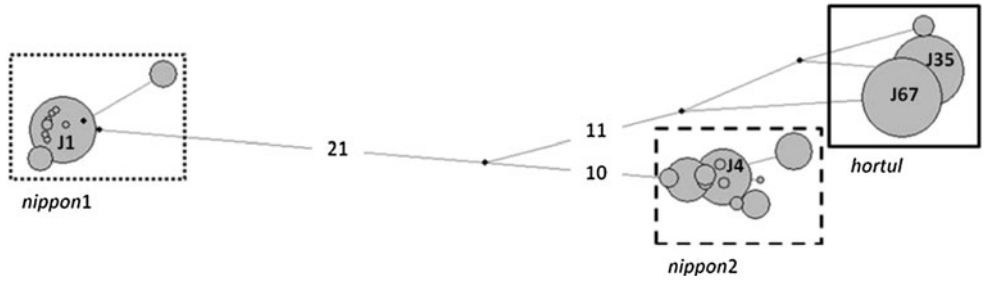

Fig. 4 Median-joining network of sika deer from the Czech Republic, Japan, and Russia, based on the cyt *b* sequences. *Numbers* of mutations between the haplotypes are indicated on the branches; the median vectors are indicated by *black dots*. The most common haplotypes are indicated in relevant *circles*

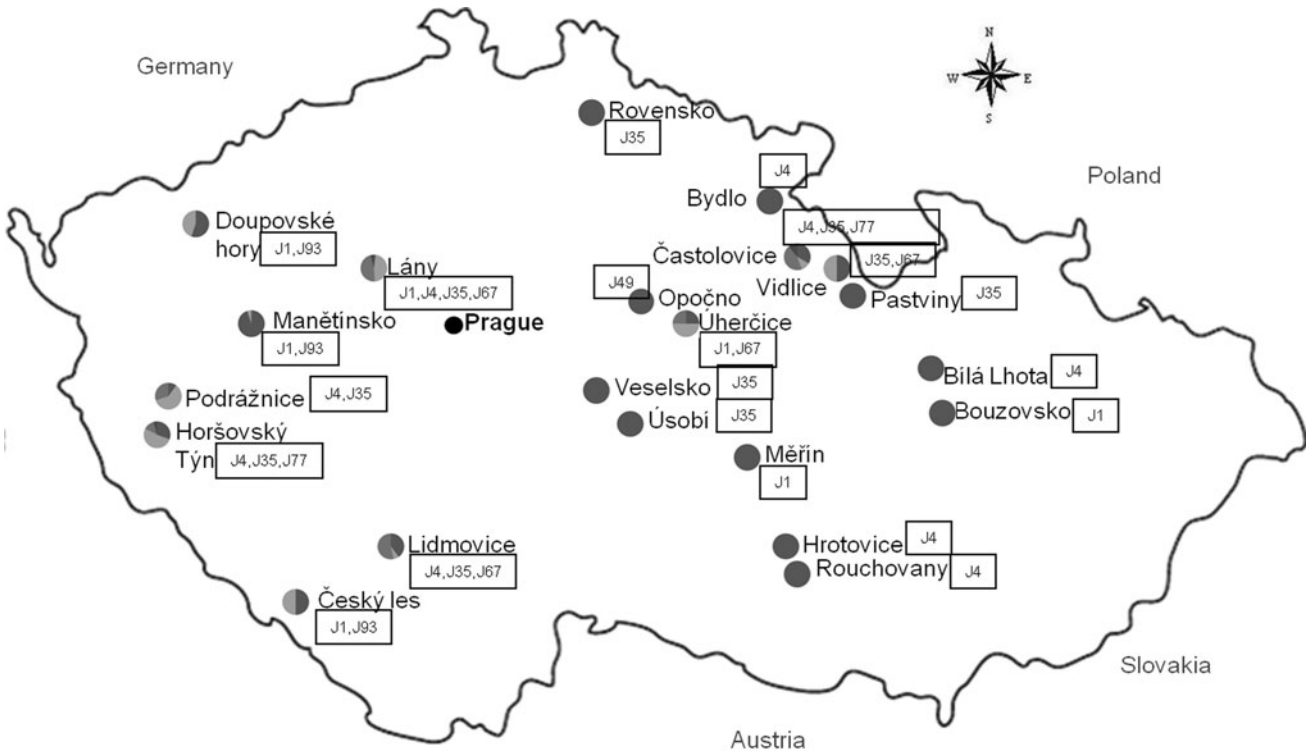

Fig. 5 Distribution and variability of CR haplotypes at the localities sampled in the Czech population

Phylogenetic reconstruction performed by ML and BE methods exclusively on the Czech samples resulted in trees with a very similar topology to the trees constructed for the complete dataset, and confirmed the presence of three haplogroups (*nippon1*, *nippon2*, *hortul*) in the Czech sika population (Fig. 3b). The haplotype diversity in the Czech sika population was higher in the samples collected from the enclosures. From the total number of eight haplotypes found in the Czech population, six haplotypes were found in the enclosures and four in the free-living populations (Table 2). Both of these populations shared two haplotypes—J1N and J4N, four other haplotypes (J35D, J49D, J67D and J173D) were found only in the enclosures, and the last two haplotypes (J93N and J203N) were only present in the samples from free-living animals. The most frequent haplotypes found in the enclosures were J35D (46.53 %) and J4N (32.64 %), and those found most often in the free-living population were J1N (69.47 %) and J93N (24.21 %). All samples within the haplogroup *hortul* originated in the enclosures, those from *nippon* haplogroups can be found in free as well as in enclosures. While the haplotypes of sika in enclosures contained 36 exclusive polymorphic mutations, only six exclusive polymorphic mutations were found in the free-living population. In total, 36 mutations shared between both populations. The average number of nucleotide differences between the free-living sika and sika in the enclosures was 36.114.

Sequence divergence of the CR region

The analyses of the CR yielded results very similar to those of the cyt *b*. Thus, we present here only the basic

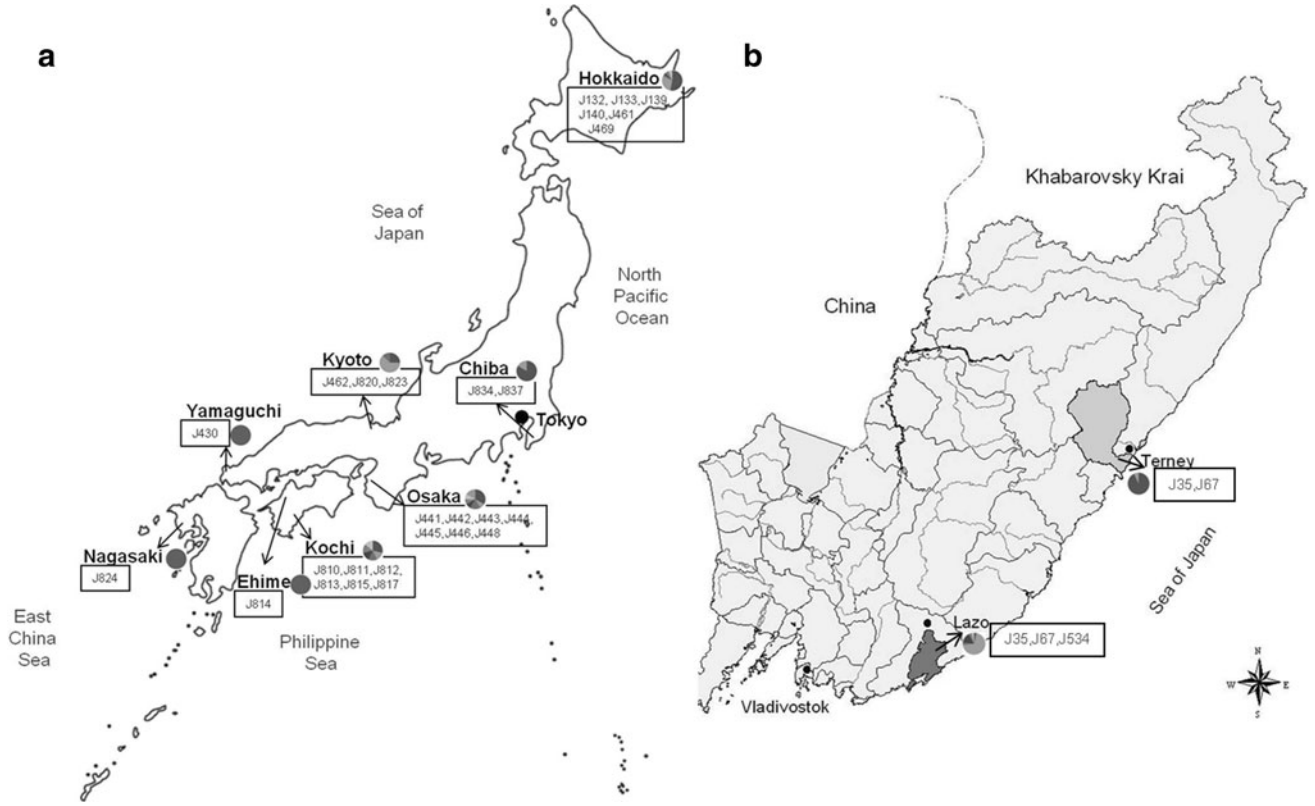

Fig. 6 Distribution and variability of CR haplotypes at the localities sampled in **a** Japan and **b** Russia

results, more detailed information can be found in the Supplementary material.

Altogether, 42 CR haplotypes were identified in the 447 samples analyzed. The length of the analyzed CR sequences varied from 975 to 1,143 bases (including one to five tandem repeat units (23 bp), found in the sequences between 285 and 443 bp). After removing the tandem repetitions, a fragment with the length of 898 bp (including gaps) remained and was used for further analyses, thus decreasing the number of analyzed haplotypes to 32. The haplotype diversity of the whole dataset was $H_D = 0.868$. The Japanese and Czech populations shared no common haplotype, while the Czech and Primorian populations shared two haplotypes. The number of haplotypes in the Czech localities varied from one to four (Fig. 5). A maximum of three haplotypes were found in the Russian localities (Fig. 6 a), while the number of haplotypes in the Japanese localities varied from one to seven (Fig. 6b).

Following the Akaike information criterion, the most suitable model for maximum likelihood and Bayesian estimation was the Hasegawa, Kishino, and Yano + invariant sites + gamma model (HKY + I + G; Hasegawa et al. 1985), with the gamma shape parameter $\Gamma = 0.5844$ and the proportion of invariable sites $I = 6522$. The analyses performed on the whole data set as well as exclusively on the Czech samples yielded phylogenetic trees with a similar topology. The topology of the CR trees indicated the presence of the same three well-defined haplotype groups that were also found in the cyt *b* tree: *nippon1*, *nippon2*, *hortul* (Fig. 7a, b). Their existence is once again also supported by the haplotype network reconstruction (Fig. 8). Haplotypes belonging to the *nippon2* and *hortul* haplogroups all contained only one repetition, while in those from the *nippon1* haplogroup one to five tandem repeats were found (Table 3).

As was also the case of cyt *b*, the CR haplotype diversity in the Czech sika population was higher in the samples collected from the enclosures (Table 4). Both populations shared two haplotypes—J1N and J4N, and samples from haplogroup *hortul* were only found in the enclosures. The average number of nucleotide differences between the free-living sika and sika from the enclosures was 39.358, and both populations were found to share 44 mutations.

Discussion

As expected, due to the higher mutation rate (Avise 1994), the number of haplotypes found within the CR sequences was higher than within the cytochrome *b* sequences. However, the overall haplotype diversity of the whole dataset was almost identical for both markers analyzed. The separate analysis on the Czech, Japanese, and Russian samples showed that these relatively high values of haplotype diversity were influenced by the inclusion of the highly diverse Japanese samples. The

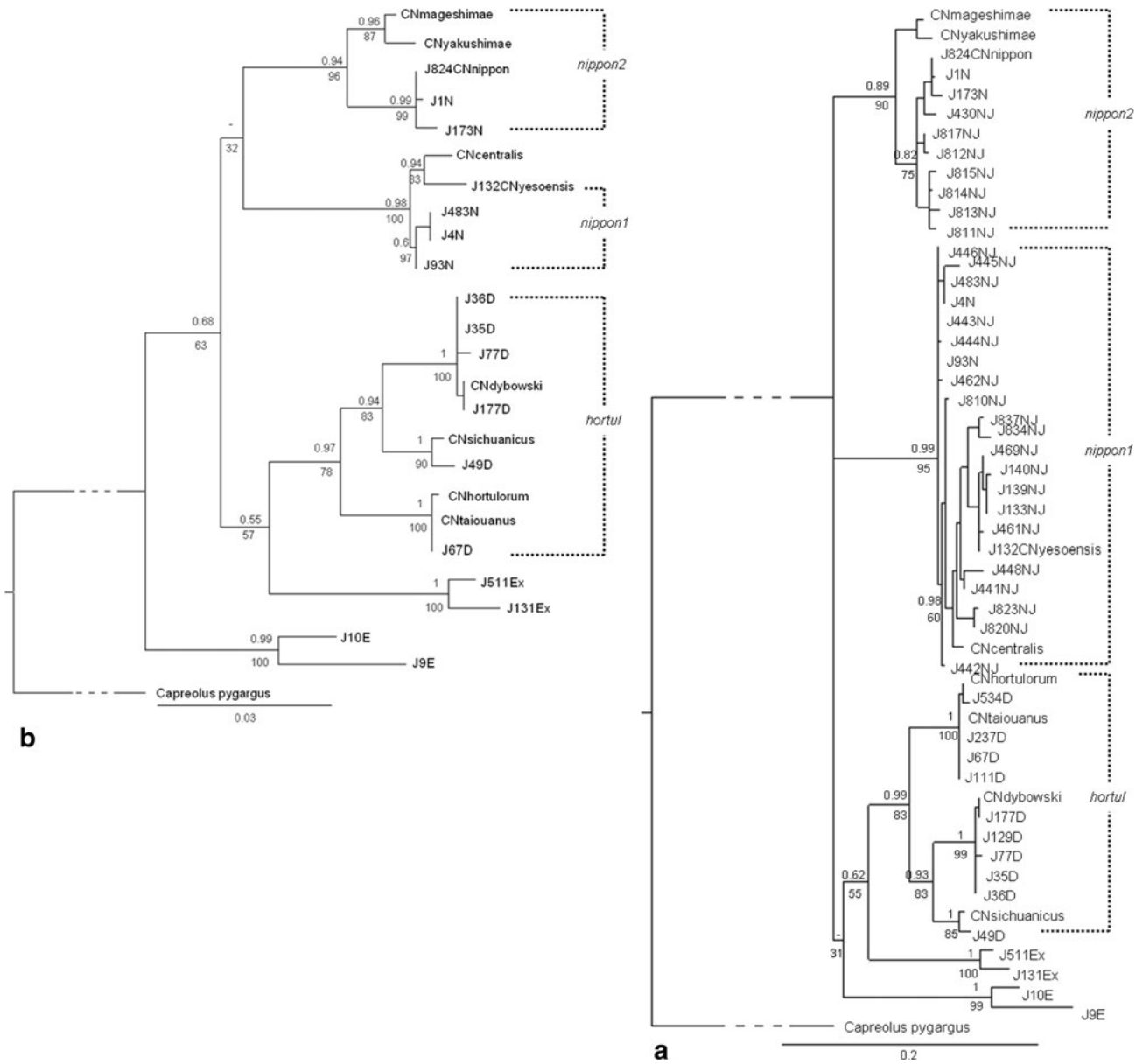

Fig. 7 Maximum likelihood trees for CR sequences. Bootstrap support of ML analysis is indicated *below the branches*, posterior probabilities of BE *above the branches*; **a** whole sample set, **b** Czech samples

haplotype diversity of the Czech sika population was lower than that of the Japanese population but higher than the diversity of the Russian population. However, the values of diversity within the Czech population were influenced by the presence of both types of haplotypes, those closely related to the Japanese population as well as those from the Russian populations, as these two native populations were not found to share common haplotypes under natural conditions. Despite this, the genetic diversity of Czech sika is low, as is typical for a recently introduced species. We also separately analyzed the haplotype diversity of Czech samples collected from the free-living population and samples collected from animals in enclosures. The free-living population contained only "Japanese-like haplotypes" (*nippon1* and *nippon2*) and its diversity was markedly lower than that of the whole Czech dataset. The low genetic diversity of the free-living population may have been caused by a founder effect, resulting from the low genetic variability of the first introduced sika combined with the relatively small number of animals that escaped from enclosures and founded the free-living population. The cytochrome *b* and CR haplotype diversities of the free-living Czech population were half of those of the native Japanese population, suggesting that the introduced individuals might have originated from restricted area(s) in Japan.

The haplotypes found within the samples from enclosures contained both *nippon* and *hortul* haplotypes, thus the diversity of sika in enclosures was higher than that of the free-living population. This may also have

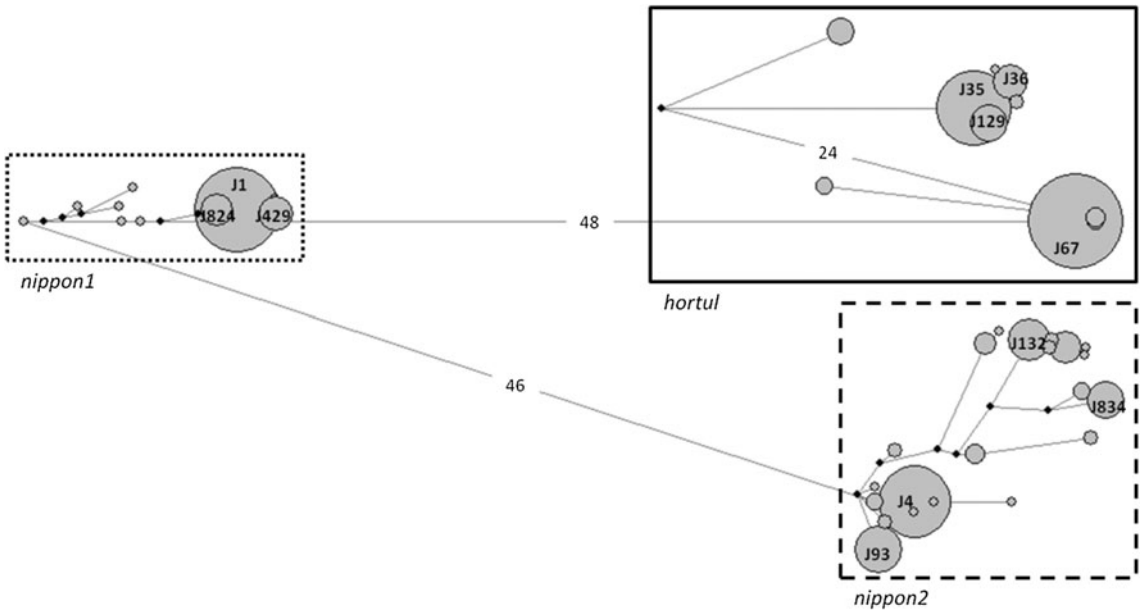

Fig. 8 Median-joining network of sika deer from the Czech Republic, Japan, and Russia, based on the CR sequences. *Numbers* of mutations between the haplotypes are indicated on the branches; the median vectors are indicated by *black dots*. The most common haplotypes are indicated in relevant *circles*

Table 3 Number of tandem repeats found within the Czech, Japanese, and Russian samples

	Number of tandem repeats			
	1	3	4	5
Czech Republic				
Free-living	×		×	×
Enclosure	×			×
Japan				
Hokkaido			×	
Honshu	×	×	×	×
Kyushu	×			
Shikoku	×	×	×	
Russia				
Lazovsky District	×			
Terneysky District	×			

Table 4 CR haplotype and nucleotide diversity of free-living sika deer and sika deer in enclosures in the Czech Republic

	N	N_H	H_D	P_i (JC)	k	S
Enclosures	146	7 (9)	0.675	0.03267	27.774	83
Free-living	95	3 (4)	0.446	0.02135	18.268	45

N number of samples, N_H number of haplotypes, H_D haplotype diversity, P_i (JC) nucleotide diversity Jukes–Cantor-corrected, k average number of nucleotide differences, S number of polymorphic sites

been partly influenced by the continuous import of individuals from Japan and Russia, as well as from other countries with introduced sika (Vach et al. 2010). Despite these new introductions, the genetic variability in the enclosures is still relatively low and the number of *hortul* haplotypes in the Czech population is almost as low as it is in the native Russian population, which suffered a significant decrease in the 1940s (Voloshina and Myslenkov 2009). According to Kokeš (unpublished data), the first confirmed introduction of *C. n. hortulorum* dates back to 1903, and it was followed by further introductions of animals from Vala Co. in Přerov Czech republic and Hagenbeck Co., Germany (Kokeš, unpublished data). Many of the introductions were unsuccessful; the fate of other introduced animals is unknown. Thus, it can be assumed that the number of founder animals of the Czech population was rather small, resulting in the low haplotype variability in the current enclosed as well as free-living population.

Despite the well-documented dates of the first sika introductions to the Czech Republic, the first introduction in 1891 to the Bor enclosure and the second introduction in 1897 to the Lipí enclosure (Kokeš, unpublished data), little is known about the origins of the introduced animals (Kokeš 1970). The small amount of remaining data indicates that the introduced animals originated from both mainland (Far East Russia) and island (Japanese Islands) populations (Kokeš 1970). In order to determine as precisely as possible the geographical origin of this allochthonous deer species in the Czech Republic, we acquired samples from both places of sika origin—Far East Russia and the Japanese Island. Two mtDNA markers, the cytochrome *b* gene and the control region, were used to determine the phylogeographical origin of the free-living and enclosed Czech sika. The topology of both cyt *b* and CR phylogenetic trees in our study showed the existence of three diverged haplogroups within the whole dataset, as well as within

the Czech population. Two of these groups, *nippon1* and *nippon2*, were found to contain samples belonging to Japanese subspecies, whereas the third group, *hortul*, was found to include Russian haplotypes.

It is generally thought that mammals migrated to the Japanese islands from mainland Asia across land bridges that were repeatedly formed between Japan and the Asian continent (Kawamura 1991). In the case of sika, the molecular analyses suggest that it colonized Japan twice. This double colonization is supported by the existence of two different mitochondrial lineages in Japan—the northern and the southern groups as reported by several studies (Nagata et al. 1995, 1999; Tamate and Tsuchiya 1995; Tamate et al. 1998). Nagata et al. (1995) studied the genetic variability of three populations of Japanese sika by sequencing the mitochondrial 12S rRNA and cytochrome *b*. Their results clearly supported the existence of two different populations of sika in northern and southern Japan. Further evidence of repeated colonization could be the closer relationship between the southern Japanese lineage and the Chinese subspecies *C. n. hortulorum* than with the northern Japanese lineage (Nagata 2009), suggesting that the divergence of both lineages probably occurred before the colonization of Japan from China (Kawamura 2009). The existence of the two mitochondrial lineages was also confirmed in the sika populations introduced to Germany and Austria (Pitra et al. 2005). Also, our results confirm the presence of these two lineages, denoted in the phylogenetic trees as *nippon1* and *nippon2,* and these can also be seen not just in the cytochrome *b* and D-loop phylogenetic trees reconstructed from the whole dataset but also in the trees reconstructed solely from the Czech samples. This suggests that the introduced sika individuals brought to the Czech Republic at the end of the 19th century and at the beginning of the 20th century may have originated from a much larger area than expected and might have belonged to two different genetic lineages. Part of the Czech sika population seems to be related to smaller bodied sika (*C. n. nippon*, *C. n. keramae*, *C. n. mageshimae*, *C. n. pulchellus*) from southern Japan, while the other part is closer to the larger bodied sika (*C. n. centralis*, *C. n. yesoensis*) from northern Japan. The haplotypes from the *hortul* group were only found within enclosures; not even one sample from the free-living animals belonged to this group. The reason for this could be that only a few animals from the *hortul* group escaped the enclosures (according to unpublished data of Kokeš, individuals of *C. n. hortulorum* bred in an enclosure in Vlkava, in Bohemia, became a part of the native fauna after its liquidation). Furthermore, another reason could be intraspecific hybridization. The existence of hybridization between sika subspecies in the Czech Republic was confirmed when 32 samples from enclosures that were thought to be *C. n. hortulorum* actually contained haplotypes belonging to Japanese subspecies (Krojerová-Prokešová et al., in prep.). Of course, this could be caused by mistakes in identification caused by the hybridization among sika subspecies, and hybridization between sika and red deer. A detailed analysis of microsatellite markers on the samples used in the study could provide further data to support, and maybe even improve, the data on the geographical origin of the Czech sika obtained from the mtDNA analyses. It should also help to evaluate the degree of interspecific (sika subspecies) and intraspecific (sika deer × red deer) introgression within the Czech sika and red deer populations.

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