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Dybowski's Sika Deer (*Cervus nippon hortulorum*): Genetic Divergence between Natural Primorian and Introduced Czech Populations

Jarmila Krojerová-Prokešová, Miroslava Barančeková, Inna Voloshina, Alexander Myslenkov, Jiří Lamka, and Petr Koubek

From the Institute of Vertebrate Biology Academy of Sciences of the Czech Republic, v.v.i., Květná 8, Brno 603 65, Czech Republic (Krojerová-Prokešová, Barančeková, and Koubek); Lazovsky State Nature Reserve, Lazo, Russia (Voloshina and Myslenkov); Charles University Prague, Faculty of Pharmacy, Hradec Králové, Czech Republic (Lamka); and the Department of Forest Protection and Game Management, Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Czech Republic (Koubek).

Address correspondence to Dr. Jarmila Krojerová-Prokešová at the address above, or e-mail: krojerova@ivb.cz.

Abstract

Dybowski's sika deer (*Cervus nippon hortulorum*) originally inhabited the majority of the Primorsky Krai in Far Eastern Russia, north-eastern China, and Korean Peninsula. At present, only the Russian population seems to be stable, even though this taxon is still classified as endangered by the Russian Federation. Almost 100 years ago, this subspecies, among others, was imported to several European countries including the Czech Republic. We used both mitochondrial (mtDNA; the cytochrome *b* gene and the control region) and nuclear DNA markers to examine the actual taxonomic status of modern Czech Dybowski's sika population and to compare the genetic diversity between the introduced and the native populations. Altogether, 124 Czech samples and 109 Primorian samples were used in the analyses. Within the samples obtained from individuals that were all morphologically classified as Dybowski's sika, we detected mtDNA haplotypes of Dybowski's sika (84 samples), as well as those belonging to other sika subspecies: northern Japanese sika (25 samples), southern Japanese sika (6 samples), and south-eastern Chinese sika (8 samples). Microsatellite analysis revealed a certain level of heterozygote deficiency and a high level of inbreeding in both populations. The high number of private alleles, factorial correspondence analysis, and Bayesian clustering analysis indicate a high level of divergence between both populations. The large degree of differentiation and the high number of population-specific alleles could be a result of a founder effect, could be a result of a previously suggested bottleneck within the Primorian population, and could also be affected by the crossbreeding of captive individuals with other sika subspecies.

Key words: *bottleneck, founder effect, genetic diversity, inbreeding, microsatellites, mtDNA*

The sika deer (*Cervus nippon* Temminck 1838) belongs to the subfamily Cervinae. It originated on the mainland of north-eastern Asia (the southern Ussuri, Korea, Manchuria, and north-eastern China) and subsequently spread to south-eastern China, Vietnam, Taiwan, and the Japanese Archipelago (Cook et al. 1999; Wilson 2000; Nagata 2009). Over the last two centuries, sika 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. 2009b).

Sika in their native range have greatly suffered due to the 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, 2009b). Based on morphological characters, all sika deer were originally classified into 13 subspecies (Whitehead 1993). Six of these subspecies are found in Japan (Ohtaishi 1986): *C. n. 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 deer can still be found in mainland Asia (McCullough et al. 2009a); these are attributed to three subspecies, *C. n. hortulorum*, *C. n. sichuanicus*, and *C. n. kopschi*. Two other mainland Asian subspecies, *C. n. pseudaxis* in Vietnam and *C. n. taiouanus* in Taiwan, were extinct in the wild; however, in Taiwan, the recovery program using animals from captivity started in 1986 (McCullough 2009b).

This 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 the southern Japanese group (Tamate and Tsuchiya 1995; Nagata et al. 1999), which does not reflect the morphological classification.

Unlike the Japanese subspecies, almost all mainland Asian subspecies, Dybowski's sika deer (*C. n. hortulorum* Swinhoe, 1864) among them, are listed as endangered taxa, and there is a general lack of data about their current abundance and distribution and their historical demography (Wilson 2000; McCullough et al. 2009b).

Dybowski's sika was originally distributed over much of the Primorsky Krai in Far Eastern Russia, in north-eastern China (adjacent to the Russian and North Korean borders), and the Korean Peninsula (Whitehead 1993; Aramilev 2009; McCullough et al. 2009a). At present, the Russian population is the most numerous one and appears to be relatively stable (Aramilev 2009). Current Chinese population is questionable and probably consists of sporadic isolated individuals that are likely dispersers across Russian and North Korean borders (McCullough et al. 2009a). The current status of North Korean population is unknown (McCullough 2009a).

According to scarce historical records, the population numbers of Dybowski's sika in Far East Russia were substantially reduced at the end of the 19th and the beginning of the 20th century, primarily by overhunting and the capture of animals for deer farms (Makovkin 1999). In the 1940s, the population was estimated to be at about 300 individuals (Bromley 1956), 160 of which were located in the present Lazovsky Reserve (Voloshina and Myslenkov 2009). The population numbers started to increase in the 1970s and by the end of the 1980s; the abundance of sika was two to three times higher within the whole area of the Primorsky Krai than it was before. Re-occupation of the original range consisted of both wild and farm-escaped sika deer, but they should all be part of the original genetic stock (Aramilev 2009). Its current population size is estimated to be more than 20 000 individuals (Aramilev 2009), 4500 of which live in the Lazovsky Reserve (Voloshina and Myslenkov 2009).

Dybowski's sika deer is considered to be the largest subspecies of sika deer (Whitehead 1993; McCullough et al. 2009b). However, based on its morphological features and its karyology, several authors have suggested a hybrid origin of this subspecies—a hybrid between Manchurian wapiti (*Cervus canadensis xanthopygus*) and sika deer (Lowe and Gardiner 1975; Bartoš and Žirovnický 1981; Herzog 1987, 1995; Bartoš 2009). This theory was also supported by the fact that the distribution ranges of sika and wapiti overlap in eastern Asia (in the area of presence of Dybowski's sika subspecies) and also by the known fact that these taxa can naturally hybridize in the wild as well as in captive and introduced populations (Mirolyubov and Ryashchenko 1948; Sokolov 1959; Bartoš and Žirovnický 1981; Geist 1999; Prisyazhnyuk 2005; Bartoš 2009). However, according to an unpublished study by Goodman S (reported in Aramilev 2009), in the rare cases, when this hybridization occurs, the hybrid individuals retain the phenotype of the Dybowski's sika or Manchurian wapiti, indicating that natural selection may be acting against hybrids with intermediate phenotypes. These results support the concept of Dybowski's sika and Manchurian wapiti as "good species," despite some difficulties with the biological species concept in the strictest sense (Aramilev 2009). Previous molecular genetic analyses based on the analysis of mitochondrial (mtDNA) markers (the cytochrome *b* gene [cyt *b*] and the control region [CR]) have also supported the taxonomic status of Dybowski's sika as a valid sika subspecies (Whitehead 1993; Tamate and Tsuchiya 1995; Cook et al. 1999; Nagata et al. 1999; Randi et al. 2001; Pitra et al. 2004).

Sika have been widely introduced beyond their native range. Outside Asia, free-ranging populations are established in Australasia (New Zealand); North America (Kentucky, Maryland, North Carolina, Texas, Virginia); and 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 on distribution, dates and history of introductions/reintroductions, and current status of sika can be found in McCullough et al. (2009b). The majority of established populations deriving from these introductions were, however, of Japanese sika (*C. n. nippon* or *C. n. centralis*); many fewer successful introductions would appear to have been made of mainland sika (*C. n. hortulorum*) (Apollonio et al. 2010).

According to such historical records as are available, the first sika were introduced into the Czech Republic in 1891 (Bartoš 2009). During the next few years, further introductions followed (Komárek 1945). Based on recent mtDNA analyses, it was revealed that introduced individuals belonged to at least three different sika subspecies: *C. n. yesoensis* and *C. n. nippon* both from Japan, and *C. n. hortulorum* from mainland Asia (Barančeková et al. 2012). The animals were bred in enclosures as hunting trophies, but during World War II, some deer parks were destroyed and a number of individuals escaped, forming two free-living populations, which are believed to be essentially of Japanese sika (Vavruněk and Wolf 1977). Populations of Dybowski's sika have been maintained until now entirely within deer parks and enclosures. The exact number of Dybowski's sika currently held in such captive populations is unknown; however, unofficial estimates mention 500–650 individuals. This population is significant because, due to many previous and recent translocations of stock, especially among the Czech Republic, Poland, Austria, and Germany, it is generally representative of the wider Central European population of Dybowski's sika (Vach et al. 2010).

This study represents the first population genetics study of the free-living Primorian and the introduced Czech Dybowski's sika population. Our aim was to examine the taxonomic status of individuals maintained as Dybowski's sika in the Czech Republic and to compare the genetic diversity of the introduced captive population with the native Primorian population. We also aimed to assess the level of differentiation between the Primorian and the introduced Czech "founder" population of Dybowski's sika.

Materials and Methods

Sampling and DNA Extraction

Tissue samples of Dybowski's sika from the Czech Republic were obtained in the form of small pieces of ear taken from animals maintained in enclosures or as small pieces of muscles from culled individuals. Altogether, 124 tissue samples of Dybowski's sika were obtained from the 17 Czech enclosures (Figure 1).

Samples of Primorian sika were obtained from carcasses (prey remains, animals that died due to exhaustion during winter, etc.) and from poached animals discovered. The samples were collected from two sampling sites in the Primorsky Krai: Lazovsky Reserve (Lazovsky District) and Sikhote-Alin Reserve (Terneysky District) between Sikhote-Alin Ridge and the Sea of Japan, the northernmost area of its distribution in Russia (Figure 1). A total of 109 tissue samples were obtained from native Primorian sika (13 from Sikhote-Alin Reserve and 96 from Lazovsky Reserve).

All tissue samples were stored in 96% ethanol. The DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) and the Jetquick Tissue DNA Spin Kit (GENOMED GmbH, Löhne, Germany) were used to isolate DNA from tissue samples, according to the manufacturer's protocols.

MtDNA

Amplification of MtDNA Markers

Two mtDNA markers, the cyt *b* and the CR, were chosen to evaluate the phylogeographic relationships between both populations and to establish the taxonomic status of the Czech population of Dybowski's sika. These markers, segments of mtDNA with different mutation rates (Douzery and Randi 1997; Randi et al. 1998), are those regularly selected for use in phylogeographic and taxonomic studies (Kuwayama and Ozawa 2000; Mahmut et al. 2002; Zachos et al. 2003; Feulner et al. 2004).

Complete cyt *b* amplification was performed using primers L14724 and H15915 (Irwin et al. 1991), and the CR was amplified using primers L15926 and H00651 (Kocher et al. 1989). Polymerase chain reaction (PCR) amplification of cyt *b*/CR was conducted in a reaction volume of 25 µL as follows: an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 40/30 s, annealing at 50/53 °C for 40/30 s, an extension at 65 °C for 90/60 s, and a final extension of 5 min at 65 °C. Each 25 µL of reaction mixture contained 4/8 µm of a particular primer, 12.5 µL of Top-Bio PPP Master Mix, and 1/2 µL of DNA template. The PCR products obtained were purified using the Qiagen QIAquick PCR Purification Kit or the GENOMED Jetquick PCR Purification Spin Kit and sequenced using BigDye Terminator sequencing chemistry on an ABI 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). The sequences were assembled and checked in Sequencher v.4.6 (Gene Codes Corp., Ann Arbor, Michigan, USA), and an alignment was created in BioEdit v.7.0.1 (Hall 1999). All haplotype sequences were submitted to the GenBank database (Supplementary Table S1).

Haplotype Data Set

We obtained 230 complete cyt *b* sequences (1140 bp) and 228 complete CR sequences (997 bp). The lengths of the haplotypes of the CR differ between particular sika deer subspecies as well as between sika and other deer species (Douzery and Randi 1997; Nagata et al. 1999; Cook et al. 1999). We also detected this variation in length. Therefore, we excluded the repetitive units from the alignment, and its final length, including the outgroup *Capreolus pygargus*, consisted of 820 characters.

The haplotype data set was completed with cyt *b* and CR sequences of Japanese and other mainland Asian sika subspecies—European red deer, Manchurian wapiti, and American wapiti (Supplementary Table S1)—to evaluate the taxonomic status of Dybowski's sika. Haplotypes of *C. n. yesoensis* (JF893474 and JF893502), *C. n. nippon* (JF893487 and JF893526), *C. elaphus* (JF893495-6 and JF893541-2), and *C. canadensis xanthopygus* (JF893493-4 and JF893539-40) were obtained from tissue samples originating from Hokkaido Island, Nagasaki region, Czech Republic, and Primorsky Krai, respectively. Other haplotypes (*C. n. sichuanicus,* JN389443; *C. n. kopschi*, HQ832482; *C. n. taiouanus,* EF058308; *C. n. mageshimae,* AB021092; *C. n. yakushimae,* AB218689; *C. canadensis nelsoni,* AY347753 and AF016961) were obtained from the GenBank database. We used white-tailed deer (*Odocoileus virginianus,* DQ379370) and Siberian roe deer (*C. pygargus,* JF893543) as outgroups for rooting of the cyt *b* and CR phylogenetic trees, respectively (Supplementary Table S1). The Latin and the common names of all taxa used in this study are given in Supplementary Table S2.

Phylogenetic Analyses

The best models of evolution were determined by MrModeltest 2.2 (Nylander 2004) under the Akaike Information Criterion. For the haplotype data set of cyt *b*, the GTR+I+G model was used, and for the haplotype data set of the CR, the HKY+I+G model of evolution was used. These models were used for calculation of sequence divergence and for all phylogenetic analyses.

Evolutionary relationships between haplotypes were firstly inferred under maximum parsimony criterion using PAUP* v.4.10b (Swofford 2000). Heuristic searching was conducted 100 times with random addition of sequences and a tree-bisection-reconnection swapping algorithm for rearrangement of branches. Resulting topology was tested using 1000 bootstrap replicates (Felsenstein 1985). Phylogeny was further investigated under the maximum likelihood

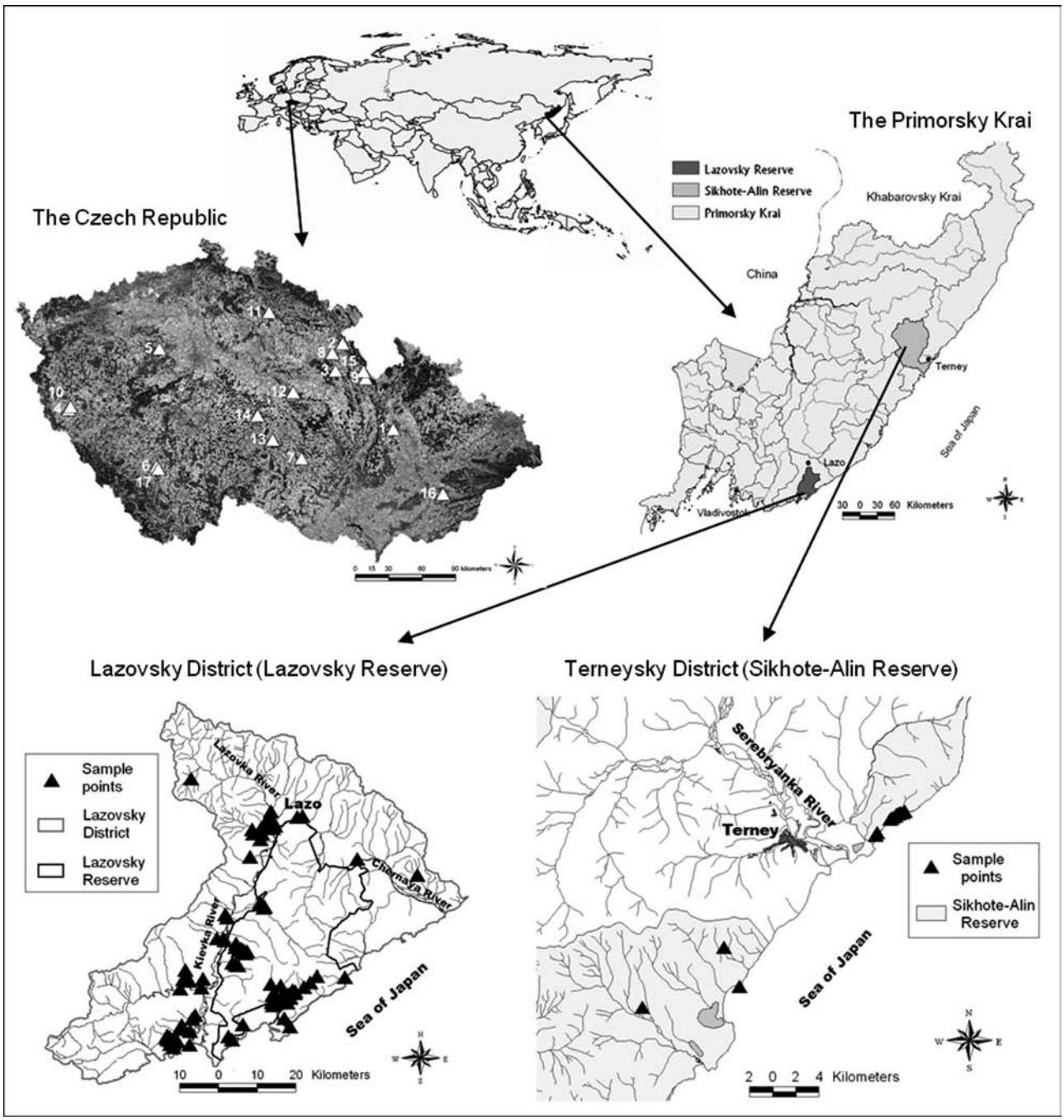

Figure 1. Sampled enclosures (1, Bila Lhota; 2, Bydlo; 3, Castolovice; 4, Horsovsky Tyn; 5, Lany; 6, Lidmovice; 7, Merin; 8, Opocno; 9, Pastviny; 10, Podraznice; 11, Rovensko; 12, Uhercice; 13, Usobi; 14, Veselsko; 15, Vidlice; 16, ZOO Zlin; 17, Vodnany) within the Czech Republic and the locations of samples taken in Lazovsky Reserve and Sikhote-Alin Reserve in Primorsky Krai, where material for the genetic analysis was obtained.

criterion using PhyML (Guindon and Gascuel 2003) and heuristic searching with parameters of the model of evolution based on the results of MrModeltest 2.2. Tree support was assessed using 1000 bootstrap replicates. Finally, phylogeny was assessed using a Bayesian approach using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Bayesian Markov chain Monte Carlo (MCMC) sampling was performed with four heated and one cold chains run for 2 000 000 iterations, using estimated model parameters as starting values. The standard deviation of split frequencies was used to assess the sufficient number of generations. The convergence of the chains to stationarity was checked using Tracer 1.5 (Rambaut and Drummond 2009), and the first 100 000 generations were discarded as burn-in. Two independent runs were conducted,

and Bayesian posterior probabilities were obtained from the 50% majority rule consensus of trees sampled every 100 generations.

Relationships between haplotypes were also calculated and visualized by constructing haplotype networks using the median-joining method available in Network v 4.611 (Bandelt et al. 1999). Haplotype diversity (H_D), nucleotide diversity (P_i), and number of polymorphic sites (S) were detected using DnaSP 4.9 (Rozas et al. 2003).

Microsatellite Loci

Amplification of Microsatellite Loci and Genotyping

The microsatellite analysis was performed using 13 nuclear loci. Each sample was amplified in a 10 µL reaction volume, containing 1 µL of DNA template, 5 µL of the Qiagen Multiplex PCR Kit, and the corresponding primer pair—each forward primer was labeled at the 5'-end with fluorescent dyes (Applied Biosystems) (see Supplementary Table S3 for more details about primer pairs and PCR conditions). PCR was performed in 35 cycles of denaturation for 30 s at 94 °C, annealing for 90 s at 55 or 60 °C, and an extension for 60 s at 72 °C, preceded by 15-min initial denaturation at 95 °C and followed by 10-min terminal extension at 72 °C. Then, 1 µL of the PCR products was added to a mixture of Size Standard GeneScan LIZ600 (Applied Biosystems) and formamide, and the mixture was denatured and run on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The DNA fragment sizes were independently scored using GENEMAPPER 3.7 software (Applied Biosystems) by two people (J.K-P. and M.B.). The PCR was repeated if no allele was amplified, when the peaks were too low, or when the allele had a high number of stutters or atypical shape.

Measures of Population Genetic Diversity

The number of alleles (N_A), the number of private alleles (P_A), allelic richness (AR) corrected for sample size, and observed (H_O) and expected (H_E) heterozygosities were estimated for each locus in both populations using FSTAT 2.9.3.2 (Goudet 2001). This program was also used to estimate the inbreeding coefficient (F_{IS}) and the pairwise index of genetic differentiation (F_{ST}). Tests of departure from the Hardy–Weinberg equilibrium (HWE) using the exact probability test and linkage tests of disequilibrium between any two loci were conducted using GENEPOP v.3.4 (Raymond and Rousset 1995). The *P*-values for multiple testing were corrected using the Bonferroni correction (Rice 1989). The frequency of null alleles was also estimated using this program. The allelic dropout was detected using Micro-Checker v.2.2.3 (van Oosterhout et al. 2004).

Analysis of Population Demographic Changes

Any recent bottlenecks in the Primorian population were tested using the BOTTLENECK program (Cornuet and Luikart 1996). We considered all three models of molecular evolution: the infinite allele model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM), which is probably closer to the true mode of mutation at most microsatellite loci (Di Rienzo et al. 1994; Piry et al. 1999). The proportion of alleles attributed to SMM under TPM was set to a default of 70%, with a variance of 30. Ten thousand iterations were used for each mutation model. The one-tailed Wilcoxon signed-rank test for heterozygote excess was applied as a test of significance (Cornuet and Luikart 1996; Piry et al. 1999), and the distribution of allele frequencies was tested against the L-shaped distribution as expected under the mutation-drift equilibrium (Luikart et al. 1998).

Further, recent and historical population decline or expansion was inferred using MSVAR v.1.3 (Beaumont 1999; Storz and Beaumont 2002), which implements a coalescent simulation-based Bayesian likelihood analysis, assumes a strict SMM, and estimates the posterior probability distribution of population parameters using MCMC simulation, based on the observed distribution of microsatellite alleles and their repeat number. Five independent tests for the linear model of decline/expansion and five tests for the exponential model were run using priors with large variances to minimize their effects on posterior distributions (based on literature records about population demographic changes of Dybowski's sika and within the range of mutation rate recommended for microsatellite loci of mammals from 10^{-5} to 10^{-2} per locus per generation [Dallas 1992, Ellegren 1995]). In every simulation, the chain was simulated for up to 2×10^9 MCMC iterations, recording parameter values for every set of 1×10^5 iterations to give 20 000 recorded parameter sets from the posterior distribution. We discarded the first 10% of recorded values for each chain (when simulations may not have stabilized) and processed the data using the scripts implemented in the R program (R Development Core Team 2006; http://www.r-project.org) . Convergence of individual chains was checked visually, and convergence among chains was tested with the Gelman and Rubin (1992) statistic using the R package.

Analysis of Genetic Structure

The genetic structure of both populations was assessed by the Bayesian clustering procedure in the STRUCTURE 2.3.3 program (Pritchard et al. 2000). The program was run with five independent simulations for each value of K from 1 to 8, with 1 000 000 permutations and an initial burn-in of 100 000 generations. In all simulations, an admixture ancestry model without using sampling locations as prior information and a correlated allele frequency model were used. The K value was estimated by Evanno's calculation (Evanno et al. 2005), which is based on the second-order rate of change in the log probability of the data between successive values of K (ΔK). Factorial correspondence analysis (FCA) was performed in the program GENETIX 4.05.2 (Belkhir et al. 1996–2004).

Results

MtDNA

Within all samples, we detected 6 haplotypes of cyt *b* and 13 haplotypes of CR. All haplotypes were submitted to the

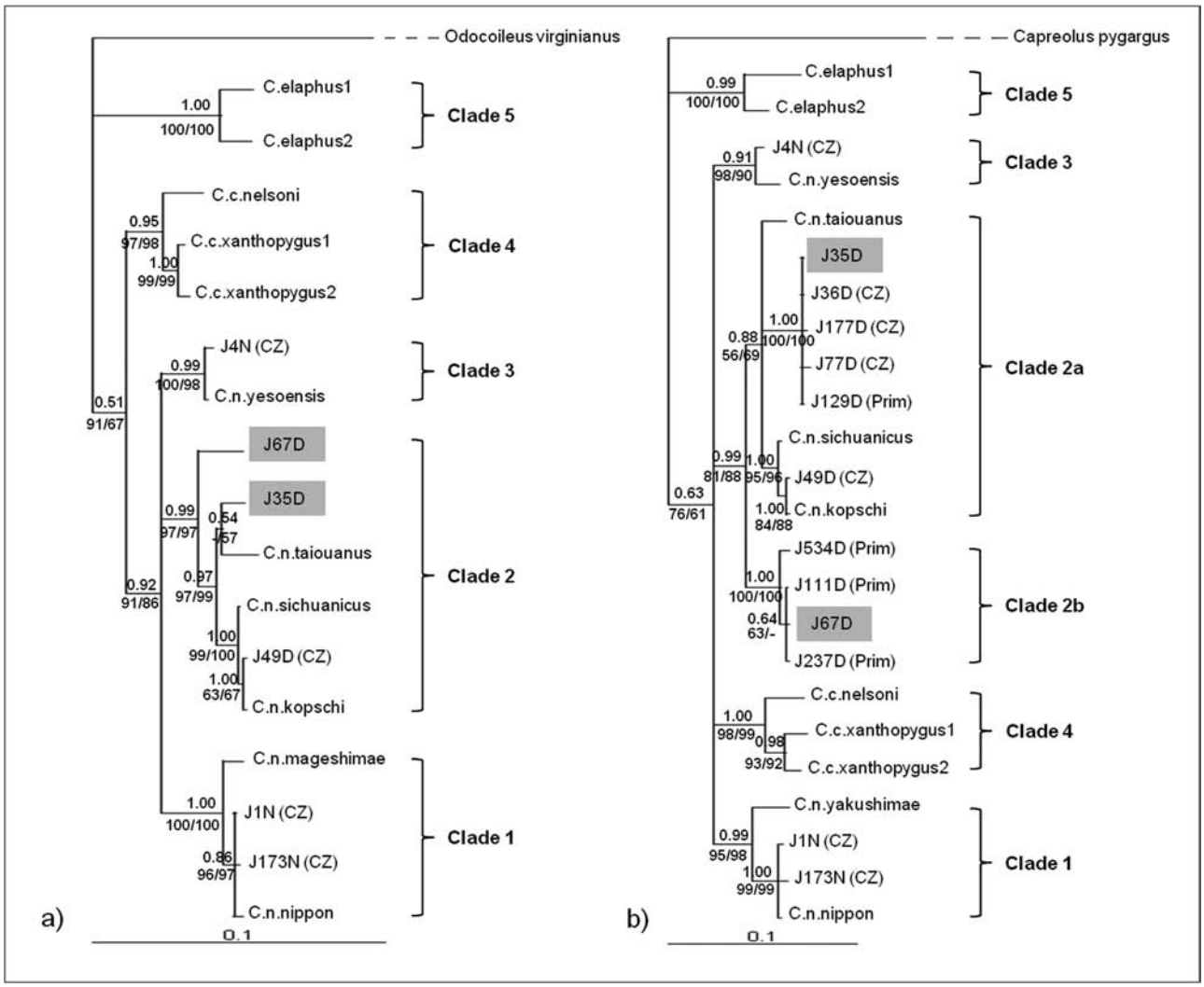

Figure 2. Bayesian 50% majority rule consensus phylogram with support for the major nodes: (**a**) cyt *b* gene and (**b**) CR. The posterior probabilities of Bayesian analysis are indicated above the branches; bootstrap support of maximum parsimony (1000 replicates)/maximum likelihood (1000 replicates) analyses is indicated below the branches. The geographic locations of haplotypes are marked in the brackets after the label: CZ, Czech Republic and Prim, Primorsky Krai. Haplotypes common for both populations are highlighted by gray color in the background of the label.

GenBank database (for accession numbers, see Supplementary Table S1). The exact number of individual haplotypes in Czech enclosures and in both sampling sites in the Primorsky Krai is given in Supplementary Table S4.

The phylogenetic analysis was done for both markers to evaluate the phylogenetic relationships among haplotypes found. In this analysis, the haplotypes of several sika subspecies (from Japan and mainland Asia), red deer (*Cervus elaphus*), and wapiti (*C. canadensis*), obtained from GenBank database or from our own samples (see more in Supplementary Table S1), were also included. The resulting trees showed similar topologies with significant bootstrap support for the major clades, and they clearly showed five distinct groups of haplotypes (Figure 2).

The sika haplotypes formed three separate clusters. The haplotypes J1N, J4N, and J173N (of cyt *b* and CR), found only in the Czech population, clustered within two clades, representing two different mitochondrial lineages of Japanese sika, southern Japan (Clade 1) and northern Japan (Clade 3), in both trees. All other found Czech haplotypes and all Primorian haplotypes were grouped with haplotypes of other mainland Asian subspecies (*C. n. sichuanicus, C. n. kopschi,* and *C. n. taiouanus*) and represented the mainland Asian sika deer group marked as the "Clade 2" in cyt *b* phylogram, respectively, "Clade 2a" and "Clade 2b" in CR phylogram. However, within this group, the haplotype J49D surprisingly clustered with haplotype of *C. n. kopschi*, an endangered sika subspecies from south-east China. This confirmed that maternal lineages of multiple mainland Asian subspecies additional to Dybowski's sika are also present in the Czech Republic. Analysis of Clade 2 further revealed two divergent lineages of Dybowski's sika—one represented by cyt *b* haplotype J67D,

respectively, by several CR haplotypes grouped within Clade 2b, and the other represented by cyt *b* haplotypes J35D and J49D, respectively, by several CR haplotypes grouped within Clade 2a. All other haplotypes of mainland sika subspecies also clustered within this second lineage. The genetic distances between these two lineages of Dybowski's sika were higher (it varied between 2.3% and 3.2%) than the distances between various mainland sika subspecies (it varied between 1.1% and 2.5%) (Supplementary Table S5).

Two other clades were formed by haplotypes of red deer and wapiti: the Clade 4 with two haplotypes of Manchurian wapiti from Primorsky Krai clustered with North American wapiti *C. c. nelsoni,* and the Clade 5 with European red deer haplotypes.

The percentage distances (minimum and maximum values) between the main clades and separately also within the Clade 2 (a and b), calculated using particular models of evolution, are provided in Supplementary Table S5. Based on the topologies of the constructed trees, bootstrap support values, and percentage distances between the main clades, we concluded that Manchurian wapiti and Dybowski's sika are phylogenetically divergent, and therefore, we confirmed the taxonomic status of Dybowski's sika as a sika deer.

The haplotypes J1N, J4N, and J173N (found only in the Czech population) clustered with haplotypes of other sika deer subspecies from the Japanese islands (Figure 2). The same haplotypes were previously found in the Czech population of Japanese sika (Barančeková et al. 2012). Similarly, the haplotype J49D found just in one Czech enclosure (Opocno) was identified as haplotype of Kopschi sika and not Dybowski's sika subspecies. Therefore, we decided to exclude 39 Czech samples with these haplotypes from further analyses in order not to increase artificially the genetic diversity of the Czech Dybowski's sika population. However, they were added to the Bayesian clustering analysis and the FCA for more detailed analysis of their relationship with the rest of the Dybowski's sika samples.

A total of 26 polymorphic sites were found in the cyt *b* gene of Dybowski's sika, giving two haplotypes J35D and J67D (Supplementary Table S6). The haplotype and nucleotide diversity of the Czech and the Primorian sika deer populations were extremely low (Table 1). The haplotypes were present in both populations. The relationships between all sika cyt *b* haplotypes were illustrated using the median-joining network (Figure 3a).

Within the alignment of CR sequences of Dybowski's sika haplotypes (997 bp), 35 characters were variable and 34 were parsimony informative, giving 9 haplotypes (Supplementary Table S7). A median-joining network was generated to infer the relationships between all CR sika haplotypes (Figure 3b). Only two haplotypes were detected in both populations. The haplotype and nucleotide diversity of the Czech population were higher than that of the Primorian population for both mtDNA markers (Table 1).

Table 1. Haplotype and nucleotide diversity of cyt *b* and CR of both studied populations

	N	N_H	H_D	P_i	K	S
Cyt b						
Czech	93	3	0.4078	0.00791	9.020	33
Primorian	106	2	0.2846	0.00649	7.401	26
CR						
Czech	93	6	0.6104	0.01088	9.322	36
Primorian	104	6	0.4455	0.00836	7.066	25

N = number of samples.

Microsatellite Loci

Microsatellite Genetic Diversity

Genotypes for 13 microsatellite loci were obtained for 84 Czech Dybowski's sika deer samples and for 109 Primorian Dybowski's sika samples. All analyzed loci were polymorphic in both sampled populations. The number of alleles varied in the Czech population from 6 to 18 (average 10, AR = 9. 98) and in the Primorian population from 4 to 20 per locus (average 10.23, AR = 10.19; Table 2). The average H_O was higher for the Primorian population (H_O = 0.617) than for the Czech population (H_O = 0.598). In contrast, the average H_E was higher for the Czech population (H_E = 0.735) than for the Primorian population (H_E = 0.710). Consequently, estimated values of Wright's fixation index (Czech F_{IS} = 0.183; Primorian F_{IS} = 0.132) indicated a certain level of heterozygote deficiency and a high level of inbreeding in both populations, especially in the captive Czech population. No evidence for allelic dropout was detected.

Each population was tested separately for linkage disequilibrium across all pairs of the 13 microsatellite loci. After Bonferroni adjustment for multiple testing, no linkage disequilibrium was found between any pairwise locus combinations in the Primorian population. On the other hand, six combinations were significant in the Czech population. Further, significant deviations from HWE after the Bonferroni correction (adjusted P = 0.00385) were observed at 5 loci for the Primorian population and at 10 loci for the Czech population (Table 2). Relatively high frequencies of null alleles were estimated for several loci in both populations, mainly in the Czech population (Table 2).

Population Differentiation

A high number of private alleles was detected within both populations (Czech: 0–7 per locus, Primorian 1–11 per locus, altogether 43 and 46 alleles per population, see Table 2), and the value of the fixation index F_{ST} = 0.086 (presented as a moderate value) indicates genetic differentiation between populations. This differentiation was also supported by the assignment of both populations by FCA (Figure 4), even though we included in the analysis the 39 samples (above) with mtDNA haplotypes, which had clustered with haplotypes of other sika subspecies.

For all of the 232 samples analyzed in the Bayesian clustering analysis, the maximum value for the "estimated likelihood of K" was found at K = 8, but for K values higher than 2, the likelihood values only showed a slight increase (Figure 5a), which usually indicates isolation-by-distance relationships in the data set (Worley et al. 2004; Frantz et al. 2006). Also, the ΔK distribution (Evanno et al. 2005) showed the highest peak at K = 2 (Figure 5b).

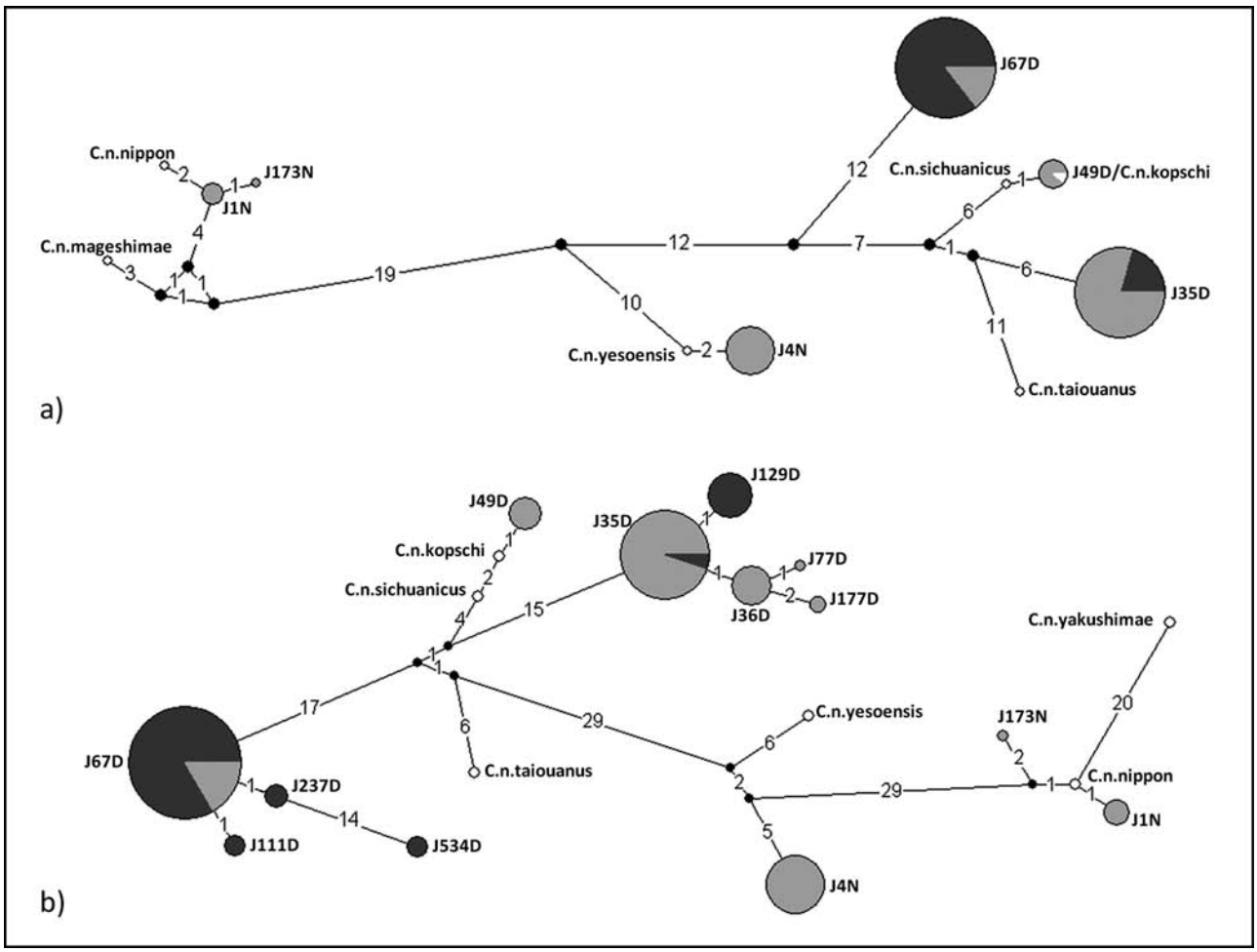

Figure 3. Median-joining network of (**a**) cyt *b* gene and (**b**) CR sika haplotypes. The number of mutations between haplotypes is related to the length of branches, and it is indicated on branches. The median vectors are indicated by black dots, and circle sizes are proportional to the number of the same haplotypes in the data set. Colors indicate geographic origin of samples: light gray, Czech population of Dybowski's sika; dark gray, Primorian population of Dybowski's sika; white, other sika subspecies from mainland Asia, Taiwan, and Japan.

The proportion of individual genotypes (q) belonging to each of these two inferred clusters clearly corresponded to predefined populations. The Czech samples were divided into two clusters: cluster 1 (q = 0.814) and cluster 2 (q = 0.186), whereas the Primorian samples belonged almost exclusively to cluster 2 (q = 0.985). Samples from the Czech Republic, which is based on mtDNA markers belonged to maternal lineages of other sika subspecies, clustered in a similar manner to the other Czech samples of Dybowski's sika within two clusters: cluster 1 (q = 0.758) and cluster 2 (q = 0.242). These samples did not form separate clusters in any of the other models, which divided samples into more clusters (K = 3–8; Figure 6).

Population Demographic Events in the Primorian Population

Using the BOTTLENECK program, a significant excess of heterozygosity was observed only in the Primorian population using IAM (Wilcoxon signed-rank test, $P = 0.024$), suggesting that there was a recent bottleneck event in this population. Two other mutational models, SMM ($P = 0.998$) and TPM ($P = 0.682$), were not significant. In addition, there was no significant deviation from the normal L-shaped distribution of allele frequencies. On the contrary, using SMM and TPM, we detected a significant heterozygosity deficiency in the Primorian population, which could indicate a serious long-lasting reduction in population abundance or a rapid population expansion after previous bottleneck event (Maruyama and Fuerst 1985).

The results of the MSVAR procedure for assessing and dating population size changes identified a signal of historical population decline (Supplementary Table S8). Under an exponential model of population size change, a decline from an estimated ancestral effective population size (N_1) of 10 471 (794–104 713) individuals to a current one (N_0) of 117 (11–1288) individuals started *c.* 3400 (265–31 774) years ago, based on the generation time of 4 years (based on Forestry Commission records; Swanson G, Hendry D, unpublished

Table 2. Summary of the N_A, P_A, AR, H_O and H_E, and *P*-values of the test for the HWE (Bonferroni-corrected $P = 0.0038$; significant deviations from Hardy–Weinberg expectations are marked in bold); values of Wright's fixation index (F_{IS}) and estimated frequencies of null alleles (F_{Null}) are given for each microsatellite locus in both populations

	Primorian population (*n* = 109)							Czech population (*n* = 84)						
Locus	N_A (P_A)	AR	H_O	H_E	P (HWE)	F_{IS}	F_{Null}	N_A (P_A)	AR	H_O	H_E	P (HWE)	F_{IS}	F_{Null}
HH064	11 (2)	10.954	0.752	0.827	0.008	0.090	0.186	10 (1)	10.000	0.821	0.839	**0.000**	0.021	0.136
BM4006	4 (1)	3.954	0.303	0.351	0.007	0.138	0.149	6 (3)	5.976	0.429	0.689	**0.000**	0.380	0.202
BOVIRBP	10 (4)	9.926	0.787	0.841	**0.003**	0.064	0.060	6 (0)	6.000	0.655	0.705	0.248	0.071	0.068
BM6438	8 (2)	8.000	0.604	0.784	**0.000**	0.230	0.151	9 (3)	9.000	0.434	0.686	**0.000**	0.369	0.167
INRA40	20 (11)	20.000	0.769	0.870	0.040	0.116	0.055	13 (4)	12.976	0.690	0.879	**0.000**	0.215	0.224
BM4513	16 (5)	15.920	0.546	0.787	**0.000**	0.306	0.139	18 (7)	17.928	0.595	0.762	**0.000**	0.220	0.243
TGLA127	11 (4)	10.943	0.636	0.721	0.511	0.120	0.035	8 (1)	8.000	0.655	0.767	**0.001**	0.147	0.084
TGLA40	8 (2)	7.998	0.706	0.792	0.011	0.108	0.047	8 (2)	8.000	0.488	0.742	**0.000**	0.343	0.207
CSSM43	7 (1)	6.963	0.796	0.769	0.302	−0.036	0.073	12 (6)	11.964	0.690	0.781	0.004	0.117	0.310
FSHB	15 (5)	14.952	0.853	0.880	0.179	0.031	0.063	12 (2)	11.976	0.726	0.828	**0.000**	0.124	0.095
INRA131	10 (3)	9.860	0.716	0.760	0.006	0.059	0.289	14 (7)	13.940	0.857	0.858	0.042	0.001	0.000
INRA5	6 (4)	5.961	0.130	0.166	**0.001**	0.221	0.088	7 (5)	6.976	0.310	0.378	**0.000**	0.181	0.071
UWCA47	7 (2)	6.999	0.421	0.684	**0.000**	0.386	0.176	7 (2)	7.000	0.429	0.646	**0.000**	0.337	0.163

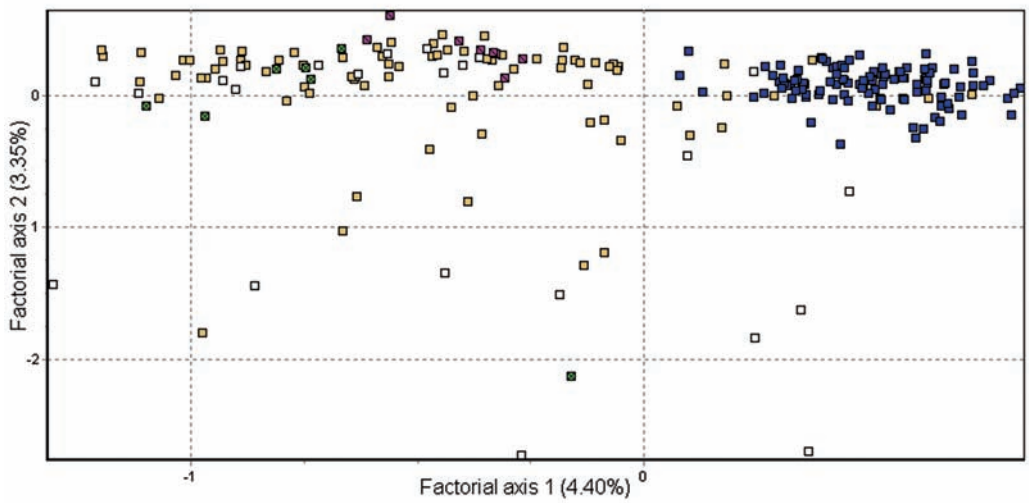

Figure 4. A biplot of the FCA performed using GENETIX based on 13 microsatellite loci. Microsatellite genotypes of Czech Dybowski's sika: 84 samples (orange squares), Primorian Dybowski's sika: 109 samples (blue squares), Czech samples with mtDNA haplotype clustered with haplotype of Kopschi sika: 8 samples (pink squares), Czech samples with mtDNA haplotypes clustered within northern Japanese sika clade: 25 samples (white squares), and Czech samples with mtDNA haplotypes clustered within southern Japanese sika clade: 6 samples (green squares).

data in Pitra and Lutz 2005. Under a linear model, a decline from 4074 (447–53 703) individuals to a current 30 (3–437) individuals started more than 6000 (728–87 511) years ago. However, the confidence intervals (limits) of estimated values (mentioned in brackets) are extremely wide, so no strong inference can be made from these results.

Discussion

Many species of large vertebrates are losing genetic diversity because of population decline (Avise and Hamrick 1996). This can lead to a reduction in fitness and increased inbreeding depression (Frankham 1995). Dybowski's sika are classified as an endangered subspecies as their population numbers have been severely impacted by hunting management during the last centuries. This study represents the first population genetics study of the free-living Primorian and the introduced Czech populations using both mtDNA and microsatellite markers.

MtDNA

Our results based on the mtDNA analysis confirmed the taxonomic status of Dybowski's sika as a member of the sika deer species group, similar to the results of previous phylogenetic studies (Cook et al. 1999; Nagata et al. 1999; Kuwayama and Ozawa 2000). Theory about the origin of this subspecies as a hybrid of sika deer and wapiti was not supported. Moreover, we did not detect any recent hybrids between

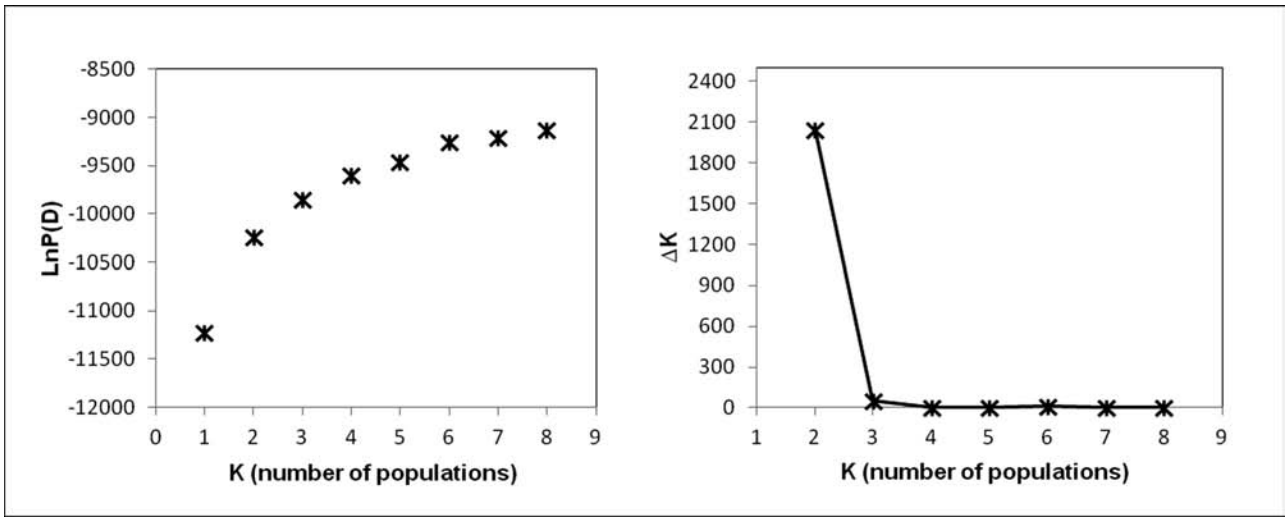

Figure 5. (**a**) Results of the Bayesian clustering analysis using the STRUCTURE program showing the log probability of the data Ln P(D) as a function of the number of assumed populations (K). (**b**) The values of Δ(K), calculated according to Evanno et al. (2005), were used for selection of the best fitting model.

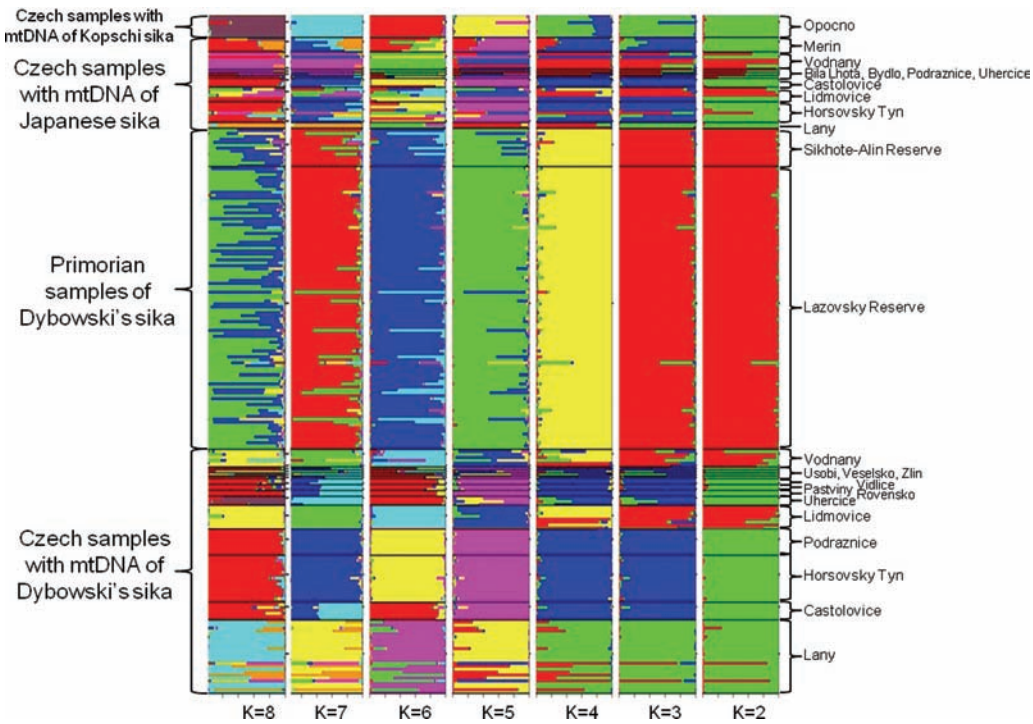

Figure 6. Output from the Bayesian clustering procedure of the STRUCTURE program for 232 samples and for K = 2–8. Particular Czech enclosures and both Primorian sampling sites are marked at the right side; individual populations are marked at the left side. Each vertical bar corresponds to one individual.

Dybowski's sika and Manchurian wapiti in the Primorsky Krai using mtDNA markers.

According to a priori expectations, the genetic diversity of maternally inherited mtDNA was found to be extremely low in both Russian and Czech populations. The low diversity coupled with the known history of the two populations means that there is evidence to infer a founder effect and possibly also the legacy of a bottleneck in the Primorian population, which was previously suggested in the study by Kholodova et al. (2007) using partial D-loop sequences (542 bp).

The analysis of mtDNA also confirmed that the majority of samples from the Czech population belonged

to the mitochondrial lineage of the Asian sika deer group and were found to be closely related to or represented the same haplotypes as the samples from the native Primorian population. However, this analysis also uncovered that almost one-third of the Czech samples contained mtDNA haplotypes of other sika subspecies, although these individuals were morphologically determined as Dybowski's sika. These haplotypes clustered with haplotypes of subspecies from northern Japan, southern Japan, and south-east China. This definitively confirms that there has been hybridization between various sika subspecies even within the enclosed populations either in the Czech Republic or amongst animals imported from other Central European collections.

The analysis of mtDNA helped to validate the taxonomic status of Dybowski's sika, but it also showed other problems in the taxonomy of mainland sika subspecies group. Analysis revealed that there are two divergent lineages of Dybowski's sika (Clades 2a and 2b), with one of these lineages (Clade 2a) clustered with all other mainland sika subspecies haplotypes. Wu et al (2004), who analyzed mtDNA genetic diversity of Chinese sika deer, also found only two different mitochondrial lineages within three recognized Chinese subspecies. It is clear that some taxonomic revision within mainland Asia sika group is required (as previously for island populations, see Tamate and Tsuchiya 1995; Nagata et al. 1999; Nagata 2009). However, to do this, more extensive collection of samples of different geographic origin is needed.

Microsatellite Loci

Contrary to the mtDNA findings, we detected a relatively high level of heterozygosity and polymorphism of all microsatellite markers in both populations. The proportion of polymorphic loci and the mean number of alleles per locus is generally expected to be low in introduced populations (Hartl and Pucek 1994); but Hartl et al. (2003) have shown that deer in enclosures revealed considerable deviations of allele frequencies from isolation-by-distance expectations, without the expected loss of genetic diversity. Moreover, microsatellites can uncover variability and differentiation in cases where the mitochondrial markers are not sensitive enough (Feulner et al. 2004; Hmwe et al. 2006).

Our results are consistent with both possibilities. The genetic variability, measured by H_E, of both populations (Czech $H_E = 0.735$, Primorian $H_E = 0.710$) was higher than values previously found for free-living native and introduced Japanese sika deer populations, for example, sika deer in Kinkazan Island $H_E = 0.516$ (Tamate et al. 2000), sika deer in Ireland $H_E = 0.21$ (McDevitt et al. 2009), and sika deer in Great Britain $H_E = 0.15$ (Senn 2009). H_E were also similar or higher than those found in some free-living (mostly bottlenecked) red deer populations, for example, Bavarian red deer $H_E = 0.68$ (Kuehn et al. 2003), Danish red deer $H_E = 0.524$ (Nielsen et al. 2008), Iberian red deer $H_E = 0.771$ (Sanchez-Fernandez et al. 2008), red deer in Sardinia $H_E = 0.6$ (Hmwe et al. 2006), or Tunisian red deer $H_E = 0.78$ (Hajji et al. 2007). However, they were lower than values usually found in abundant deer populations, for example, Carpathian red deer $H_E = 0.81-0.9$ (Feulner et al. 2004).

In the Czech population of Dybowski's sika, the majority of analyzed loci showed significant deviations from Hardy–Weinberg expectations (10 loci out of a total of 13 loci), and also linkage disequilibrium was detected for six pairwise loci combinations. The level of linkage disequilibrium is influenced by a number of factors, including genetic linkage, selection, the rate of recombination, the rate of mutation, genetic drift, nonrandom mating, and population substructure (Hill and Robertson 1968; Pritchard and Rosenberg 1999; Pritchard et al. 1999; Gaut and Long 2003; Mueller 2004). Similarly, there are several possible explanations for deviations from HWE: the translocations of animals into the population, which has the same effect as immigration (whose absence is one of the premises of HWE), population substructure, and the presence of null alleles. All these factors may cause loci to deviate in the Czech population. Significant numbers of animals are translocated between enclosures each year and in many, but not all cases, the loci with significant deviations from HWE matched those for which the GENEPOP analysis suggested the high possibility of null alleles. Estimated frequencies of null alleles for at least five loci for the Czech population were high (from 20% up to 30%, Table 2). However, recent hybridization among several subspecies could also cause loci to deviate from HWE and linkage equilibrium (Goodman et al. 1999).

In the Primorian population, too, several loci showed deviations from HWE. Similarly to the Czech population, the higher values of F_{IS} and the lower than expected values of H_O (heterozygosity deficiency) provide evidence of inbreeding in the population (Genlous and Björn 2003). Although an effect of null alleles as suggested by our analysis using GENEPOP should not be excluded (estimated frequencies of null alleles for loci deviated from HWE were 6–17.6%), it seems to be less likely because these calculations are primarily based on discrepancies between H_O and H_E, which are also influenced by inbreeding. Therefore, we consider inbreeding as more likely to explain the low proportion of heterozygotes and the observed Hardy–Weinberg nonequilibrium.

Further, using microsatellite markers, we detected a high degree of genetic differentiation between the introduced and the native population of Dybowski's sika. Even though we detected relatively high frequencies of null alleles, which could have affected the measures of population differentiation by overestimation of F_{ST} and genetic distances (Chapuis and Estoup 2007), genetic population differentiation was also supported by the Bayesian clustering analysis and by the separation of both populations by the FCA. A lack of gene flow, the strong effect of genetic drift due to inbreeding, and the fast differentiation between source and founder populations through random sampling (founder effect) will certainly have influenced the level of differentiation during the breeding of introduced individuals and their offspring in captivity over a number of generations. In addition, given suggestions of a genetic bottleneck occurring within the Primorian population due to the population reductions during last century (Aramilev 2009), it is also possible that the Czech population could also retain alleles that were lost from the Primorian population at that time. However, the high differentiation

and high number of private alleles within the Czech samples could also be affected by previous crossbreeding within enclosures (Thévenon et al. 2004), confirmed in our own study by mtDNA analyses, which revealed the presence of maternal lineages of other sika subspecies in the captive Dybowski's sika population.

Heterozygosity-based bottleneck tests (Cornuet and Luikart 1996) did not show a signal of population decline of the Primorian population. We detected a recent bottleneck event only using the IAM model of evolution. However, the SMM and the TPM models did not show heterozygosity excess, that is, evidence for a population decline. On the contrary, using these two models, we detected a significant heterozygosity deficiency in the Primorian population, which could indicate a serious, previous long-lasting reduction in population abundance or a rapid population expansion after previous bottleneck event (Maruyama and Fuerst 1985), which would support the historical records about demographic changes in Dybowski's sika population (Aramilev 2009; Voloshina and Myslenkov 2009).

Even though MSVAR simulations, which are very efficient at detecting historical population declines and expansions (Girod et al. 2011), imply that Dybowski's sika population decline might have started much earlier than the 19th century, the confidence limits of the estimated values were so wide that we did not feel these results were particularly meaningful. Several factors could have biased the estimated values, for example, relatively small sample size, unknown microsatellite mutation rates, unsubstantiated estimation of generation time, population admixture (according to historical records the present Primorian population was re-established also by farm-escaped sika deer individuals [Aramilev 2009]), and hybridization with Manchurian wapiti, which was not found by our results but also cannot be rejected, as male wapiti–female sika hybrids would leave a signature within the nuclear genes but not mtDNA (Beaumont 1999; Storz and Beaumont 2002; Girod et al. 2011).

Management Implications

MtDNA analysis confirmed the close relationship between the native Primorian and the introduced Czech population, but at the same time, it revealed hybridization with other sika deer subspecies within the Czech population. Only five enclosures contained just individuals with mtDNA of Dybowski's sika; however, from these enclosures, only one or two samples were obtained (for more details, see Supplementary Table S4). MtDNA haplotypes of individuals from 9 of 17 sampled enclosures belonged to both Japanese sika and Dybowski's sika haplogroups; those from enclosure Merin belonged just to the Japanese sika haplogroup. In the enclosure Opocno, we detected just the haplotype of Kopschi sika subspecies, even though no historical records mention introduction of animals from south-eastern China to the Czech Republic.

Consequently, the current distinction of sika subspecies based on their morphology is very uncertain in the Czech Republic. Unfortunately, as a result of many previous and recent translocations of individuals between enclosures within the Czech Republic as well as between the Czech Republic and neighboring countries, it is probable that hybrid individuals may exist in many captive populations throughout the Central European region. Based on these results, the taxonomic status of all captive Dybowski's sika deer should be verified and, if efforts are to be made to restore the purity of such populations, only genetic pure individuals should be mated together in the future. Our results suggest that the purity of captive individuals should be verified not only by mtDNA analysis but primarily also using nuclear markers.

Our results also revealed a high level of inbreeding and relatively low genetic diversity in the Primorian population, which could have negatively affected fitness, adaptive diversity, and population viability (inbreeding depression). Therefore, this population may require some degree of management intervention, at least a suitable conservation plans and consistent monitoring of population density and fitness, even though the possible population increase in the last decades can indicate that the viability of the population was not affected by historical population abundance changes.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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Corresponding Editor: Jennifer Jackson