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# Admixture of Eastern and Western European Red Deer Lineages as a Result of Postglacial Recolonization of the Czech Republic (Central Europe)

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## Abstract

Due to a restriction of the distributional range of European red deer (*Cervus elaphus* L.) during the Quaternary and subsequent recolonization of Europe from different refugia, a clear phylogeographical pattern in genetic structure has been revealed using mitochondrial DNA markers. In Central Europe, 2 distinct, eastern and western, lineages of European red deer are present; however, admixture between them has not yet been studied in detail. We used mitochondrial DNA (control region and cytochrome *b* gene) sequences and 22 microsatellite loci from 522 individuals to investigate the genetic diversity of red deer in what might be expected to be an intermediate zone. We discovered a high number of unique mtDNA haplotypes belonging to each lineage and high levels of genetic diversity (cyt *b* $H = 0.867$, D-loop $H = 0.914$). The same structuring of red deer populations was also revealed by microsatellite analysis, with results from both analyses thus suggesting a suture zone between the 2 lineages. Despite the fact that postglacial recolonization of Central Europe by red deer occurred more than 10 000 years ago, the degree of admixture between the 2 lineages is relatively small, with only 10.8% admixed individuals detected. Direct translocations of animals by humans have slightly blurred the pattern in this region; however, this blurring was more apparent when using maternally inherited markers than nuclear markers.

**Subject areas:** Population structure and phylogeography, Molecular systematics and phylogenetics
**Key words:** Gene introgression, hybridization, microsatellites, mtDNA, Quaternary, suture zone

As with most temperate European mammal species, current patterns of genetic variability of red deer (*Cervus elaphus* L.) may be the consequence of Quaternary contraction and expansion events due to climatic fluctuations (Hewitt 1999; Hewitt 2004). Based on fossil evidence, it is considered that the species was relatively widely distributed in the southern part of Central Europe 60 000–25 000 years ago, but subsequently restricted to several refugial areas south of the permafrost in southern Europe during the last glacial maximum (LGM) and the early Late Glacial (25 000–14 700 years ago) (Hewitt 1996; Hewitt 2000; Sommer and Nadachowski 2006;

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Sommer and Zachos 2009; Linnell and Zachos 2011; Apollonio et al. 2014). Refugial populations that evolved in allopatry accumulated independent genetic differences that may be used as genetic markers to trace postglacial expansion routes during recolonization in the Late Pleistocene and Holocene (Sommer and Zachos 2009).

In many cases, only a fraction of genetic diversity present in the refuge is found in recolonized areas, leading to a relative genetic depletion in Northern and Central Europe (Hewitt 2000). However, if particular areas have been recolonized from a number of different refugia, the admixture of different genetic lineages can lead to higher genetic variability, thus an admixture zone might be confused with a former refugial area (Provan and Bennett 2008). In the admixture area, the different refugial genomes meet and can form hybrid (=suture, admixture) zones of different width (Nichols and Hewitt 1994). These secondary hybrid zones can provide useful insights into evolutionary mechanisms of reproductive isolation, selection, and the genetic basis of local adaptation. In addition, spatial patterns of cline widths and slopes provide detailed information about the extent of linkage disequilibrium, heterozygote deficits, and a balance between selection and migration (Kawakami and Butlin 2012). Such hybrid zones have been identified in several species, that is, grasshopper, hedgehog, bear, newt, shrew, and mouse, and may well exist also in other species (Hewitt 1989).

The red deer (*C. elaphus*) is one of the most widespread and most important game species in Europe (Apollonio et al. 2010). As a result, red deer populations have been subjected to a variety of anthropogenic interventions (historic or relatively recent) that have potentially affected the genetic structure of local populations (Hartl et al. 2003; Pérez-Espona et al. 2009; Linnell and Zachos 2011; Sykes and Putman 2014). The red deer is probably the species that has undergone the most extensive translocations, mostly in attempts at improving trophy quality or establishing hunting grounds (Niethammer 1963; Hartl et al. 2003). Translocations of alien genotypes into native populations had already started in the Neolithic period and are the most significant type of human intervention, expected to have had major genetic consequences (Hartl et al. 2003; Frantz et al. 2006; Linnell and Zachos 2011). Particularly in Central Europe, it is uncertain if there are any truly native populations left; as red deer have great dispersal abilities, introduced animals and/or their offspring may disperse into other populations of the region (Linnell and Zachos 2011). The genetic impact of translocations increases with the distance over which animals are moved and, at least at a regional scale, the natural genetic pattern can be blurred or even destroyed (Linnell and Zachos 2011).

Selective harvesting of red deer populations is also a management practice which is long-established; selective harvesting could significantly influence the genetic variability and fitness parameters of natural populations (Milner et al. 2007; Linnell and Zachos 2011; Mysterud 2014). Most commonly it is driven by the desire to shoot males with large "trophies" (Geist 1986) and avoid shooting females with offspring to enhance population growth (Milner et al. 2011). This can considerably affect sex ratio, age structure, and effective size of the population and consequently influence survival, growth, and reproduction of red deer (Proaktor et al. 2007; Mysterud 2014). The effects of selective hunting pressure on the population are difficult to predict since the genetic variability of the population may be affected by changes in population size, migration, and mating selection pattern (Mysterud 2014).

While translocations and selective hunting have been influencing the genetic diversity of European red deer for centuries, increasing landscape fragmentation (due to fenced motorways, channels, and huge human settlements) is a more recent phenomenon which, by influencing gene flow between populations, or isolating particular populations now adds a further major factor influencing population genetics and may become the most important factor in the future (Pérez-Espona et al. 2008; Niedziałkowska et al. 2012b). Especially in Central Europe, expanding human infrastructure causes populations to become more and more isolated from each other. As a consequence, gene flow is reduced, effective population size decreases and genetic drift and inbreeding in small and discontinuously distributed populations results in the loss of genetic variability (Zachos et al. 2007; Linnell and Zachos 2011; Apollonio et al. 2014).

Based on previous studies of mtDNA, at least 3 distinct postglacial lineages of European red deer are known (western, eastern, and North-African/Sardinian) (Ludt et al. 2004; Pitra et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011). The western lineage is believed to be indicative of a south-western glacial refuge in Iberian Peninsula and southern France. This mtDNA lineage currently covers Western Europe including the British Isles, Scandinavia, and north-western Poland. The eastern lineage is believed to be indicative of refuge/ia in the Balkans and perhaps somewhere eastern to the Carpathian region (possibly Moldova, around the Black Sea, and up to Crimea, etc.). It is assumed that contact between these eastern refugia was not interrupted during Pleistocene (Sommer et al. 2008; Skog et al. 2009; Linnell and Zachos 2011; Stankovich et al. 2011; Karaiskou et al. 2014). The eastern lineage is primarily found in the Carpathians and the Balkans, south-east and Central Europe (Niedziałkowska et al. 2011). It is presumed that eastern and western lineages probably meet somewhere in south-eastern Germany (Sommer et al. 2008); however, only small numbers of samples from that part of Central Europe where the 2 lineages might meet, have been included in previous phylogeographic analyses (Ludt et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011). Therefore, it is still unknown where exactly the lineages meet and how wide may be the admixture zone between them (Linnell and Zachos 2011). The influence of human activities makes the interpretation of global phylogeographic patterns difficult and it sometimes remains unclear whether natural migration or human-engineered translocation has occurred in certain areas (Niedziałkowska et al. 2011, 2012a; Carden et al. 2012; Apollonio et al. 2014). Nevertheless, despite many past translocations of alien genotypes into the native populations of red deer across Europe, the phylogeographic analysis of Skog et al. (2009) identified only 4 red deer individuals out of 600 analyzed which showed discordances between their geographical location and their genetic lineage, with 2 additional discrepancies found by Nussey et al. (2006) on the Isle of Rum, Scotland and by Carden et al. (2012) in Ireland. This indicates that most of the extensive translocations in red deer—at least as far as maternal lineage is concerned—were carried out within observed mtDNA red deer lineages rather than between them (Skog et al. 2009; Niedziałkowska et al. 2011; 2012a).

Studies of the distribution of mitochondrial DNA haplotypes (cytochrome *b* gene and D-loop) suggest that the modern red deer population remains primarily genetically structured as a result of postglacial recolonization (Polziehn and Strobeck 1998; Polziehn and Strobeck 2002; Kuwayama and Ozawa 2000; Ludt et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011; Zachos and Hartl 2011). However, this structuring of mtDNA may only be the result of red deer female philopatry (Skog et al. 2009) and potentially reveals little about wider genetic structuring. The dispersion of red deer males is higher (Albon and Langvatn 1992) and in species with male-biased dispersal, postglacial recolonization patterns typically

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differ when nuclear DNA markers are included (Waits et al. 2000; Prugnolle and de Meeûs 2002). Microsatellites are popular markers for the study of geographical structure and gene flow because of their high levels of polymorphism and biparental inheritance in a Mendelian fashion (Jarne and Lagoda 1996). To date no detailed study has been done on the possible admixture zone between western and eastern European red deer lineages using nuclear (microsatellite) markers. However, some unpublished results from the Norwegian and north German red deer populations showed less structuring when using microsatellite markers than revealed by mtDNA (A. Skog and F. E. Zachos, unpublished results cited in Skog et al. 2009).

Central Europe represents a crossroads of postglacial colonization routes for many terrestrial species (Hewitt 2004). Within the territory of the Czech Republic, situated in the center of this region, hybrid zones are present at (sub-)species level for several European species as a result of the meeting of different mtDNA lineages originating in different postglacial refugia [e.g., *Mus musculus musculus/domesticus* (Macholán et al. 2012), *Erinaceus europeus/roumanicus* (Bolfíková and Hulva 2012), *Corvus corone corone/cornix* (Brodin et al. 2013)]. However, to date only small number of Czech red deer samples have been used in wider studies of the phylogeography of European red deer, assigning the Czech Republic to the eastern European red deer lineage (Skog et al. 2009; Niedziałkowska et al. 2011). The existence of western lineage within the Czech Republic was previously reported only by Fickel et al. (2012) from the Bohemian–Bavarian border area. Using a larger sample size, we present in this study an analysis of the genetic diversity of the Czech red deer population using both mtDNA [the coding cytochrome *b* (cyt *b*) gene and the noncoding control region (D-loop)] and microsatellite markers, interpreting results in relation to the wider postglacial recolonization pattern reported for European red deer. High sampling intensity allowed us also to investigate the possible impact of human translocations on the blurring the macrogeographical pattern at a local scale. While focusing on samples from the Czech Republic, we also analyzed several samples from 2 neighboring countries, Slovakia and Hungary, to fill gaps in red deer phylogeographic studies in the Central European region.

## Materials and Methods

### Sampling and DNA Extraction

We obtained tissue samples from 486 free-living red deer legally culled during the hunting seasons 2009–2011 in different parts of the Czech Republic (Figure 1)—choosing sample sites representative of the distribution of red deer in the Czech Republic (Supplementary Figure S1 online, Anděra and Červený 2009). Some reference samples (tissue and hair) were collected in Slovakia (13 samples—all used in microsatellite analysis; 11 used in mtDNA analysis) and in Hungary (23 samples—all used in microsatellite analysis; 6 used in mtDNA analysis).

Tissue samples were stored in 96% ethanol, hair samples were fixed using silica gel. The DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), the Jetquick Tissue DNA Spin Kit (Genomed GmbH, Löhne, Germany), and the Genomic DNA Mini kit Tissue (Geneaid Biotech Ltd., New Taipei City, Taiwan) were used to isolate DNA from tissue samples, according to the manufacturers' protocols.

### Mitochondrial DNA

#### Amplification of mtDNA Markers

Two mtDNA markers were chosen to evaluate the phylogeographic relationships of the Czech red deer population to different European red deer lineages: D-loop and cytochrome *b* gene (cyt *b*, for a subset

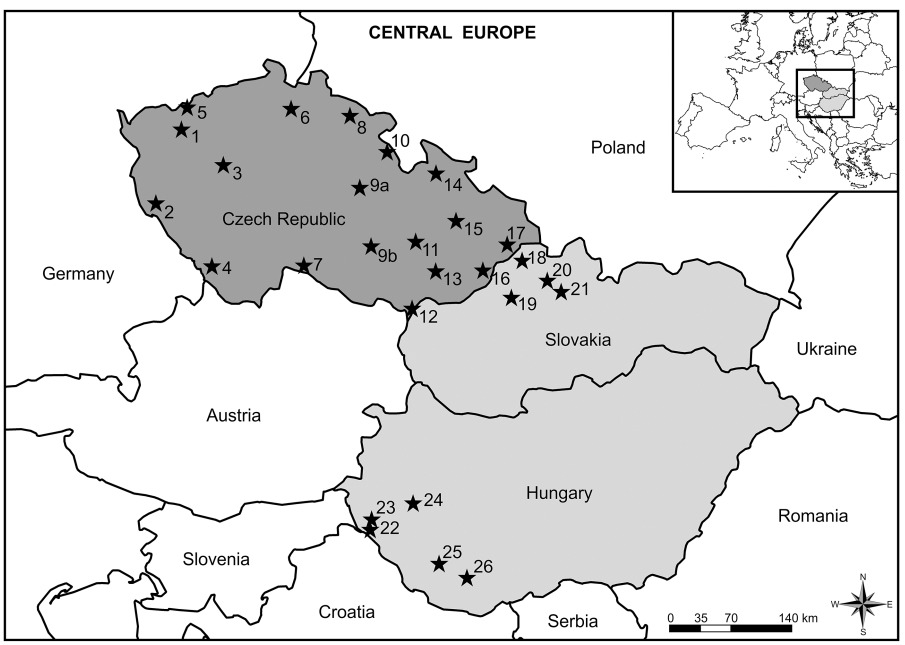


**Figure 1.** Sampled locations in the Czech Republic, Hungary, and Slovakia. (1: Doupovské hory Mts (99 samples), 2: Bohemian Forest (33 samples), 3: Křivoklátsko (29 samples), 4: Šumava Mts (71 samples), 5: Krušné hory Mts (27 samples), 6: Central Bohemian Upland (17 samples), 7: Javořická vrchovina Upland (2 samples), 8: Krkonoše Mts (18 samples), 9a+b: Bohemian-Moravian Highlands (6 samples), 10: Orlické hory Mts (4 samples), 11: Drahanská vrchovina Upland (17 samples), 12: South-Moravian lowlands (12 samples), 13: Chřiby and Buchlovské vrchy Highlands (35 samples), 14: Jeseníky Mts (57 samples), 15: Oderské vrchy Highlands (21 samples), 16: Bílé Karpaty (22 samples), 17: Moravian–Silesian Beskids (16 samples), 18: Javorníky (1 sample), 19: Strážovské Hills (1 sample), 20: Malá Fatra (7 samples), 21: Veľká Fatra (4 samples), 22: Bánokszentgyörgy (9 samples), 23: Szentpéterfölde (4 samples), 24: Keszthely Mts (4 samples), 25: Vörösalma (3 samples), 26: Nagymáté (3 samples)].

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of individuals only). PCR reaction conditions and primer pairs are described in detail in Krojerová-Prokešová et al. (2013). The PCR products obtained were purified using the Qiagen QIAquick PCR Purification Kit, the Genomed Jetquick PCR Purification Spin Kit, or Gel/PCR DNA Fragments Extraction kit (Geneaid Biotech Ltd., Taiwan) and sequenced using BigDye Terminator sequencing chemistry on an ABI 3730 Genetic Analyzer (Applied Biosystems). The sequences were assembled and checked in Sequencher v.4.6 (Gene Codes) and an alignment was created in BioEdit v.7.0.1 (Hall 1999).

### Haplotype Dataset

D-loop sequences (815 bp) were obtained for all 486 Czech samples, and cyt *b* sequences (1140 bp) were obtained for 255 Czech samples (stratified to include at least 50% of samples from each study area). D-loop/cyt *b* sequences were obtained also for 6/6 Hungarian and 13/11 Slovak samples. All cyt *b* and D-loop haplotypes obtained were compared with existing red deer haplotypes registered within the GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST. All new haplotypes were submitted to the Genbank (See Supplementary Table S1 online) (We used only those haplotypes from Genbank which were of similar size. There are many shorter ones (ca 300–500 bp) in the database but we did not want to reduce the resolution offered by the full fragment length of the fragments analyzed in our own studies.).

The haplotype data set was completed with cyt *b* and D-loop sequences (obtained from the GenBank database or from own datasets—for details see Supplementary Table S1 online) of different sika subspecies, Manchurian wapiti, American wapiti, Siberian wapiti, and red deer. We used Siberian roe deer (*Capreolus pygargus*) as outgroups for rooting the cyt *b* and D-loop phylogenetic trees, respectively (See Supplementary Table S1 online). The length of cyt *b*/D-loop sequences in analyzed data sets was 1140 bp/815 bp.

### Phylogenetic Analyses

The best models of evolution were determined by MrModeltest 2.2 (Nylander 2004) under the Akaike Information Criterion (AIC). For the haplotype data set of cyt *b* as well as of the D-loop, the HKY+I+G model of evolution was used. The model was used for calculation of haplotype divergence as well as for all phylogenetic analyses.

Evolutionary relationships between haplotypes were firstly inferred under maximum parsimony criterion using PAUP* v.4.10b (Swofford 2003). Heuristic searching was conducted 100 times with random addition of sequences and a tree-bisection reconnection (TBR) swapping algorithm for rearrangement of branches. The resulting topology was tested using 1000 bootstrap replicates (Felsenstein 1985). Phylogeny was further investigated under the maximum likelihood criterion using PhyML (Guindon and Gascuel 2003) using 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 4 heated and 1 cold chains run for 2 000 000 (cyt *b*) and 5 000 000 (D-loop) 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 all detected Czech 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), nucleotide diversity ($\pi$) and number of polymorphic sites (S) were calculated using DnaSP 4.9 (Rozas et al. 2003).

## Microsatellite Loci

### Amplification of Microsatellite Loci and Genotyping

The microsatellite analysis was performed using 22 nuclear loci. Each sample was amplified in a 10-μL reaction volume, containing 1 μL DNA template, 5 μL of the Qiagen Multiplex PCR Kit, and corresponding primer pair—each forward primer was labeled at the 5′-end with fluorescent dye (Applied Biosystems) (see Supplementary Table S2 online for more details about primer pairs and PCR conditions). Polymerase chain reaction was performed in 35 cycles of denaturation for 30 s at 94 °C, annealing for 90 s at particular temperatures, 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 2 people (J. K.-P., 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 allelic richness corrected for sample size (AR), and the observed ($H_O$), and expected ($H_E$) heterozygosity were estimated for each locus using FSTAT 2.9.3.2 (Goudet 2001). Tests of departure from the Hardy–Weinberg equilibrium (HWE) were conducted using GENEPOP version 3.4 (Raymond and Rousset 1995) using the exact probability test and linkage tests of disequilibrium between any 2 loci. 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 version 2.2.3 (van Oosterhout et al. 2004).

### Analysis of Genetic Structure

The genetic structure of the Czech population was assessed by the Bayesian clustering procedure in the STRUCTURE 2.3.3 program (Pritchard et al. 2000). The program was run with 5 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 was used without using sampling locations as prior information, together with a correlated allele frequency model. 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 ($\Delta$K). Factorial correspondence analysis (FCA) was performed in the program GENETIX 4.05.2 (Belkhir et al. 1996–2004). The program GENEPOP version 3.4 (Raymond and Rousset 1995) was used to estimate the inbreeding coefficients ($F_{IS}$) and the pairwise index of genetic differentiation ($F_{ST}$). The test for significance of population differentiation (assuming no HWE) was performed by means of a log-likelihood G-test, according to Goudet et al. (1996).

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In fulfilment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses. Alignments of cyt *b* and D-loop haplotypes used for phylogenetic analyses and microsatellite data file are available in Dryad (doi: 10.5061/dryad.v7c54). New cyt *b* and D-loop haplotypes are registered within the GenBank database under accession numbers KM410099-KM410150.

## Results

### Mitochondrial DNA

A total of 34 polymorphic sites were found in the cyt *b* gene of Czech red deer, giving 15 haplotypes (Table 1). Within the alignment of D-loop sequences of the Czech red deer haplotypes, 49 characters were variable and 38 parsimony-informative, giving 44 different haplotypes. Nine cyt *b* and 33 D-loop haplotypes were detected for the first time. The haplotype as well as nucleotide diversity of the Czech population was high (Table 1). The exact numbers of individual haplotypes detected at different sampling sites in the Czech Republic are given in Supplementary Table S3 online.

Phylogenetic analysis was performed for both markers to evaluate the phylogenetic relationships among haplotypes found. The resulting trees showed similar topologies with significant bootstrap support for the major clades and they clearly showed 2 distinct groups of Czech red deer haplotypes (See Supplementary Figure S2 online), thus confirming the presence of both, eastern and western, postglacial mtDNA lineages of European red deer previously described by Skog et al. (2009). The genetic distances between these 2 lineages varied between 1.6 and 2.2% for cyt *b* and 2.4 and 3.7% for D-loop (See Supplementary Table S4 online). Two cyt *b* haplotypes of red deer from Iran and Turkey (obtained from Genbank; previously used in Ludt et al. 2004, See Supplementary Table S1 online) formed separate lineage from eastern and western European red deer lineages, although they were more closely related to "eastern" clade. Genetic distance of these haplotypes to other European red deer haplotypes was 1.1–1.4% to eastern and 1.7–2.2 % to western haplotypes, indicating the possible existence of further European red deer lineage in the Caucasus region. A separate cluster was also formed in both phylogenetic trees by North African/Sardinian haplotypes. The haplotypes of Tarim red deer from the Asia (*C.e. bactrianus* and *yarkandensis*) formed a basal branch to the red deer tree with all European red deer haplotypes forming 1 main "red deer" (*C. elaphus*) cluster. Haplotypes of sika deer (*C. nippon*) and wapiti (*C. canadensis*) formed separate clusters in both trees. Relationship between cyt *b* and D-loop haplotypes within the "red deer" cluster were confirmed using the median-joining networks (Figure 2).

Distribution of mtDNA haplotypes within the Czech Republic showed a distinct geographic pattern (Figure 3a). The haplotypes assigned to western lineage dominated in western part of the Czech Republic and those assigned to eastern lineages dominated in eastern part of the Czech Republic. At 2 sites, Šumava Mts and Central Bohemian Upland, both lineages were present together. The proportion of western European red deer haplotypes also showed slight increase in the north-eastern part of the Czech Republic near the border with Poland and Slovakia and this western haplotype was also found in 2 of 11 Slovak samples. All 6 Hungarian samples belonged to the eastern lineage.

**Table 1.** Haplotype and nucleotide diversity of cyt *b* and D-loop of the Czech red deer population

| | *N* | $N_H$ | *H* | π | *k* | *S* |
|---|---|---|---|---|---|---|
| cyt *b* | 255 | 15 | 0.867 | 0.00914 | 10.421 | 34 |
| D-loop | 486 | 44 | 0.914 | 0.01402 | 11.311 | 49 |

*N*, number of samples; $N_H$, number of haplotypes; *H*, haplotype diversity; π, nucleotide diversity; *k*, average number of nucleotide differences; *S*, number of variable sites.

### Microsatellite Loci

#### Microsatellite Genetic Diversity

Genotypes for 22 microsatellite loci were obtained for 484 Czech red deer samples out of 486, for 13 Slovak samples, and for 23 Hungarian samples (together 520 samples). No evidence for allelic dropout was detected. After Bonferroni adjustment ($P = 0.000216$) for multiple testing, no linkage disequilibrium was found between any pairwise locus combinations. All analyzed loci were polymorphic. The number of alleles varied from 3 to 84 (average 22, AR = 21.69, See Supplementary Table S5 online). The average observed heterozygosity (Ho = 0.602) was lower than the average expected heterozygosity (He = 0.710). Consequently, estimated values of Wright's fixation index ($F_{is} = 0.165$) indicated a certain level of heterozygote deficiency. Further, significant deviations from HWE after the Bonferroni correction (adjusted $P = 0.002273$) were observed at 17 loci (See Supplementary Table S5 online). Relatively high frequencies of null alleles, a common cause of HW disequilibrium (Pemberton et al. 1995), were estimated only for 6 loci of these loci (ranging between 10 and 27%, See Supplementary Table S5 online).

#### Population Substructuring

Deviations from Hardy–Weinberg equilibrium at many loci and the reduction in Ho compared with He both indicated non-random mating and substructuring of the Czech red deer population (Wahlund´s effect). The Bayesian clustering analysis for all 520 red deer samples (including Slovak and Hungarian ones) detected the maximum value for the "estimated likelihood of *K*" at $K = 8$, but for *K* values higher than 2 the likelihood values showed only a slight increase (See Supplementary Figure S3a online), 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 $K = 2$ (See Supplementary Figure S3b online).

The divergences among samples clearly corresponded to the geographic pattern established by mtDNA haplotypes: the samples from the western part of the Czech Republic were assigned predominantly to the first cluster and the samples from the eastern part of the Czech Republic, Slovakia, and Hungary were assigned predominantly to the second cluster (Figure 4).

A threshold value of $q \geq 0.1$ (Vahä and Primmer 2006) was used to detect admixed individuals. Based on this criterion we calculated the proportion of "pure" (western or eastern) and admixed individuals for particular sampling sites (Figure 3b). From all 520 samples 228 individuals belonged to western genotype, 236 individuals belonged to eastern genotype, and 56 showed admixture. The value of the fixation index ($F_{ST} = 0.026$, $P < 0.001$) indicates a small but significant genetic differentiation between these 2 subpopulations. The differentiation of samples was also supported by factorial correspondence analysis, performed both with admixed individuals included (See Supplementary Figure S4a online) and excluded (See Supplementary Figure S4b online). The reference samples from Slovakia and Hungary belonged predominantly to eastern European red deer lineage even though some degree of admixture was present in several Hungarian samples (Figures 3b and 4).

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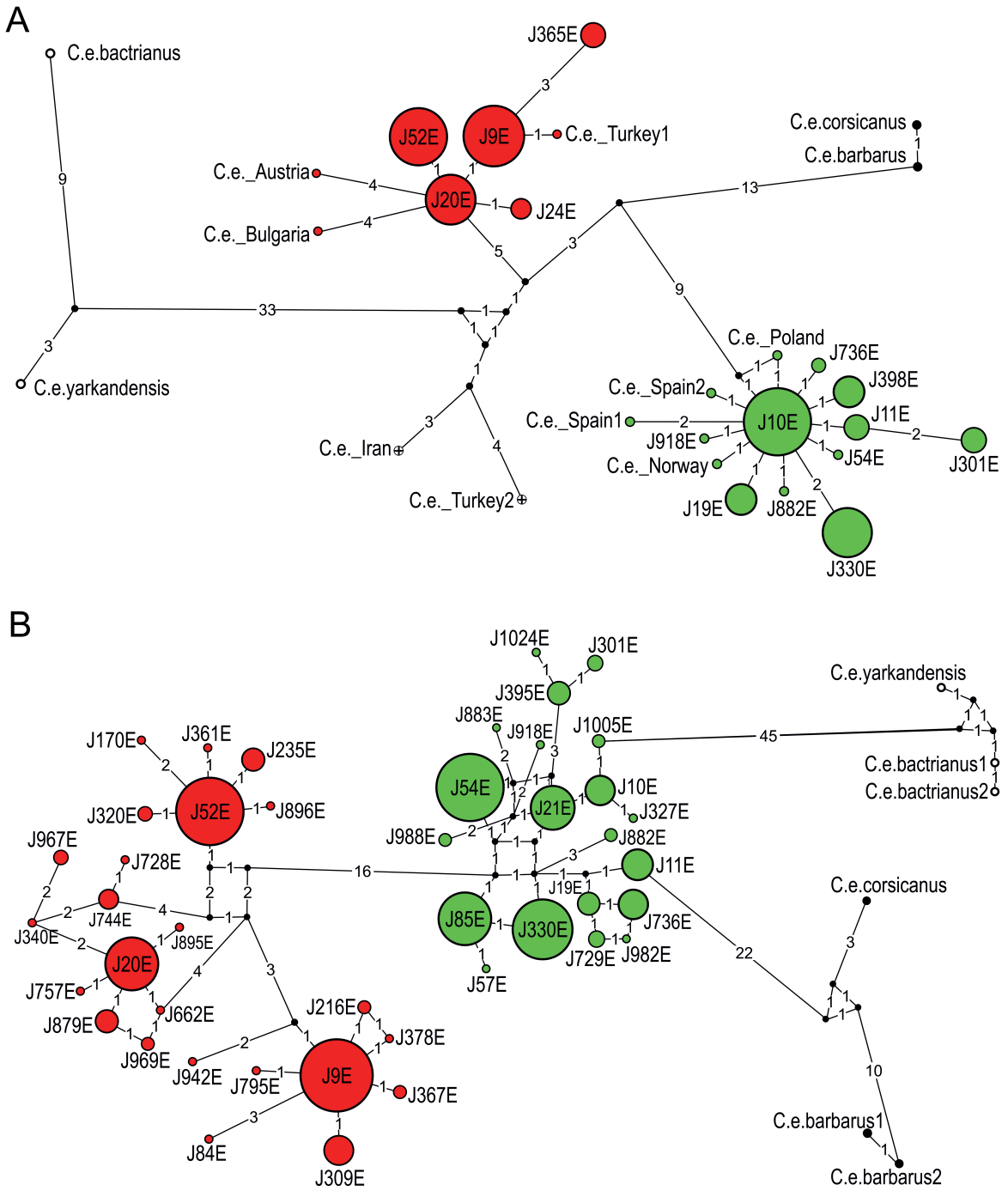


**Figure 2.** Median-joining network of (**a**) cyt *b* gene and (**b**) D-loop red deer haplotypes. The number of mutations between haplotypes is related to the length of branches and it is indicated on branches. The median vectors are indicated by black dots and circle sizes are proportional to the number of the same haplotypes in the data set. Colors indicate geographical origin of haplotypes: light grey/green—western European red deer haplotypes, dark grey/red—eastern European red deer haplotypes, black—haplotypes from Sardinia and North Africa, white double-crossed—haplotypes from Turkey and Iran (used previously in Ludt et al. 2004), white—haplotypes of Tarim red deer.

## Discussion

During the LGM "sensu lato", the climate in Central Europe was extremely cold and dry and most of this region was covered by steppe tundra (Hewitt 1999). The distribution of red deer during this time lay to the south of the permafrost area, suggesting that this might have been a decisive factor restricting the northward expansion of the species (Sommer and Zachos 2009). Recolonization of Central and Northern Europe started during the Greenland Interstadial 1 (Bølling/Allerød warming, c. 14 700–11 600 years ago). Western locations appear to have been recolonized earlier than the Central European region, where the reoccurrence of red deer was confirmed by fossils from early Holocene, indicating that recolonization from the Iberian Peninsula and from South-Western France (Gascony, Dordogne, Languedoc) began earlier and/or was faster than that from the Balkans and other eastern refugia (Sommer and Zachos 2009, Meiri et al. 2013). Based on fossil evidence and modern phylogeographic studies, the Carpathians did not serve as a refuge during LGM and were not a significant barrier between western and eastern

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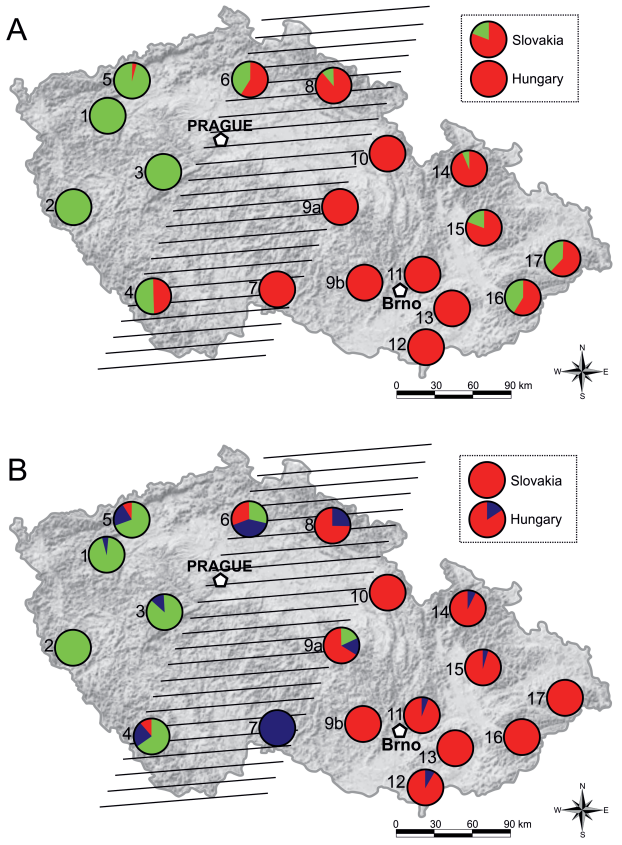


**Figure 3.** The pie charts representing the proportion of (**a**) samples with D-loop haplotypes belonging to western (light grey/green color) and eastern (dark grey/red color) European red deer lineages; (**b**) samples based on microsatellite cluster membership inferred from STRUCTURE for $K = 2$ belonging to western (light grey/green color), eastern (grey/red color) and admixed (dark grey/blue color) individuals at particular Czech sampling sites (1: Doupovské hory Mts, 2: Bohemian Forest, 3: Křivoklátsko, 4: Šumava Mts, 5: Krušné hory Mts, 6: Central Bohemian Upland, 7: Javořická vrchovina Upland, 8: Krkonoše Mts, 9a+b: Bohemian-Moravian Highlands, 10: Orlické hory Mts, 11: Drahanská vrchovina Upland, 12: South-Moravian lowlands, 13: Chřiby and Buchlovské vrchy Highlands, 14: Jeseníky Mts, 15: Oderské vrchy Highlands, 16: Bílé Karpaty, 17: Moravian–Silesian Beskids). The pie charts for Slovak and Hungarian samples are given in insets. The cross-hatched area represents the possible admixture zone in the Czech Republic.

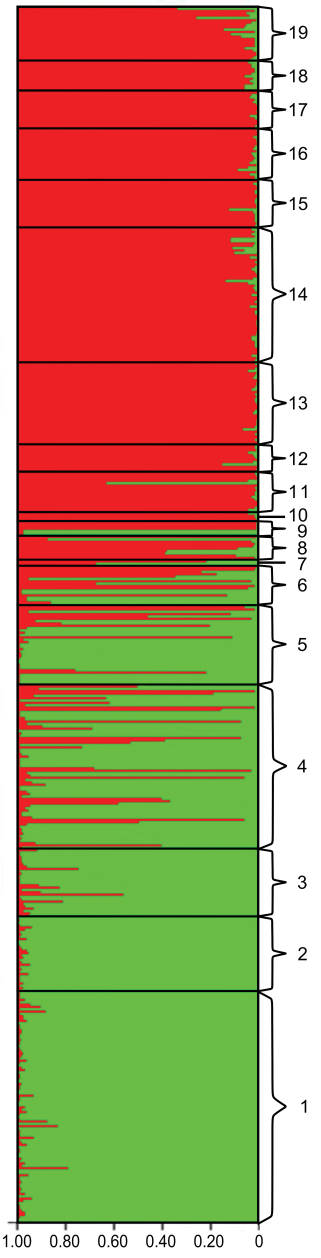


**Figure 4.** Graphical output of Bayesian clustering analysis of 520 red deer samples performed using STRUCTURE for model $K = 2$. Each horizontal bar corresponds to a single individual partitioned into 2 colored segments (light grey/green color-western European red deer, dark grey/red color—eastern European red deer), the length of each color being proportional to the estimated membership coefficient. Particular Czech sampling sites (1: Doupovské hory Mts, 2: Bohemian Forest, 3: Křivoklátsko, 4: Šumava Mts, 5: Krušné hory Mts, 6: Central Bohemian Upland, 7: Javořická vrchovina Upland, 8: Krkonoše Mts, 9a+b: Bohemian-Moravian Highlands, 10: Orlické hory Mts, 11: Drahanská vrchovina Upland, 12: South-Moravian lowlands, 13: Chřiby and Buchlovské vrchy Highlands, 14: Jeseníky Mts, 15: Oderské vrchy Highlands, 16: Bílé Karpaty, 17: Moravian–Silesian Beskids) as well as samples from Slovakia (18) and from Hungary (19) are marked at the right side.

recolonization routes (Skog et al. 2009; Niedziałkowska et al. 2011; Meiri et al. 2013); however, the possibility that the Alps imposed a barrier in the recolonization process could not be rejected (Ludt et al. 2004; Skog et al. 2009).

Even though the red deer has been the subject of several studies with a phylogeographic focus at local (Kuehn et al. 2003; Zachos et al. 2003; Feulner et al. 2004; Nussey et al. 2006; Hmwe et al. 2006; Hajji et al. 2007; Pérez-Espona et al. 2008; Fickel et al. 2012; Karaiskou et al. 2014) as well as at larger geographical scales (Ludt et al. 2004; Pitra et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011), the question how far the eastern/western haplogroups extend has not yet been resolved, due to the lack of red deer samples from Central and Eastern Europe. It has been assumed that the border between these 2 recolonization lineages probably lies somewhere in Germany or in Eastern Central Europe, in correspondence with Taberlets' second suture-zone between populations of the Iberic and Balcanic refuge (Taberlet et al. 1998). Our research, as a detailed study done at intermediate geographical scale in Central Europe, aims to fill the gap in knowledge of phylogeographical pattern of red deer and tries to resolve the question about the location of the suture zone between eastern and western lineages in this region.

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### Mitochondrial and Microsatellite Genetic Diversity of the Czech Population

Thanks to intensive sampling, we detected the presence of both lineages of red deer in the Czech Republic, in contrast to the findings of several previous studies with more limited sampling in the region (Ludt et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011). One of the consequences of this coexistence of both lineages was high mtDNA haplotype diversity within the Czech population (cyt *b* $H$ = 0.867, D-loop $H$ = 0.914). Genetic diversity of the Czech red deer was higher than that found in other genetic surveys of European red deer populations (Feulner et al. 2004 (D-loop)—H = up to 0.810, Pérez-Espona et al. 2009 (D-loop)—$H$ = 0.252–0.836, Niedziałkowska et al. 2011 (D-loop)—$H$ = 0.32–0.79) and almost as high as that previously detected, in Europe as a whole (Skog et al. 2009 (cyt *b*) $H$ = 0.95, (D-loop) $H$ = 0.96). The population of red deer in the Czech Republic appears to be one of the most genetically diverse populations in Europe (at least in relation to maternal inherited mtDNA). We have to agree with Niedziałkowska et al. (2011) that more rare haplotypes occur in Central Europe than in other parts of Europe. This could support the idea of genetic differentiation of Carpathian red deer and the existence of Carpathian glacial refuge (Feulner et al. 2004). However, the possible existence of a Carpathian (or other eastern) refuge (already discounted by Skog et al. 2009; Niedziałkowska et al. 2011; Meiri et al. 2013) seems to be supported in our study only by the structuring of eastern European red deer lineage within the D-loop tree (See Supplementary Figure S2b online) and within the median-joining network (Figure 3b). The trichotomy of the eastern European red deer clade was significantly supported by high bootstrap values. However, the geographic distribution of haplotypes clustered within this clade (from Slovakia, Czech Republic, Hungary, Italy, and Greece) did not show any clear geographic pattern and further research, especially within the Carpathians, is necessary to explain it fully. The cyt *b* tree did not reveal the same structuring (See Supplementary Figure S2a online).

Contrary to mtDNA findings, genetic variability among Czech red deer revealed by expected heterozygosity was not so high. Expected heterozygosity He = 0.710 was similar to those found in free-living mostly bottlenecked red deer populations (e.g., Bavarian red deer: He = 0.680, Kuehn et al. 2003; Danish red deer: He = 0.524, Nielsen et al. 2008; Iberian red deer: He = 0.771, Sanchéz-Fernandéz et al. 2008; red deer in Sardinia: He = 0.6, Hmwe et al. 2006; or Tunisian red deer: He = 0.78, Hajji et al. 2007). However, He was lower than the level reported for more abundant red deer populations (e.g., Carpathian red deer: He = 0.81–0.9, Feulner et al. 2004; or Polish red deer: He = 0.83, Niedziałkowska et al. 2012b). Further, Ho was lower than He and 17 loci (from 22 analysed) showed deviation from HW equilibrium (See Supplementary Table S5 online). Both these, heterozygosity deficiency and deviations from HWE, indicate nonrandom mating and significant substructuring of the Czech red deer population (Wahlund's effect) which was also suggested by Bayesian clustering analysis (Figure 4) as well as by Factorial correspondence analysis (See Supplementary Figure S4 online).

### Admixture Zone Between Eastern and Western European Red Deer

Previous analyses of red deer phylogeography have concentrated only on analysis of mitochondrial lineages. Our results show that the structuring of Czech red deer population suggested by mtDNA is supported also by neutral nuclear (microsatellite) markers. The combined analysis of 22 microsatellite loci corroborated the major findings of mtDNA about the presence and admixture of 2 European red deer lineages in the Czech Republic. However, our results did not support expectations of a less clear structuring of red deer population when microsatellite loci are involved (Skog et al. 2009).

Based on the results from both types of DNA markers (mtDNA and microsatellites), the position of the admixture zone in the Czech Republic was located in the area between E14° and E15°45′. Our results were in agreement with those of Fickel et al. (2012) who identified the Bohemian–Bavarian forest as a part of a suture zone between both lineages. This border between the lineages corresponds to 2 big rivers Vltava and Elbe, which intersect the Czech Republic from north to south. Identification of this zone as an area of admixture of different red deer lineages was in accordance with results from an earlier study on European hedgehogs, which also identified this area as a suture zone between the west European hedgehog and northern white-breasted hedgehog (Bolfíková and Hulva 2012). Unfortunately, the Czech red deer suture zone could not be identified more precisely within this study due to the low density of red deer in the presumed admixture zone (and thus the low number of samples obtained).

Hybridization, either between species or between distinct populations of the same species, is a common natural phenomenon important in the process of speciation (Barton 2001; Seehausen 2004). In general, the accuracy of detecting hybrids increases with increasing $q$ value threshold; however at the same time, the efficiency of detecting hybrids decreases due to incorrect classification of hybrid individuals as purebred ones (Vahä and Primmer 2006). Vahä and Primmer (2006) recommended $q \geq 0.1$ as the best value for efficient identification of admixture. Pérez-Espona et al. (2013) also used this value successfully for identification of admixture within Scottish red deer populations. Using Bayesian clustering analysis with this same threshold value at $q \geq 0.1$ we detected 56 individuals of admixed genetic type within 520 red deer individuals sampled (10.8%). The individuals classified as admixed had usually higher admixture rate than 20%, implying that the slight increase of $q$ value to $q \geq 0.2$ would not affect significantly the proportion of hybrid individuals detected. However, when we decreased the value to $q \geq 0.05$, the number of individuals classified as hybrid almost doubled.

Admixed individuals were observed at different distances from the possible suture zone. Their occurrence can be the result of natural admixture (immigration); however, the influence of human-engineered translocations cannot be excluded. Despite this, the level of genetic introgression of western genotype into the eastern genotype or vice versa was relatively low and did not blur the global geographic pattern significantly. We detected only 11 individuals (all belonging to the eastern lineage) classified as purebred but located in areas where the other (western) lineage currently dominates. Within these individuals, in agreement with sex-biased dispersal of red deer, males dominated (10 samples) and only 1 sample belonged to a female.

A number of secondary hybrid zones of European mammals and birds have remained more or less unchanged during the Holocene since their post-glacial formation (Hewitt 1999). There are several processes which form and maintain the hybrid zones: prezygotic mechanisms, which reduce the frequency at which gametes combine to form a zygote, postzygotic mechanisms, which reduce the fitness of hybrid individuals, ecological adaptation to different environments, and/or behavioral differences that cause assortative mating between hybridizing taxa (Mayr 1963; Dobzhansky 1970). The hybrid zone between western and eastern European red deer lineages has not previously been studied in detail and the mechanisms which contribute

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to its formation are unknown. In the study of sex-based dispersal from the Scottish Highlands (Pérez-Espona et al. 2010), it was detected that the dispersal could significantly vary between different locations depending on age structure, sex ratio, and dispersal behavior of the populations studied. The distribution range of red deer in the Czech Republic is currently largely fragmented due to landscape fragmentation and deer are restricted predominantly to the border areas. However, landscape fragmentation could have influenced the level of red deer migration and admixture only during the last few decades and cannot explain the existence and maintenance of hybrid zone for several thousand years from the past recolonization of this region at the beginning of Holocene. Therefore, other processes must have played an important role in maintenance of the hybrid zone during the whole period of its existence. We know that both lineages can mate without any prezygotic or postzygotic barriers, therefore assortative mating (Asmussen et al. 1987; Arnold et al. 1988) could be responsible for maintaining the suture zone between western and eastern European red deer lineages, as in other species (e.g., crows: Brodin et al. 2013, see more in M'Gonigle and FitzJohn 2010).

### Effect of Human-Engineered Red Deer Translocations

Translocations by humans may have significantly influenced and blurred the natural phylogeographic pattern of red deer, one of the most translocated and manipulated species in Europe (Apollonio et al. 2014). In this study, we detected the presence of western mtDNA haplotypes in the north-eastern part of the Czech Republic as well as in 2 of 13 Slovak samples included. The presence of both lineages in the Polish Carpathians and in Ukraine was previously confirmed by Niedziałkowska et al. (2011). According to such scarce historical records as exist, there were several introductions of west-European red deer to Slovakia during 17th–19th century (Bališ 1971; Niethammer 1963; Mošanský 1971). Similarly, there are several documented records of translocations of red deer at the end of the 19th and beginning of the 20th century's from Germany to Poland (Niedziałkowska et al. 2011). The presence of western mtDNA haplotypes and evidence of some small introgression of western genotypes in nuclear DNA in north-eastern part of the Czech Republic is probably the result of these translocations to Poland and/or to Slovakia and the subsequent natural immigration of relatively small numbers of individuals (whether purebred or already admixed) to the Czech Republic.

## Supplementary Material

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

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