Phylogeography of red deer (Cervus elaphus) in Europe


ABSTRACT

Aim To investigate the phylogeographical patterns of red deer (Cervus elaphus) in Europe, and to disentangle the influence of ancient (e.g. Pleistocene ice ages) from more recent processes (e.g. human translocations).

Location Europe.

Methods In this study we provide by far the most extensive analysis of genetic structure in European red deer, based on analyses of variation at two mitochondrial markers (cyt b and D-loop) in a large number of individuals from 39 locations. Relationships of mitochondrial DNA haplotypes were determined using minimum spanning networks and phylogenetic analyses. Population structure was examined by analyses of molecular variance. Historical processes shaping the present patterns were inferred from nested clade analysis and nucleotide diversity statistics.

Results Within Europe, we detected three deeply divergent mitochondrial DNA lineages. The three lineages displayed a phylogeographical pattern dividing individuals into western European, eastern European and Mediterranean (Sardinia, Spain and Africa) groups, suggesting contraction into three separate refugia during the last glaciation. Few haplotypes were shared among these three groups, a finding also confirmed by FST values. Calculations of divergence times suggest that the groups probably split during the Pleistocene.

Main conclusions The observed pattern is interpreted to result from isolation in different refugia during the last glaciation. The western and eastern European lineages could be linked to an Iberian and Balkan refugium, respectively. The third lineage might originate from a Sardinian or African refugium. We link local phylogeographical patterns observed in Europe to the post-glacial recolonization process, shaped by the geographical localization of refugia and barriers to gene flow. Regardless of the importance of red deer as a game species and the tradition of translocating red deer in Europe, we detected few individuals that did not match the trichotomous pattern, suggesting that translocations have occurred mainly at smaller spatial scales.

Keywords Cervus elaphus, cyt b, D-loop, glacial refugia, mitochondrial DNA, phylogeography, recolonization, translocation.

INTRODUCTION

The patterns of genetic variation of present-day European terrestrial fauna and flora have been influenced by repetitive fluctuations between major glacial and interglacial climatic cycles that have occurred during the Quaternary period (Hewitt, 2004, and references therein). The isolation of species into one or more refugia during the glaciations (Hewitt, 1996) and the mode of post-glacial colonization (Ibrahim et al., 1996) are regarded as processes that are particularly influential...
on contemporary genetic variation and structure of populations. In general, five major European refugia have been identified: the Balkans, Iberia, Italy, the Carpathians and the Caspian/Caucasus region (Taberlet et al., 1998; Hewitt, 2004). Depending on the refugia, different species met different barriers during post-glacial expansion into previously occupied areas (Hewitt, 2004, and references therein). For species with multiple refugia, the contribution of each refugium to recolonization seems to vary, and many extant species essentially originate from a few, or even a single, refugium (Taberlet et al., 1998).

The most important and widely distributed game species in Europe today is arguably the red deer, *Cervus elaphus* L. 1758 (Milner et al., 2006). A comprehensive phylogeographical study of this species by Ludt et al. (2004) focused on identifying the origin of red deer, and disentangling the relationship between three closely related deer species in the northern Hemisphere: the red deer, the sika deer (*Cervus nippon* Temminck 1838) and the wapiti (*Cervus canadensis* Erxleben 1777). Here we aim to investigate the ancient and recent evolutionary history of red deer in Europe. While Ludt et al. (2004) were able to identify four substantially divergent subgroups in Europe, they concluded that further research into the European history of the species would be necessary due to their limited European sampling (Ludt et al., 2004, p. 1075), and they only briefly addressed some possible reasons for the subdivision. We wanted to localize European refugia and recolonization routes for red deer and to assess the potential influence of human actions (particularly translocations) on the species’ genetic structure.

Red deer utilize a variety of different habitats. They are typically found in deciduous woodlands of various types (Ahlen, 1965), but they are also generalists found in coniferous forests (Catt & Staines, 1987) and open habitat, as in Scotland (Conradt et al., 1999). Moreover, the red deer is classified as a mixed or intermediate feeder (Hofmann, 1989), alternately browsing and grazing according to the available habitat (Gebert & Verheyden-Triexier, 2001; Lister, 2004). This range of behaviours may have enabled red deer to benefit from the landscape changes brought about by glaciations, deglaciations, megafaunal extinctions and, in recent times, human disturbance (Geist, 1999; Lister, 2004). Being an important game species, the red deer has been extensively managed, reintroduced, restocked, and selectively hunted throughout its history and distribution area (Hartl et al., 2003, and references therein; Milner et al., 2006), which may have influenced the species’ spatial genetic structure (Hartl et al., 2003).

The study by Ludt et al. (2004) focused on the red deer over a large geographical scale, i.e. Eurasia. Other studies have addressed the genetic structure in local parts of Europe and have shown genetic differentiation among specific geographical regions in the European red deer population (Hartl et al., 1993, 1995, 2005; Perez et al., 1998; Zachos et al., 2003; Feulner et al., 2004; Lorenzini et al., 2005; Hmwe et al., 2006a,b). No group of researchers has yet performed a detailed investigation of genetic variation across the entire European range of red deer. In the present study, we combine new and previously generated data (Zachos et al., 2003; Feulner et al., 2004; Ludt et al., 2004; Hmwe et al., 2006a,b) in order to provide a detailed and more comprehensive analysis of the phylogeographical history and present population structure of the European red deer. This level of sampling intensity has also allowed us to investigate the possible impact of human translocations. In order to obtain enhanced resolution, we have analysed the coding cytochrome b (cyt b) region and the non-coding control region (D-loop) – two mitochondrial DNA markers having different evolutionary rates in mammals (Moritz et al., 1987). Compared with previously published large-scale studies, the D-loop, having the highest mutation rate, allows for a more finely scaled picture of red deer populations in Europe. We have evaluated the extent to which the observed spatial patterns can be attributed either to human translocation or to isolation in several refugia during the Pleistocene climatic cycles followed by post-glacial recolonization.

**MATERIALS AND METHODS**

**Samples**

Samples from 587 individuals from 11 countries throughout Europe (Fig. 1) were tested for genetic polymorphism in the D-loop region. In addition, one D-loop sequence representing five Algerian red deer was downloaded from GenBank (AF296807/08). Further, 34 individuals from six countries were sequenced for variation in the cyt b gene, and 20 sequences representing 145 individuals from 18 countries were downloaded from GenBank (see Table S1 in Supporting Information for accession numbers and more detailed information). This means that the D-loop and cyt b sequences presented here do not necessarily originate from the same individuals.

**DNA extraction, PCR and sequencing**

DNA was isolated from tissue by a standard phenol–chloroform isolation procedure (Sambrook et al., 1989); by chelex extraction (Walsh et al., 1991); or by using the SuperQuickGene-Extraction kit (Genetic Analytical Testing Center, Denver, CO, USA).

Polymerase chain reaction (PCR) amplifications of the mtDNA D-loop were carried out for all samples using either primer pair LD5 and HD6 (Nagata et al., 1998), or Pro-L and Phe-Hb (Zachos et al., 2003). PCR conditions were established according to either Nagata et al. (1998) or Zachos et al. (2003). The cyt b region was amplified using primers cytoB A1 and cytoB B2 (Ludt et al., 2004), and Cytb-1 and Cytb-2 (Janczewski et al., 1995). The PCR protocol for these markers followed Ludt et al. (2004).

DNA sequencing was performed on ABI373A, ABI3730 (Applied Biosystems, Foster City, CA, USA), or MegaBACE1000 (Molecular Dynamics, Sunnyvale, CA, USA) automatic sequencers. Sequencing primers were either LD5 or...
Figure 1 Map showing sampling sites and haplotype distributions. Details about sampling sites are given in Table S1. Open circles, 
cyt b haplogroup found in the corresponding locality: A; haplogroup indicated in red; B; haplogroup in orange; C; in blue and purple; the 
C. e. bactrianus sample is indicated in pink (for more clade details see Figs 2 & 3). Full circles, frequency of D-loop haplotypes at different 
localities; shades of pink and red, haplogroup A; shades of blue and green, haplogroup C; yellow circles, Sardinian D-loop haplotypes (B) 
and African haplotype.
Pro-L for the D-loop region, and either cytoB A1 or Cyth-I for the cyt b region.

The sequences were edited and aligned by eye using the alignment explorer in MEGA ver. 3.1 (Kumar et al., 2004). Although the complete sequences were amplified and sequenced for each of the two mtDNA regions, poor sequence quality in some of the samples caused us to restrict analyses to a 332-base pair (bp) fragment for the D-loop and 1118 bp of cyt b, for which complete sequence was obtained for all samples. All sequences were deposited in GenBank, and alignments are available on request.

Evaluating haplotype relationships

Minimum spanning networks (MSN) of the haplotypes (D-loop and cyt b separately) were constructed according to Templeton et al. (1992) using tcs ver. 1.20 (Clement et al., 2000), treating gaps in the D-loop as a fifth state. Cladogram ambiguities (loops) were solved by favouring connections with frequent haplotypes over connections with singletone/er or rare haplotypes (Posada & Crandall, 2001). To investigate further the relationships between haplotypes as detected in the minimum spanning networks, and to evaluate their relative age, phylogenetic analyses were carried out using the neighbour-joining (NJ, Saitou & Nei, 1987) and maximum likelihood (ML) algorithms as implemented in paup ver. 4.0 (Swofford, 1998). To test the substitution model that best fitted the two separate data sets (D-loop and cyt b), we used the program modeltest (Posada & Crandall, 1998). The optimal model chosen using Akaike’s information criterion (AIC; Akaike, 1974) was TrN + I + G (Tamura & Nei, 1993, with rate heterogeneity and gamma distributed rates) for the D-loop data, while the optimal model for cyt b was TVM + I (transversional model with rate heterogeneity). The ML analyses were done by heuristic search (random stepwise addition with random seed) using the models of evolution selected by modeltest. Sequences of sika deer (D-loop AB012381/cyt b AB035876) and wapiti (D-loop AY970666/cyt b AY347753) were used as outgroups. All paup ver. 4.0 and modeltest analyses were performed at http://www.bioportal.uio.no.

Identifying population structure

Patterns of genetic divergence across Europe, for both the D-loop and cyt b data, were assessed by analysis of molecular variance (AMOVA; Excoffier et al., 1992) using ARLEQUIN ver. 2.000 (Schneider et al., 1997) with 10,000 permutations. To assess structuring within the data, the sampling sites were grouped as one pan-European population. To further identify patterns of genetic divergence, we applied the spatial AMOVA procedure using SAMOVA ver. 1.0 (Dupanloup et al., 2002). This procedure assigns populations to groups based on geographical vicinity and sequence similarity. The most likely structuring was considered to be the one that maximized among-group variation, \( F_{CT} \). SAMOVA identified the grouping of sampling sites that maximized the \( F_{CT} \) value based on 100 simulated annealing steps for the instructed \( K = 2 \) to \( K = 8 \) partitions of sampling sites.

To investigate whether the structuring in the D-loop data could be explained by isolation by distance, a Mantel test was conducted (Mantel, 1967), as implemented in ARLEQUIN (Schneider et al., 1997). Correlation coefficients between \( F_{ST} \) and geographical distance, \( \log(km) \), between each pair of sampling sites were calculated and the test used to evaluate whether the slope of the resulting regression was steeper than expected under the null hypothesis of panmixia.

Phylogeographical inferences

A nested clade analysis (NCA) was carried out using the D-loop data to differentiate population history from population structure (Templeton, 1998). The haplotype networks created by the tcs program were nested by hand according to the rules of Templeton et al. (1987) and Templeton & Sing (1993). The nested design was used to calculate geographical distances among haplotypes, which in turn were used to test for geographical associations between clades. Two distance measures were used: \( D_h \) and \( D_n \). Whereas \( D_h \) measures the geographical range of a clade, \( D_n \) measures the geographical distance between a haplotype and the geographical centre of the haplotypes at the next level of nesting (Templeton et al., 1995). Comparisons between tip and interior clades were made to determine the potential causes of geographical differentiation (Templeton et al., 1995). Latitudinal and longitudinal coordinates of sampling sites were used for calculating the geographical distances. The association between the geographical location and genetic distances was tested statistically with 10,000 random permutations using the program GEODIS ver. 2.2 (Posada et al., 2000). Inferences about the historical population structure were made on the basis of Templeton’s inference key of 11 November 2005 (http://darwin.uvigo.es/download/geodisKey_11Nov05.pdf).

Genetic diversity and divergence times

Genetic diversity within sampling sites (and haplogroups) for the D-loop data was measured as haplotype diversity, \( H_t \) (Nei, 1987) and nucleotide diversity, \( \pi_n \) (Tajima, 1983; Nei, 1987). All diversity measures were calculated using a TrN (Tamura & Nei, 1993) correction, applying the program ARLEQUIN. To investigate whether refugial populations have higher diversity than non-refugial populations, all sampling sites were grouped according to suitable and non-suitable habitat during the last glaciation. This was done by grouping populations north and south of the permafrost border from Andersen & Borns (1997), and diversity measures were calculated and compared. An evolutionary rate for cyt b in Cervinae was calculated by Pitra et al. (2004). Calibration of the mutation rate of D-loop was done by comparing divergence in the D-loop region with divergence in a coding region (cyt b) in pairwise comparisons of closely related deer species (Peters et al., 2005) using MEGA and the TrN model of evolution for divergence rates. The
| Table 1 Percentage nucleotide divergence within and between lineages of Cervus, and the relative rate difference between coding and non-coding regions of mtDNA. |
|-----------------------------------------------|-----------------|-----------------|
|                                  | Coding (cyt b)  | Non-coding (D-loop) | Relative rate difference between coding and non-coding regions |
| Sika deer – between lineages               | 2.70*           | 4.40†            | 1.6 |
| Between sika and wapiti                   | 2.80†           | 4.70§            | 1.7 |
| Within sika                               | 1.90**          | 3.80‡           | 2.0 |
| Within red deer                           | 1.40¶           | 3.80¶           | 2.7 |
| Average rate difference                   |                 |                 | 2.0 |

*Between southern and northern Japanese lineages, AB002470–AB002477, Tamate et al. (1999).†Between southern and northern Japanese lineages, AB012364–AB012385, Nagata et al. (1999).‡Silk deer, AB002470–AB002477, Tamate et al. (1999); wapiti, AF423198, AB021096, unpublished data.§Silk deer, AB012364–AB12385, Nagata et al. (1999); wapiti, AF005196–AF005200, AF016951–AF016980, Polziehn & Strobeck (1998).¶This study.**AB002470–AB002477, Tamate et al. (1998).††AB012364–AB12385, Nagata et al. (1999).§§Between sika and wapiti, compared with sequences from Asian individuals (A1C, sampling site 3; C1C, sampling site 2; C2C, sampling site 4; Fig. 1), while the difference in age of haplogroup AC and CC could not be resolved. The same phylogenetic pattern was evident from the tree of D-loop haplotypes, using wapiti as outgroups (Fig. 2b). Both trees suggest that haplogroup B diverged before groups A and C evolved from a common ancestor. The mean and 95% highest posterior density interval for each divergence time are indicated in Table 2. Divergence times for the haplogroups differ between the two markers. The split between haplogroup A/A_C and haplogroup B/B_C seems to have occurred first in both markers, followed by divergence between haplogroup B/B_C and C/C_C and haplogroup A/A_C and C/C_C. The estimated divergence times indicate that haplogroups are relatively old, at least 100,000 years.

**Phylogeny and divergence times of haplogroups**

The divergence point of haplogroup B_C was inferred to be the deepest (the most ancient) European cyt b branch (Fig. 2a) compared with sequences from Asian individuals (A1C, sampling site 3; C1C, sampling site 2; C2C, sampling site 4; Fig. 1), while the divergence in age of haplogroup A_C and C_C could not be resolved. The same phylogenetic pattern was evident from the tree of D-loop haplotypes, using wapiti and sika deer as outgroups (Fig. 2b). Both trees suggest that haplogroup B diverged before groups A and C evolved from a common ancestor.

**A refined phylogeographical pattern revealed by minimum spanning networks and nested clade analysis**

The three main haplogroups detected in the phylogenetic analysis were further confirmed by an MSN based on D-loop haplotypes (Fig. 3). As not all haplotypes could unambiguously be incorporated into a single network under the 95% probability criterion established at seven steps for this data set, haplogroups A and C were analysed separately in the NCA (for details see Table S2). Haplogroup B was omitted from this analysis due to lack of geographical differentiation: only one individual (in Spanish site 35, Fig. 1; Table S1) was found outside Sardinia, and the African haplotype could not be connected to the other B haplotypes within the 95% probability criterion. Six loops were excluded from the network. All loops were broken by favouring connections between frequent haplotypes over less frequent ones.

The central haplotype (assumed to be ancestral due to high frequency and many connections, and supported by the

**RESULTS**

Geographical distribution of haplogroups and haplotypes

In total, 17 unique haplotypes were found among the 179 European and West Asian individuals sequenced for the cyt b region (cf. Table S1; Fig. 1). Phylogenetic analysis divided these 17 haplotypes into three main clades, here called haplogroups A_C, B_C and C_C (Fig. 2a). Each haplogroup showed a distinct geographical distribution (Fig. 1, open circles): haplogroup A_C was mainly distributed along a south–north axis in western Europe, while haplogroup C_C had an eastern and central European distribution. Haplogroup B_C was found only in Sardinia and in Africa. In only one sampling site did we find haplotypes belonging to different haplogroups (Italy site 27, Fig. 1). Four sampling sites (sites 13, 14, 27 and 36, Table S1) showed variation on the haplotype level (i.e. displayed more than one haplotype).

Analysis of the D-loop data revealed additional differentiation of the European sampling sites. Fifty-eight haplotypes were found, and only seven of the 28 sampling sites were monomorphic with regard to haplotypes. The phylogenetic analysis indicated the same clades as detected in the cyt b data, denoted haplogroups A, B and C. However, the A clade has low bootstrap support in the D-loop tree, probably due to the deviating AD 3 sequence (grouping with the C clade, but without support).

The mean and 95% highest posterior density interval for each divergence time are indicated in Table 2. Divergence times for the haplogroups differ between the two markers. The split between haplogroup A/A_C and haplogroup B/B_C seems to have occurred first in both markers, followed by divergence between haplogroup B/B_C and C/C_C and haplogroup A/A_C and C/C_C. The estimated divergence times indicate that haplogroups are relatively old, at least 100,000 years.
Figure 2 Phylogenetic relationships of the cyt b (a) and D-loop haplotypes (b). Numbers on branches indicate bootstrap support for neighbour-joining (10,000 replicates) and maximum likelihood (1000 replicates for cyt b; 100 replicates for D-loop) algorithms. Main clades indicated by colour codes as in Fig. 1.
We analysed the highest level of nesting to obtain an overview of the historical processes behind the distribution patterns of the whole sampling area. Haplogroup C showed significant range expansion (Fig. 3; Table S2), while no significant inference could be made for the entire haplogroup A. The two highest levels of nesting within clade A were inferred to be shaped by long-distance colonization (clade 4-1, Fig. 3) and contiguous range expansion (clade 4-2, Fig. 3), respectively.

The MSN grouped cyt b haplotypes into three groups (not shown). However, these three haplogroups did not connect at the 95% limit, established at 14 steps. The haplotypes could be joined together in a single network only after the connection criterion was relaxed to 90% (21 steps).

### Population structuring of European red deer

Geographical structuring among D-loop haplotypes within Europe was significantly supported by AMOVA results where all European sampling sites were treated as a single group ($F_{ST} = 0.84$, $P < 0.01$). SAMOVA was used to identify the subdivision that is most likely to explain the D-loop structure observed in the European red deer population. The $F_{CT}$ value increased asymptotically with increasing number of groups, levelling out at three groups. When the number of groups exceeded three, the additional groups represented single sampling sites (Table S3). Consequently, the data were best explained assuming three groups of European red deer. The three groups identified ($F_{CT} = 0.63$, $P < 0.01$) were consistent with those shown by D-loop (Fig. 3) and cyt b haplotype networks and the phylogenetic trees in Fig. 2 (west, sampling sites 19–24, 26, 31–39; east, sites 6–11, 17, 27; Sardinia–Africa, sites 28 and 30 in Fig. 1). Applying SAMOVA to the cyt b data (Table S3), the same pattern was detected when the number of groups was set to three ($F_{CT} = 0.90$, $P < 0.01$), with western (numbers 12, 18, 21–22, 24–27, 31–38), eastern (5–7, 10, 13–17) and African/Sardinian (28–29) sampling sites each grouping together. For four and more groups, the $F_{CT}$ value increased only marginally and the groups started to correspond to single sample sites again.

A Mantel test suggested that $F_{ST}$ for our D-loop data was significantly but not strongly correlated with geographical distance ($r = 0.27$, $P < 0.01$). Performing the test separately for each haplogroup, no significant correlation between geographical and genetic distances was found (A: $r = 0.04$, $P = 0.36$, C: $r = 0.03$, $P = 0.40$).

### Geographical distribution of genetic variation

Ten singletons were detected among the 58 D-loop haplotypes as compared with none for the cyt b data. Both markers showed high haplotype diversity (D-loop: $H = 0.96$, SD ± 0.00; cyt b: $H = 0.95$, SD ± 0.01) and low nucleotide diversity (D-loop: $\pi_n = 0.03$, SD ± 0.02; cyt b: $\pi_n = 0.02$, SD ± 0.01). D-loop diversity indices observed in various European sampling sites are given in Table S4. Nucleotide diversity (Table 2) Bayesian coalescent estimates of divergence times (years) between red deer haplogroups.

<table>
<thead>
<tr>
<th>Haplogroup</th>
<th>Mean</th>
<th>95% HDP lower</th>
<th>95% HDP upper</th>
<th>ESS</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-loop</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/C</td>
<td>272,300</td>
<td>175,300</td>
<td>384,700</td>
<td>4384</td>
</tr>
<tr>
<td>A/B</td>
<td>303,700</td>
<td>209,100</td>
<td>411,000</td>
<td>7123</td>
</tr>
<tr>
<td>B/C</td>
<td>295,200</td>
<td>207,100</td>
<td>398,600</td>
<td>6613</td>
</tr>
<tr>
<td>cyt b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac/Cc</td>
<td>162,200</td>
<td>113,600</td>
<td>213,000</td>
<td>17,000</td>
</tr>
<tr>
<td>Ac/Bc</td>
<td>237,800</td>
<td>165,000</td>
<td>315,600</td>
<td>13,370</td>
</tr>
<tr>
<td>Bc/Cc</td>
<td>221,400</td>
<td>159,100</td>
<td>284,100</td>
<td>15,800</td>
</tr>
</tbody>
</table>

Calculations were done using a mutation rate of $1.03 \times 10^{-7}$ nucleotide substitutions per site per year for the D-loop region, and $5.14 \times 10^{-8}$ nucleotide substitutions per site per year for the cyt b region. Mean and 95% highest posterior density interval (HDP) together with effective samples sizes (ESS) are indicated.
diversity values were generally higher in the southern than in the northern region, with the exception of two Scottish sampling sites (38 and 39, Fig. 1), which exhibited values similar to the southern sampling sites. Haplotype diversity was generally high throughout Europe, with similar values observed in northern and southern sites. Diversity measures calculated for the different haplogroups (A, B, C) showed that haplogroup A exhibited both the highest haplotype and nucleotide diversity. Dividing each haplogroup into two groups comprising suitable and non-suitable areas during the last glaciation (Table S4) yielded no significant differences in nucleotide or haplotype diversity (pairwise t-tests).

**DISCUSSION**

A western, eastern and southern clade reflecting three main glacial refugia

In line with Ludt et al. (2004), our genetic analyses showed a large-scale structuring, indicating that red deer in western Europe, eastern Europe and the Mediterranean region are more or less geographically isolated with regard to matrilines. Deep phylogeographic structuring is usually consistent with long-term isolation in multiple refugia. Furthermore, the three clades exhibited an evident phylogeographical pattern (Fig. 1).
similar to that detected for many other European mammals from an overall point of view (Hewitt, 2004, and references therein). Based on the present geographical distribution of haplogroups, it can be assumed that haplogroups A and C descend from glacial refugial populations in the Iberian Peninsula and the Balkans, respectively. These are known refugial regions for many species (Taberlet et al., 1998; Hewitt, 2004) and coincide well with the fossil record of red deer (Sommer & Nadachowski, 2006). The high nucleotide diversity in Spain (Fig. 1; Table S4) further supports Iberia as a refugium for haplogroup A, and may account for the difference in diversity between regions north and south of the permafrost line (Table S4). This also shows that the Pyrenees did not obstruct northward expansions from the Iberian refugium, as haplogroup A dominates western and northern Europe today. The colonization route from the Iberian refugium towards central and northern Europe is also well established for other mammals (Taberlet et al., 1998). However, Italy is also regarded as a refugium for many European species (Taberlet et al., 1998; Hewitt, 2004).

Some of the individuals analysed here were sampled from the only indigenous red deer population left in Italy, Mesola (site 19, Fig. 1). These individuals all had D-loop haplotype AD3. The placement of AD3, although uncertain in both the phylogenetic tree (Fig. 2b) and the MSN (Fig. 3), indicates an ancient origin of this haplotype. Therefore, we cannot rule out the possibility that the Italian Peninsula served as a western (haplogroup A) refugium. The low genetic diversity for Mesola (Table S4) contradicts this hypothesis, but is easily explained by drift due to a severe decline in population size in Italy during the early 20th century caused by over-harvesting (Lorenzini et al., 2005). On the other hand, this region may have been colonized later from an Iberian refugium, and the divergence of AD3 might be a result of prolonged isolation of the Italian Peninsula caused by the Alps (Taberlet et al., 1998). At other sites in mainland Italy (sites 17 and 27, Fig. 1) we found D-loop and cyt b haplotypes typical of red deer from eastern Europe, consistent with the origin of translocated and immigrated individuals from central/eastern Europe (Zachos et al., 2003; Lorenzini et al., 2005).

We suggest the Balkan region as a possible refugium for red deer haplogroup C. However, the geographical localization of this refugium may also be further to the south-east (Turkey/Middle-East). Haplogroup C was less structured than haplogroup A, and no significant difference in diversity was found between the populations north and south of the permafrost line. This could be an effect of a denser sampling in this region of Europe. Our results from NCA (Fig. 3; Table S2) indicate that haplogroup C (the eastern European populations) has been stable over a longer period than haplogroup A (the western European populations), and that range expansion of haplogroup C caused ancestral haplotypes to become geographically widespread, homogenizing the populations. Haplo-type diversity is large in populations from the Carpathian region. As red deer fossils dating from the Last Glacial Maximum have also been found in this region (Sommer & Nadachowski, 2006), it may have been a refugium. However, our sampling is too small to draw definitive conclusions about this putative refugium.

Ludt et al. (2004) proposed the Alps and Carpathians as barriers in Europe. Although the eastern haplotypes (C) are roughly confined within the borders created by the Carpathian mountain range (Fig. 1), there is no conspicuous difference between the populations in the Carpathians and the adjacent lowland populations (sampling site 7 vs. 8; sampling site 13 vs. 11 and 14), indicating that this mountain range is not a barrier to the adjacent south-western lowlands.

The last haplogroup of the trichotomy, haplogroup B, has a very restricted distribution (Sardinia/Corsica, North Africa and southern Spain). For both D-loop and cyt b data, the B haplogroups belong to the most basal branches in the phylogenetic trees (Fig. 2). The origin of haplogroup B clade is thus older than haplogroups A and C, and, based on our data, haplogroup B is likely to represent the oldest clade of red deer in Europe. The current data are inconclusive as to whether it originates from Sardinia/Corsica or from Africa (Hmwe et al., 2006a).

Ludt et al. (2004) reported four groups of Western red deer based on cyt b data alone: Western Europe, Balkan, Middle East and Africa. Although we do not provide new data from the Middle East, individuals from this region are included in our analyses and are shown to group within haplogroup C (C1C and C2C, Fig. 2a), yet forming a separate subclade. Although not discussed by Ludt et al. (2004), this fourth group may reflect a historical isolation in the Caucasus (sites 2 and 4, Fig. 1).

Divergence times for main haplogroups pre-date the latest Pleistocene glaciation

The cyt b phylogeny suggests three independent pre-glacial immigration routes of red deer from Asia, as put forward by Ludt et al. (2004). We detected a similar deep divergence between the three D-loop haplogroups, which may be linked to isolation in multiple refugia in combination with several independent pre-glacial immigration routes. The estimated divergence times for the different haplogroups detected here ranged from 162,000 to 238,000 for the cyt b data, and from 272,000 to 304,000 for the D-loop data (Table 2), suggesting that haplogroup divergences pre-dated the last glaciation (a general phenomenon for species in Europe, as assessed by mtDNA; Taberlet et al., 1998). All divergences were within the Pleistocene, which is typical of mammalian intraspecific phylogroups (Avise et al., 1998). Thus, these haplogroups evolved before the most recent recolonization of Europe. The present-day pattern, showing three well defined European haplogroups, developed further during the glaciations and was reinforced by post-glacial refugial effects (such as drift, as there is a high probability of reciprocal monophyly between isolated groups after 4Ne generations; Avise, 2000) and range expansion.

The distinctive structuring of mtDNA haplotypes may also be the result of female philopatry in red deer, as male red deer
Limited phylogeographical impact of human long-distance translocations

For cervids, being important game species, there are generally many records of long-distance human-induced movements (e.g. Forsyth & Duncan, 2001), possibly influencing genetic structuring. There has been (and still is) a long tradition of translocating red deer (Hartl et al., 2003; Frantz et al., 2006). The more recent translocations have mainly been reintroductions to regions where red deer populations had gone extinct. Our results do not indicate a large impact of human long-distance translocations. Of the 58 D-loop haplotypes detected in Europe, only a few samples fall outside the geographical pattern defined by the three haplogroups (sampling sites 7, 19, 27, 28 and 35, Fig. 1). One such exception is the Mesola red deer, belonging to haplogroup A, while all other haplotypes detected in Italy belong to haplogroup C. However, rather than indicating a translocation, this finding may represent the evolutionary history of Italian red deer (see above).

The resemblance of the Sardinian haplotypes to those of North Africa (Fig. 2) has led to suggestions that red deer were introduced from these islands (Sardinia/Corsica) to the African mainland or vice versa (Ludt et al., 2004; Hmwe et al., 2006a), for example by the Romans, as suggested by Dobson (1998). In addition, some individuals in southern Spain also belong to this group (Figs 1 & 3). Alternatively, they might represent animals of ancient origin — at some time being spread over much of the Mediterranean region — introduced to the islands from the Italian mainland (Zachos et al., 2003; Hmwe et al., 2006a). The fact that we found two haplogroups (A and B) on Sardinia is perhaps the result of independent introductions/colonizations. Thus, the distribution of haplogroup B could have been influenced by human activities, as translocations in this part of the Mediterranean region are known to have occurred (Niethammer, 1963).

There are also historical tales of human translocations of red deer in the North Sea region by the Vikings (Langvatn, 1999). Our results may be interpreted as evidence that these translocations to a large extent included animals from the same haplogroup (A) and thus contributed to a homogenization within the region (England, France and Scandinavia; Figs 1 & 3), rather than leading to different phylogeographical patterns at the haplogroup level. Additional genetic markers, such as microsatellites, are needed to yield a more detailed history of red deer translocations in Europe.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Table S1** Sampling information.

**Table S2** Nested clad analysis results.

**Table S3** Results from the *SAMOVA* analysis.

**Table S4** D-loop haplotype diversity.

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**BIOSKETCH**

Anna Skog is an evolutionary geneticist. This work was carried out as a part of her PhD thesis on large-scale phylogeographical patterns in Northern Hemisphere vertebrates.

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