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Global phylogeography of the genus *Capreolus* (Artiodactyla: Cervidae), a Palaearctic meso-mammal

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Areas of sympatry and hybridization of closely related species can be difficult to assess through morphological differences alone. Species which coexist and are similar morphologically may be distinguished only with molecular techniques. The roe deer (*Capreolus* spp.) is a meso-mammal having a Palaearctic distribution, with two closely related species: the European *C. capreolus* and the Siberian *C. pygargus*. We analysed mtDNA sequences from 245 individuals, sampled through all the entire range of the genus, to investigate the distribution of genetic lineages and outline phylogeographical patterns. We found that: (1) a *C. pygargus* lineage occurs in Poland and Lithuania, much farther west than the area which so far was believed its westernmost limit; (2) no haplotype of this *C. pygargus* lineage matches any found in East Europe and Asia – this should rule out human introductions and may indicate Pleistocene–Holocene migrations from the east; (3) no geographical structuring of *C. pygargus* lineages occurs, questioning the existence of putative subspecies; (4) several genetic lineages of *C. capreolus* can be recognized, consistent with the existence of two subspecies, respectively in central–southern Italy and southern Spain. Coalescence times suggest that intraspecific variation in *C. capreolus* and *C. pygargus* developed approximately 100–10 kya. The extant mitochondrial lineages pre-dated the Last Glacial Maximum. *Capreolus pygargus* must have moved westward to Central Europe, where at least one genetic lineage still survives, coexisting with *C. capreolus*.

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ADDITIONAL KEYWORDS: Bayesian inference – biogeography – European roe deer – mtDNA control region – phylogenetic reconstruction – Siberian roe deer.

INTRODUCTION

Areas of sympatry and hybridization in closely related species of mammals can be difficult to assess when species are widely distributed. Nevertheless, identifying areas of overlap is not only important to understand the dispersal of phylogenetically close taxa, but also for conservation purposes. Until the advent of molecular technology, overlapping or contact areas were identified through the geographical distribution of individuals with interspecific morphological differences. This method can be misleading if species are closely related, as clinal variation may account for the observed differences. Morphoclines are graded series of morphotypes, frequent among ungulates and arising through dispersal. If so, determining both the geographical and the morphological

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boundaries may be difficult without information on the populations' genetic background. DNA technology has helped to understand the dynamics of hybridization and introgression (Arnold, 2006). Signatures of hybridization and introgression can be determined through an extensive sampling in the hypothetical contact zone and a good coverage of genomic markers. This can be difficult for widely distributed meso- and large mammals.

The roe deer (*Capreolus* spp.) is a meso-ungulate with a wide Palaearctic distribution range, from far Eastern Russia across Central Asia (China and Siberia) and across Europe, from the Caucasus to the Mediterranean peninsulas and Scandinavia (Danilkin, 1996), showing a morphoclinal trend (Geist, 1987). This ruminant is a generalist browser, termed as a 'concentrate selector' (Hoffmann, 1989), with a broad niche. It makes use of grasses and sedges (Duncan *et al*., 1998), thriving in deciduous wooded areas with glades and clearings, as well as in fields interspersed with wood patches, mostly in temperate climates (Andersen *et al*., 1998).

The European roe deer, *C. capreolus* (Linnaeus 1758), is closely related to the larger Siberian roe deer, *C. pygargus* Pallas 1771. Their contact zone appears to lie in far Eastern Europe, in a narrow range between the rivers Volga and Don (Danilkin, 1996). Whether the two species may generate viable fertile offspring and where the suture zone actually lies are issues that deserve attention (Lister, Grubb & Summer, 1998). Unfortunately, no comprehensive palaeontological information is available to shed light on these subjects (cf. Hufthammer & Aaris-Sørensen, 1998; Lister *et al*., 1998; Sommer *et al*., 2009). Nor have the genetic structures of European and Siberian roe deer been compared throughout all their distribution range. Their speciation occurred somewhere in the temperate zones of Central or Eastern Asia (cf. Sokolov & Gromov, 1990) in the late Pliocene/early Pleistocene, some 2–3 Mya (Danilkin, 1996; Randi, Pierpaoli & Danilkin, 1998). These two species have probably lived in allopatry for most of their evolutionary history (Hewison & Danilkin, 2001). In prehistoric times, because of alternating contractions and expansions of their overlapping areas they must have come into contact more than once (Hewison & Danilkin, 2001).

Since the 19th century, Siberian roe deer were introduced, sometimes in high numbers, to the distribution range of the European roe deer (e.g. Germany, Slovakia, Ukraine, some north-western regions of the former Soviet Union) for hunting purposes (Danilkin, 1996). Hewison & Danilkin (2001) stated that introgression and persistence of the Siberian form hardly occurs in the wild because of the strong reproductive barriers developed during and after speciation. The small females of *C. capreolus* often die in childbirth when they mate with the larger *C. pygargus* males. F1 hybrids obtained in captivity are partially or totally infertile (Sokolov & Gromov, 1990), possibly because of their highly divergent karyotypes (Danilkin, 1996). Siberian genotypes, unable to cope with the high numbers of the European species, are lost in the areas of introduction (Hewison & Danilkin, 2001). Thus, these releases do not seem to have left signs in the resident European species.

The geographical distribution and subspecific taxonomy of Siberian roe deer have been inferred from morphological data, but the issues remain of debate. From one to three subspecies are recognized [Danilkin, 1996: *C. p. pygargus*, distributed in the westernmost part of the Asiatic range (from the Volga River to Siberia), *C. p. tianschanicus* (or *bedfordi*), present in far Eastern Russia and Mongolia, and *C. p. melanotis* in Tibet and China (Vorobieva *et al*., 2011)]. A fourth subspecies has also been suggested in Korea (Koh & Randi, 2001; Wilson & Reeder, 2005). Patterns of intraspecific genetic variability have been poorly investigated and the degree of population structuring is largely unknown (Xiao *et al*., 2007; Vorobieva *et al*., 2011).

Molecular phylogeographical investigations have been carried out on the European roe deer (Lorenzini, Lovari & Masseti, 2002; Vernesi *et al*., 2002; Lorenzini *et al*., 2003; Randi *et al*., 2004; Lorenzini & Lovari, 2006; Royo *et al*., 2007). Both mitochondrial and nuclear markers have revealed high genetic variation and a complex population structure across its entire range. Most of the extant genetic lineages are thought to have originated before the Last Glacial Maximum (Vernesi *et al*., 2002; Randi *et al*., 2004; Royo *et al*., 2007), and their distribution would result from population dispersal during the Pleistocene (cf. Hewitt, 2000). A clear geographical segregation of lineages has been observed for the subspecies *C. c. italicus* (Festa, 1925) in Central and Southern Italy (Lorenzini *et al*., 2002; Randi *et al*., 2004), and for the Andalusian population, which is confined to Southern Spain (Lorenzini *et al*., 2003; Lorenzini & Lovari, 2006). Each of these Mediterranean peninsulas are believed to have functioned as Pleistocene refugia (e.g. Lorenzini & Lovari, 2006).

We have studied the present intra- and interspecific genetic differentiation in *C. capreolus* and *C. pygargus*. Variation at complete mitochondrial DNA (mtDNA) control region (CR) sequences has been used (1) to examine relationships between haplotypes, (2) to assess the timing of genetic lineages, and (3) to clarify the phylogeography of *Capreolus* under the traditional models of Quaternary radiation of populations (cf. Hewitt, 1996). Genetic variation in roe deer across such a broad spatial scale was analysed to determine

population dispersal in the Pleistocene and the influence of historical and contemporary processes on the structure of the genus.

MATERIAL AND METHODS

Sampling and laboratory protocols

A total of 245 roe deer samples were collected from 18 sites across the *C. capreolus* and *C. pygargus* ranges (Table 1, Fig. 1). Specimens were collected from allegedly native populations or from locations where (re)introductions have never been documented. Tissue and blood samples were obtained either from legally shot roe deer or from live animals caught in protected areas for research purposes. Total genomic DNA was isolated using the Qiagen QIAamp DNA Mini Kit or, alternatively, the DNA IQ™ Casework Sample Kit and the Maxwell 16 LEV System (Promega). A fragment of approximately 1000 bp of mtDNA, encompassing the CR was amplified by PCR using the external primers H-Phe (Jäger, Hecht & Herzog, 1992) and CST2 (Polziehn & Strobeck, 2002). PCR conditions followed either Polziehn & Strobeck (2002) or Lorenzini & Lovari (2006). Internal sequencing primers were CST25, CST29 (Polziehn & Strobeck, 2002), LD13r (5′TTAATGCGCTTATAGTACATT3′) and HD6r (5′CTACCATTATGGGGATGCTC3′).

Sequence alignment and phylogenetic analyses

Sequences were aligned and edited using the multiple alignment program included in the package Vector NTI v. 9.1 (Invitrogen) and the software BioEdit (Hall, 1999). After inspection by eye, a contig alignment of 925 nt was obtained, which included four indels. To ensure that our sequences were not pseudogenes of nuclear origin, we verified their mitochondrial authenticity by comparing the internal organization in domains of the entire CR with data reported for roe deer and other cervids (Douzery & Randi, 1997), and with single positions in the alignment from Randi *et al*. (1998). The TIM3 transition model of nucleotide substitution with a proportion of invariable sites (I) and rate variation among sites (G) with four categories was selected by jModeltest v. 0.1.1 (Posada, 2008) as the best-fit model for our dataset, using the Akaike Information Criterion corrected for small sample sizes (AICc). The estimated base frequencies were A = 0.310, C = 0.219, G = 0.144 and T = 0.327; the gamma-shaped distribution for variable sites was $\alpha = 0.62$, and the proportion of

Table 1. Distribution of 54 *C. capreolus* (Cc) and *C. pygargus* (Cp) haplotypes from 245 Eurasian samples

Code	Location	*N*	Haplotypes
AUS	Austria (Styria, Lower Austria)	6	Cc14 (1), Cc18 (4), Cc23 (1)
E-AL	Eastern Italian Alps (Val Rendena)	13	Cc8 (2), Cc9 (2), Cc10 (3), Cc17 (4), Cc18 (2)
CRI	Crimea	3	Cc15 (1), Cc16 (2)
DEN	Denmark (Kalø)	10	Cc14 (7), Cc22 (3)
FRA	France (Aquitaine, Chizé)	12	Cc7 (3), Cc12 (3), Cc14 (6)
GRE	Greece	1	Cc33 (1)
ITA	Central-southern Italy (Castelporziano Estate, Gargano and Pollino National Parks, Grosseto province)	42	Cc27 (10), Cc28 (10), Cc29 (14), Cc30 (8)
SWE	Sweden	6	Cc24 (6)
ROM	Romania	10	Cc19 (1), Cc26 (2), Cc31 (1), Cc35 (2), Cc36 (2), Cc37 (2)
N-SP	Northern Spain (Asturias, Lugo, Basque Country, Soria, Guadalajara, Segovia)	51	Cc1 (3), Cc4 (2), Cc7 (4), Cc11 (1), Cc13 (4), Cc14 (11), Cc20 (1), Cc21 (25)
CS-SP	Central-southern Spain (Toledo, Cáceres, Ciudad Real, Cádiz)	52	Cc2 (15), Cc3 (3), Cc5 (24), Cc6 (10)
POL	Poland (Białowieza, Bieszczady Mountains)	8	Cc25 (1), Cc32 (1), Cp38 (1), Cp39 (2), Cp40 (3)
LIT	Lithuania (Panevezys, Jurbarkas, Moletai, Sirvintos, Kaunas)	13	Cc34 (10), Cp40 (3)
NE-CH	North-eastern China (Inner Mongolia, Heilongjiang)	3	Cp48 (1), Cp49 (1), Cp50 (1)
CE-CH	Central-eastern China (Hebei, Tianjin, Shanxi)	4	Cp51 (2), Cp52 (1), Cp54 (1)
KYR	Kyrgyzstan	1	Cp53 (1)
E-RUS	Eastern Russia (Lazo)	7	Cp43 (1), Cp44 (1), Cp45 (1), Cp46 (2), Cp47 (2)
W-RUS	Western Russia (Orenburg)	3	Cp41 (2), Cp42 (1)

Absolute frequencies are in parentheses. *N*, sample size. Acronyms as in Figure 1.

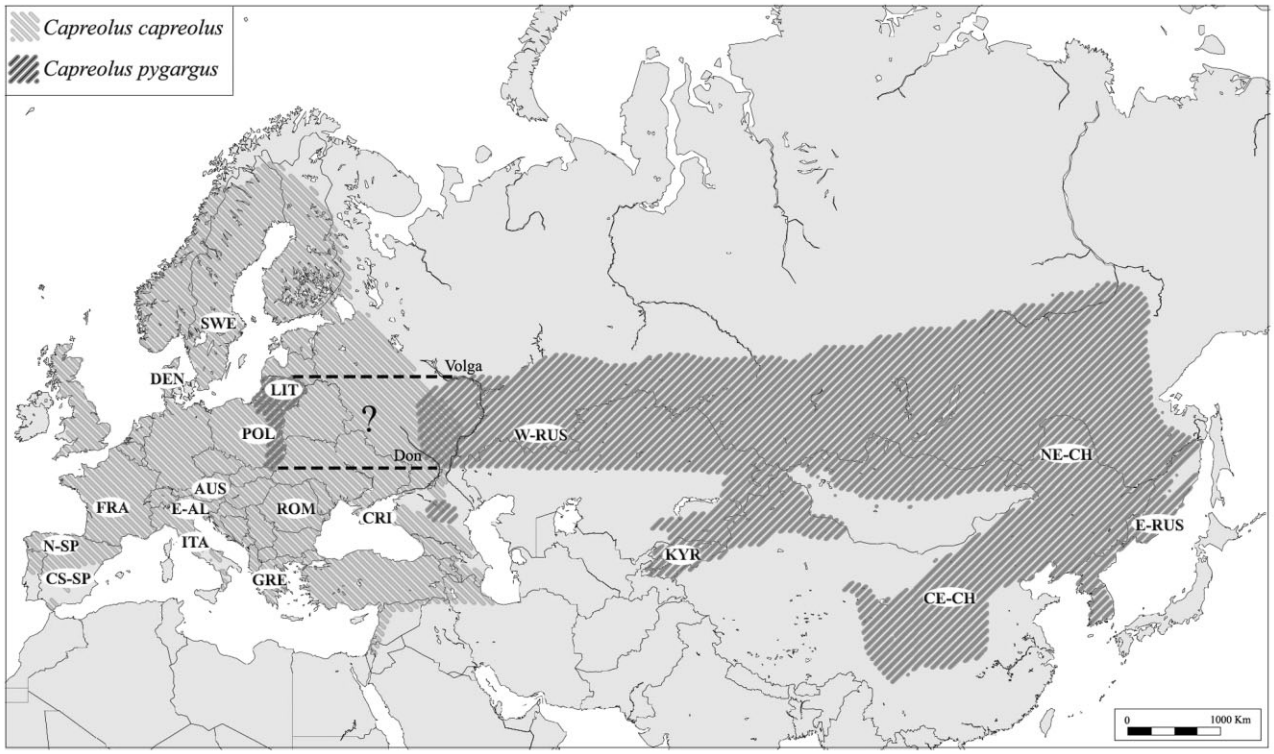

Figure 1. Distribution ranges for *C. capreolus* and *C. pygargus*, and the area of putative current sympatry between the rivers Volga and Don (Hewison & Danilkin, 2001). The question mark indicates the area of further putative presence of *C. pygargus*. Collection sites are as follows: (FRA) France, (SWE) Sweden, (DEN) Denmark, (AUS) Austria, (ITA) Central-southern Italy, (E-AL) Eastern Italian Alps, (N-SP) Northern Spain, (CS-SP) Central-southern Spain, (GRE) Greece, (ROM) Romania, (POL) Poland, (LIT) Lithuania, (CRI) Crimea, (W-RUS) Western Russia, (KYR) Kyrgyzstan, (NE-CH) North-eastern China, (CE-CH) Central-eastern China, (E-RUS) Eastern Russia. Multiple sampling sites for one population are indicated by the same acronym. See Table 1 for details on precise sampling locations and sample sizes.

invariant sites was 0.777. Transitions and transversions were observed at markedly different rates: AC = CG = 0.220, whereas AG = 21.989, AT = 1.0, and CT = 11.762, scaled to GT = 1.0.

A phylogenetic tree was obtained using MEGA v. 4.1 (Kumar *et al*., 2008) with the neighbour-joining (NJ) procedure under the TN93 genetic distance model (Tamura & Nei, 1993), using the CR sequence of *Cervus elaphus* as an outgroup. Support at the nodes was assessed by 1000 bootstrap resamplings. Model-free maximum-parsimony (MP) trees with characters unweighted were constructed by MEGA with the close-neighbour-interchange algorithm. One thousand replicates were applied to infer the final MP consensus tree. The program DNAML in the package PHYLIP v. 3.69 (Felsenstein, 2005) was used to derive a maximum-likelihood (ML) tree. Reliability of the nodes was assessed by running the program SEQBOOT in the PHYLIP package to obtain 250 bootstrap replicates. A Bayesian approach was applied for phylogenetic inference using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003). To include gaps in the analyses, two unlinked partitions of the data were simultaneously input: one consisting of 921 nt sites under the TIM3 evolution model, and the second consisting of the gaps used as characters under a simple F81-like model for binary data (absence of gaps coded as 0 and presence coded as 1). Four independent runs of 4×10^6 generations were performed to check for convergence of the ln likelihood values (< 0.008), with trees sampled every 100 generations. The initial 25% of trees were discarded as burn in. Three heated chains and a single cold chain were used in the Markov chain Monte Carlo (MCMC) analyses. Posterior probabilities at the nodes were used to estimate a 50% majority-rule consensus tree.

Phylogenetic relationships were also assessed via the median-joining network method (Bandelt, Forster & Rohl, 1999) using Network v. 4.6.0.0 (http://www.fluxus-engineering.com). Networks differ from phylogenetic bifurcating methods because they

allow for ancestral (or unsampled) sequences and alternative connections, which is particularly favourable when genetic distances between haplotypes are low, as usually occurs with intraspecific sequence data. To avoid introducing severe bias due to large dissimilarities in sample sizes, only different haplotypes, and not their frequencies, were used to construct the network.

The program Arlequin v. 3.5.1.2 (Excoffier, Laval & Schneider, 2005) was used to assess haplotype diversity within species, h, and nucleotide diversity among haplotypes, π. As most samples were small, estimates of genetic variability at the population level were not calculated to avoid unreliable results.

Timing and lineage divergence

Divergence times between the mtDNA lineages recovered from the phylogenetic analyses were derived following the coalescence method (Gaggiotti & Excoffier, 2000). Demographic bottlenecks, occurring when populations diverge, lead to a rapid increase of the genetic distances. This approach aims at removing the effect of unequal population sizes that otherwise may result in severe overestimations of divergence times. We applied a mutation rate of 0.04–0.08 substitutions per site per Myr within lineages (Randi *et al*., 1998, 2004; cf. also Vernesi *et al*., 2002; Royo *et al*., 2007). We also tested for evidence of population-level episodes of expansion from the mismatch distribution of the observed number of pairwise differences between individual haplotypes under the sudden expansion model (Rogers & Harpending, 1992), as implemented in Arlequin. Mismatch distributions are usually unimodal in populations having experienced a recent demographic growth, but multimodal in populations at equilibrium (Rogers & Harpending, 1992). Approximate expansion times were calculated in each of the two species globally, grouping haplotypes according to their respective clades (see Results). To translate mutational units into years, we used a generation time of 2 years, which is the age of first reproduction of most female roe deer (77.2%; Hewison, 1996). Time-since-expansion values were estimated using the online spreadsheet tool for the application of the mismatch analysis, available at the web site (http://www.uni-graz.at/zoowww/mismatchcalc/index.php, Schenekar & Weiss, 2011).

RESULTS

Mitochondrial DNA sequence diversity

The alignment of 245 mitochondrial CR sequences (925 nt) from European and Siberian roe deer resulted in 54 haplotypes (Table 1), defined by 96 polymorphic sites and 98 mutations (considering each gap as a single mutation), of which 79 (80.6%) were transitions, 15 (15.3%) transversions, and 4 (4.1%) indels. Complete sequences have been deposited in GenBank with accession numbers KF700100-KF700111, KF724414-KF724455. There were 62 parsimony-informative sites, of which two showed three variants. Twenty-four (25%) of the 96 observed variable sites were fixed differences between species, for a total of 24 mutations, of which 18 (75.0%) were transitions, four (16.7%) transversions, and two (8.3%) gaps. mtDNA diversity, h, was high in both species: 0.943 (SD = 0.031) and 0.942 (SD = 0.006) in *C. pygargus* and *C. capreolus*, respectively. Conversely, sequence divergence was low: nucleotide diversity, π, was 0.0108 (SD = 0.0057) for *C. pygargus* and 0.0095 (SD = 0.0049) for *C. capreolus*. The value of π between species was 0.0479, about four times higher than the corresponding intraspecific value.

Phylogenetic analysis of mitochondrial haplotypes

NJ, MP, ML and Bayesian analyses generated trees with similar patterns of the major branches. The most resolved topology was yielded by the Bayesian procedure (Fig. 2). Two major clades of haplotypes have been identified, one for *C. capreolus* and the other for *C. pygargus*. Both clades are supported by high statistical confidence in all trees (except the *capreolus* clade in the Bayesian tree, where some nodes are collapsed into a polytomy, see below). The *C. pygargus* clade included three major haplogroups (or lineages) which did not show any obvious underlying geographical structure. (1) Haplogroup A, highly supported in all analyses (≥ 92%), comprises haplotypes from Western Russia, Kyrgyzstan, and Central-Eastern China. Surprisingly, it also includes haplotypes from the putative *C. capreolus* range, i.e. Lithuania and Eastern Poland, which occur at the notable frequency of 43% (nine *pygargus* haplotypes out of 21 roe deer sampled). (2) Haplogroup B, with haplotypes from Eastern Russia and others from North-eastern China, is placed as a sister group of haplogroup A, although it receives support only from the Bayesian and NJ analyses. As a consequence of low statistical robustness of haplogroup B, the topology at the inner node (the one from which stem haplogroups A and B) is not highly stable. (3) The remaining haplotypes from Eastern Russia, North- and Central-Eastern China (haplogroup C) were placed in a basal position as ancestral non-monophyletic haplotypes in the ML and Bayesian trees. In the latter, they occur as a polytomy due to low support at the nodes. In the NJ and MP trees (not shown), all haplotypes from North- and Central-Eastern China (except those included in haplogroup

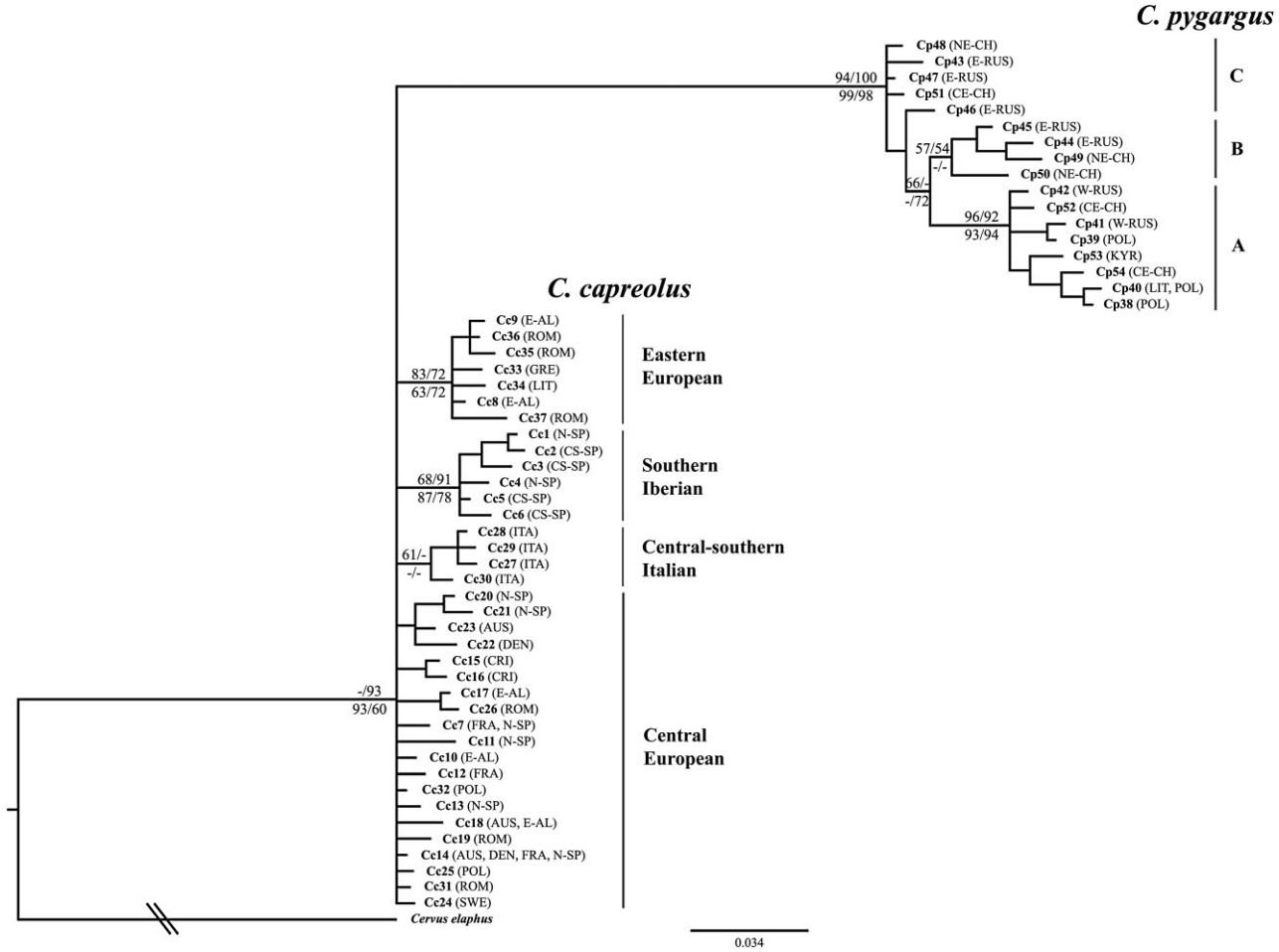

Figure 2. Phylogeny of *Capreolus* obtained from the Bayesian analysis of 54 mtDNA haplotypes (925 nt) under the TIM3 model of sequence evolution. Branch length units are expected substitutions per site. Bayesian posterior probability and bootstrap values for NJ, MP and ML, respectively, are shown for the main haplogroups (inner nodes) shared by all trees. Only values over 50% are indicated. A mitochondrial CR sequence of *Cervus elaphus* was used as an outgroup.

A) clustered with those from Eastern Russia in a single haplogroup, albeit weakly supported (bootstrap < 50% in both trees). Thus, in the *C. pygargus* cluster, only haplogroup A, which included haplotypes from Eastern Europe, Kyrgyzstan, and Central-Eastern China, is strongly supported by all phylogenetic analyses, whereas the majority of haplotypes from North- and Central-Eastern China, as well as those from Eastern Russia, clustered together, either in one or in two haplogroups (both topologies weakly supported), according to NJ and MP or Bayesian and ML trees, respectively.

In the *C. capreolus* clade, four main haplogroups were identified with high bootstrap support in all analyses (except the Central-southern Italian, see below). Three showed also a strong geographical signature. One haplogroup, hereafter referred to as the Southern Iberian haplogroup, comprised haplotypes from Central-southern Spain (with the exception of two haplotypes, Cc1 and Cc4, collected occasionally in Alava and Asturias, Northern Spain, but see Lorenzini *et al*., 2003 for more details), where the subspecies *C. c. garganta* was suggested (Meunier, 1983). A second Eastern European haplogroup joined haplotypes of Eastern Europe, from Greece to Lithuania, through the Eastern Italian Alps and Romania. The Central-southern Italian (corresponding to the subspecies *C. c. italicus*) was inferred as a third geographically restricted haplogroup, which received bootstrap support > 50% only from the Bayesian analysis. The reason for this is twofold: haplotype Cc30, sampled in Gargano National Park, was weakly linked to the other *C. c. italicus* haplotypes because of divergence at three sites (outside the CR hypervariable domain I). Moreover, this haplogroup (Cc30 included) contained one of the four nucleotide

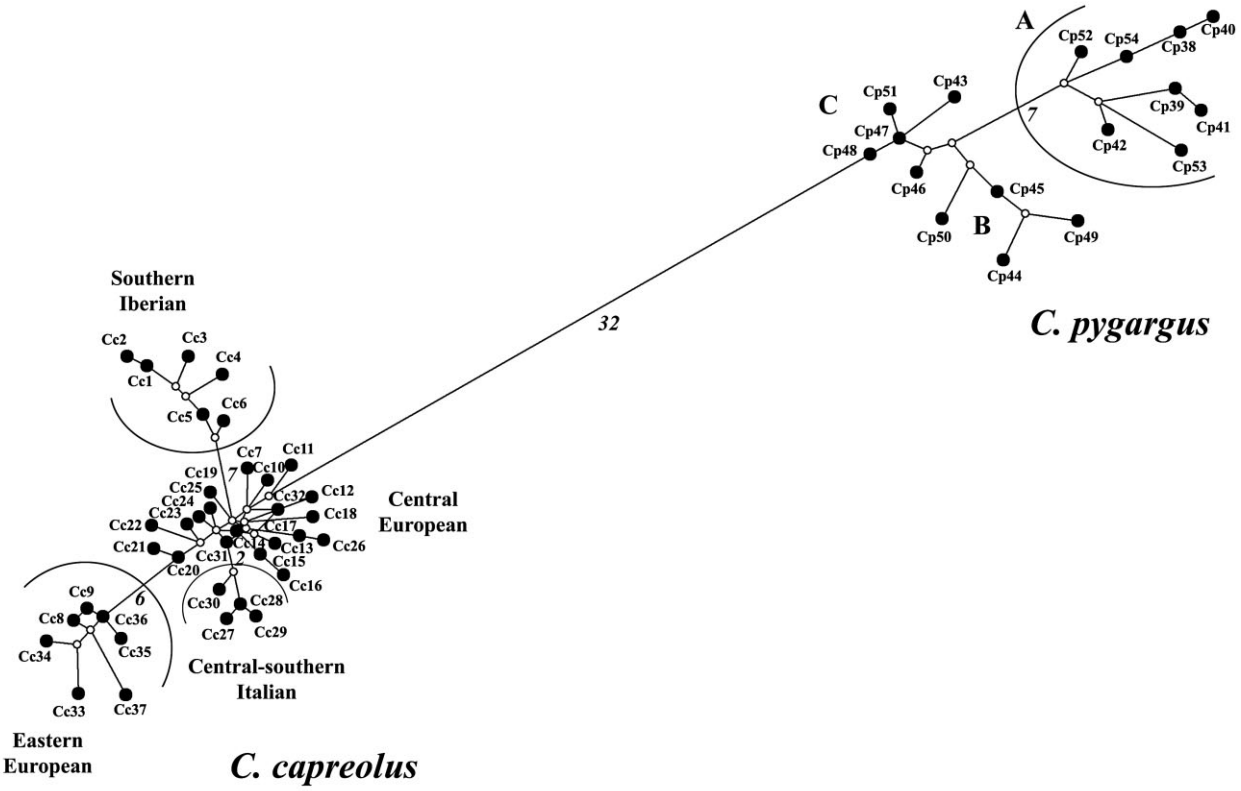

Figure 3. Median-joining network based on the data set of *C. capreolus* and *C. pygargus* haplotypes. Branch lengths are approximately scaled to the number of nucleotide substitutions occurring along the branches. White circles represent missing haplotypes. The main haplogroups are indicated by curved lines. The number of mutations are reported only for the main haplogroups.

deletions in the entire alignment. As the gaps were removed by a fixed option in all phylogenetic procedures except the Bayesian, this led to either low support (< 50%) of the *italicus* haplogroup, or the scattering of the *italicus* haplotypes into different sister haplogroups. Conversely, the Bayesian analysis can optionally consider the gaps as characters, and this yielded the separation of a supported *C. c. italicus* haplogroup in the tree. A fourth Central European haplogroup was not geographically restricted, including haplotypes widely distributed across all Europe and Crimea, with the exception of Lithuania. Poor resolution of this haplogroup was due to low divergence of haplotypes; unreliable branches were either collapsed into extended polytomies in the Bayesian tree or poorly supported (bootstraps < 50%) at the outer sub-haplogroups in the bifurcating trees (not shown).

The median-joining network (Fig. 3) contained many missing (either ancestral or unsampled) sequences. The majority of extant haplotypes stood on the tips of the network, suggesting recent differentiation, without obvious reticulations, and central ancient haplotypes could not be recognized. Haplotype relationships were congruent with those reported by the Bayesian tree. Two clades, separated by 32 mutational steps, were consistent with splitting of the species. Within the *C. capreolus* clade, haplotypes of the Southern Iberian, Eastern European, and Central-southern Italian lineages are split into three well-defined haplogroups, separated, respectively, by seven, six and two mutations from the Central European lineage. Haplotypes from the latter fell into an unresolved topology, in full agreement with phylogenetic trees. In the *C. pygargus* cluster, haplotypes from Western Russia, Poland, Lithuania, Kyrgyzstan and Central-Eastern China were maintained as a monophyletic lineage (A), separated by seven mutations from a sister haplogroup of scattered Eastern Russian and Chinese haplotypes.

Unexpectedly, our original alignment showed that some haplotypes sampled in the *C. capreolus* range,

Table 2. Approximate divergence times (kya) derived with the coalescence method of Gaggiotti & Excoffier (2000), between the *C. capreolus* and *C. pygargus* mtDNA lineages identified in this study

	C. capreolus			*C. pygargus*		
	Central European	Southern Iberian	Central-southern Italian		A	B
Central European				A		
Southern Iberian	70–35 (5.15)			B	373–37 (5.41)	
Central-southern Italian	17–8 (1.25)	108–54 (8.00)		C	84–42 (6.24)	18–9 (1.31)
Eastern European	48–24 (3.56)	99–50 (7.34)	71–36 (5.27)			

Intervals are obtained using a fast (0.08) and slow (0.04) mutation rate. Values of τ are in parentheses.

i.e. Lithuania and Poland, were assigned by all analyses to the *C. pygargus* clade (Figs 2, 3). We derived a Bayesian tree from a shorter alignment (425 nt) by pooling our data set with additional published sequences of Eastern European (Poland, Slovakia, Bulgaria) roe deer (Wiehler & Tiedemann, 1998; accession numbers AJ287365, AJ287368–70, AJ287372, and AJ287380–83). The new tree was constructed to verify if any sequence from literature fell within the *pygargus* clade. Data of roe deer from China (Xiao *et al*., 2007) and Siberia (Koh & Randi, 2001; Vorobieva *et al*., 2011) were also included to obtain more exhaustive haplotype sampling for *C. pygargus*. In the resulting Bayesian tree (not shown), no new sequences from Eastern Europe joined our *C. pygargus* clade: they all fell in the *capreolus* clade, either included in the Eastern European haplogroup (Bulgaria), or scattered throughout both the Eastern and the Central European haplogroups (Poland, Slovakia). No geographical structure of haplotypes was detected in the *C. pygargus* sequences from China and Siberia. The addition of the new sequences has left the topology of our original tree totally unchanged (Fig. 2).

Divergence estimates and test for demographic expansions

Divergence times (Table 2) were estimated between the main genetic lineages identified in each species. Within *C. capreolus*, divergence ranged from 108 to 8 kya, according to the pair of haplogroups considered and the fast/slow mutation rate that was assumed. As expected from both the Bayesian tree and the network, the Southern Iberian lineage showed the oldest divergence time (70–35 kya) from the Central European haplogroup, followed by the Eastern European, which separated some 48–24 kya. The *C. c. italicus* diverged from Central haplotypes more recently, about 17–8 kya. Early isolation and genetic drift may be responsible for increased genetic distances of the peripheral Southern Iberian and Central-southern Italian lineages. The coalescence method may not have totally removed the bottleneck effect, with possible overestimation of the divergence times. *Capreolus pygargus* showed comparable divergence times of lineages, with estimates ranging from 84 to 9 kya. Haplogroups C and B diverged about 84–37 kya before the most recent haplogroup A, which comprises also haplotypes from Eastern Europe.

Evidence for recent demographic growth was obtained in both major clades, with the observed distribution of pairwise differences which did not deviate from the distribution expected under a model of sudden demographic expansion ($P > 0.05$ for both the sum of squared deviations and the raggedness index). One main peak of expansion in *C. capreolus* occurred at $\tau = 10.91$ [95% confidence interval (CI) 6.00–14.87] mutational units, which translates into approximately 74 kya (95% CI 41–100 kya), or, when considering a slow mutation rate, into about 147 kya (95% CI 81–201 kya). However, two peaks at $\tau = 9$ and $\tau = 13$ were clearly detectable with respect to an underlying slightly ragged distribution (not shown), suggesting that two possible expansion events for the European roe deer populations could have occurred (cf. Randi *et al*., 2004; Royo *et al*., 2007) at about 88 and 61 kya, respectively or, according to a slow mutation rate, at about 176 and 122 kya. Similarly, one main peak occurred for the Siberian roe deer at $\tau = 13.64$ (95% CI 7.48–17.97), with demographic expansion dating at about 92 kya (95% CI 51–121 kya) and 184 kya (95% CI 101–243 kya), using a fast and slow mutation rate, respectively.

DISCUSSION

Phylogenetic relationships of mitochondrial haplotypes

Some mtDNA haplotypes that belong to the *C. pygargus* clade with high support were found at a frequency of nearly 50% in Poland and Lithuania,

which are areas of the alleged range of *C. capreolus*. Currently, the Don and Volga rivers are said to mark the putative area of hybridization/coexistence of the two roe deer species (Danilkin, 1996), which is farther East than Lithuania and Poland. Conversely, no mtDNA haplotypes previously found in Eastern Europe (Bulgaria and Slovakia included, cf. Wiehler & Tiedemann, 1998) belonged to *C. pygargus*. The original topology of our tree has been maintained, even after shortening the initial alignment (from 925 to 425 nt) to include additional sequences. Thus, over its entire European range *C. capreolus* is non-monophyletic for mtDNA.

Introductions of *C. pygargus* to the range of *C. capreolus*, which were meant to increase body weight and antlers of the latter, might explain these results (Danilkin, 1996; Hewison & Danilkin, 2001). Danilkin (1996) stated that, in the large majority of cases, these introductions have not led to viable populations of *C. pygargus*, and assumed hybrids have been rare. *C. capreolus* has a constant chromosome number $2n = 70$, while *C. pygargus* has a variable number of additional B chromosomes in its distribution range, clinally growing from West to East (1 to 14). This feature, together with body size, could act as a strong reproductive barrier (Hewison & Danilkin, 2001).

In our study, no *C. pygargus* haplotype found in Poland and Lithuania was recorded in areas previously sampled by other authors, and by ourselves as well, from Korea and far Eastern Russia, across China to West Siberia (Koh & Randi, 2001; Xiao *et al*., 2007; Vorobieva *et al*., 2011). *Capreolus pygargus* may have moved westward into the range of *C. capreolus* (see below). Genetic consequences of natural migrations – or introductions – are clearly visible at the mtDNA level and *C. pygargus* genotypes are present in Eastern Europe.

The same samples of roe deer from Poland and Lithuania, which correspond to our current *C. pygargus* haplotypes, were previously analysed in a European framework (Lorenzini & Lovari, 2006). Restriction fragment length polymorphisms of mtDNA CR assigned them to a separate branch (Haplogroup I of fig. 2 in Lorenzini & Lovari, 2006), highly divergent from all the remaining European haplotypes. The lack of comparison with Siberian samples, not included in that survey, did not allow us to identify them as belonging to *C. pygargus*. On the contrary, microsatellite variation and Bayesian tests of assignment (see fig. 4 and table 4 in Lorenzini & Lovari 2006) ruled out any recent admixture, suggesting an ancient hybridization between these species. Lack of congruence between mitochondrial and nuclear markers is not surprising in historically mixed populations. Lower levels of population substructuring for short tandem repeat (STR) loci than for mtDNA can be explained by female phylopatry and maternal inheritance of the mitochondrial genome, as well as by gene flow mediated mainly by male dispersal and different modes of evolution of the two marker systems. To date, no STR-based exhaustive genetic survey has been conducted on populations of *C. pygargus* and the genetic structure of its populations at the nuclear level remains unknown. Thus, the presence of *C. pygargus* haplotypes in Northeastern Europe may be due to an ancient introgression through hybridization between highly divergent lineages during population expansion(s) in contiguous areas of the species' range (see below).

Our phylogenetic analyses failed to identify highly supported haplogroups of Siberian roe deer, with underlying geographical structuring, suggesting an uninterrupted gene flow among populations (see below). The most supported genetic lineage (haplogroup A) included haplotypes sampled in extremely distant areas: Eastern Europe and Western Russia, as well as Kyrgyzstan and Central–Eastern China. No relationship could be established between genetic lineages and geographical distribution. Nor could any particular haplogroup corresponding to the proposed subspecies (*C. p. pygargus*, *C. p. tianschanicus*, *C. p. melanotis*, Danilkin, 1996) be detected. They therefore cannot be confirmed. In line with this, Koh & Randi (2001) reported that roe deer from Korea belong to the same genetic lineage as populations from Western Siberia, in spite of its geographical distance, while they were highly divergent from the roe deer of the geographically much closer Amur region. Phylogenetic data from modern and ancient (up to 50 kyr old) Siberian roe deer also indicated the lack of either any genetic subspecies structure, or any geographical segregation of the genetic lineages detected (Vorobieva *et al*., 2011).

The Bayesian topology of *C. capreolus* contains a branching pattern that is partly supported by high posterior probabilities and partly collapsed into polytomies in correspondence to unreliable nodes. This can be the case when either the sequence divergence of recently differentiated haplotypes is low or the genetic structuring of populations is lacking due to high migration rates. Both the Bayesian and the other phylogenetic procedures, as well as the network, showed four mitochondrial lineages. Three of these lineages revealed a strong geographical signature: (1) an Eastern European lineage, with haplotypes sampled in Greece, Romania, Eastern Italian Alps, and Lithuania, as well as Poland, Bulgaria, and Slovakia, if we include sequences from literature; (2) a Southern Iberian lineage, with haplotypes mainly from Central-southern Spain; (3) the *C. c. italicus* lineage, corresponding to the subspecies dwelling in Central-southern Italy

(Lorenzini *et al*., 2002; Randi *et al*., 2004); (4) all remaining haplotypes, throughout Europe and Crimea, are placed in the collapsed portion of the Bayesian tree, where the phylogenetic relationships are poorly resolved. Low statistical support was also obtained for other phylogenies on *C. capreolus* (Vernesi *et al*., 2002; Randi *et al*., 2004). Being recurrent, this feature may derive from a genuine lack of high genetic structuring (rather than from insufficient or inappropriate sampling schemes), perhaps due to high rates of migration: in contrast to most polygynous ungulates, both sexes of roe deer show high rates of dispersal (Linnell, Wahlstroem & Gaillard, 1998).

Our figure of genetic structuring is largely consistent with previous phylogenetic studies on European roe deer (Vernesi *et al*., 2002; Randi *et al*., 2004; Royo *et al*., 2007). Randi *et al*. (2004) reported that roe deer from Central and Southern Spain contained haplotypes belonging to different and distantly related genetic lineages, and stated that their molecular data did not support the monophyly of subspecies *C. c. garganta*. Conversely, our current and previous results indicate that roe deer of Central and Southern Spain (our sample with the greatest size, $N = 52$) carried only haplotypes from the Southern Iberian haplogroup (Lorenzini & Lovari, 2006), and showed microsatellite genotypes that are highly divergent from those occurring in Northern Spain and in the rest of Europe (Lorenzini *et al*., 2003). This pattern of variation suggests that Southern Iberian populations belong to a phylogenetically distinct ancient lineage (cf. below), and, according to the ESU criterion (*sensu* Moritz, 1994), may deserve a subspecific status, possibly *C. c. garganta* (cf. Meunier, 1983).

Divergence times

Divergence times of European and Siberian populations, obtained from a coalescence method, indicated that most of the current intraspecific variation of haplotypes may have originated in the range of about 100–10 kya. Most of the variation probably accumulated before the Last Glacial Maximum (around 20 kya). The Southern Iberian lineage appears to be the most ancient within *C. capreolus*. It diverged from the other lineages on average some 92–46 kya. A similar phylogenetic pattern has been found in the Iberian red deer, where a Southern lineage diverged early, before a Northern lineage (Skog *et al*., 2009), possibly during a pre-glacial Pleistocene immigration route from Asia (cf. Ludt *et al*., 2004). It may represent the oldest clade of red deer in Europe (Skog *et al*., 2009).

The oldest lineages B and C of *C. pygargus* split on average 79–40 kya from the most recent lineage A, which included also haplotypes from Poland and Lithuania. This suggests that *C. pygargus* may have moved from Asia westward to Central Europe (cf. below), where at least one of its genetic lineages still survives and coexists with the resident *C. capreolus* lineages. Age estimates of demographic growth based on the mismatch distribution analyses in both *Capreolus* species have yielded divergence times that were slightly older than those obtained with a coalescent method. Two waves of population expansion may have occurred for the European roe deer, one ranging approximately from 122 to 61 kya (possibly in correspondence to the Eemian interglacial, 130 kya), and an earlier one, from 176 to 88 kya. Almost coeval with the earliest expansion of the European populations, Siberian counterparts may have experienced one main growth between 184 and 92 kya. Our results on divergence times are fairly consistent with those from other phylogenetic studies on the European roe deer (107–2 kya, Vernesi *et al*., 2002; 244–78 kya, Randi *et al*., 2004; 221–40 kya, Royo *et al*., 2007), and approximately congruent with those for the Siberian roe deer, for which greater divergence times have been obtained (370–190 kya, Randi *et al*., 1998). Age estimates should be taken with caution because of uncertainties associated with the use of tests to derive divergence times or population expansion, e.g. risk of underestimation due to recurrent mutations, typical of hypervariable regions of the mitochondrial control region. Stochastic factors, e.g. lineage sorting, as well as the use of substitution rates derived from phylogenetic studies, possibly inappropriate for population-level analyses, may lead to rough approximations of the timing of evolutionary events.

Biogeography and phylogeographical patterns

The genus *Capreolus* probably descended from the genus *Procapreolus*, based on skull, tooth, and antler morphologies (Groves, 2007). The transition seems to have occurred during the late Pliocene (Valli, 2010). About 3 Mya, the genus *Capreolus* originated in temperate areas where both its living species are distributed today, e.g. Western Trans-Baikal Russia (*C. pygargus*), and Moldavia–Slovakia (*C. capreolus*). Contemporary *C. pygargus* preserves many more primitive morphological traits than *C. capreolus* (Lister *et al*., 1998). Substantial genetic differences and partial reproductive isolation have developed since the split of the two species (Danilkin, 1996). About 10 kya, the two species seem to have occupied approximately their modern distribution ranges (Hufthammer & Aaris-Sørensen, 1998), i.e. *C. pygargus* mainly in Central Asia and *C. capreolus* in Europe (Danilkin, 1996). During the cold stages of the

Pleistocene *C. capreolus* was excluded from the northern regions of Europe (Sommer & Zachos, 2009). During these cold episodes, fossil remains and genetic data indicate the presence of roe deer in the Mediterranean region and several other Eastern European refuge areas (cf. Lorenzini & Lovari, 2006; Sommer *et al*., 2009). In the interglacials, roe deer populations recolonized Central–Northern Europe. Lister *et al*. (1998) reported that, at the last glaciation/interglacial transition, roe deer moved north accompanying the spread of woodland, shortly before the beginning of the present interglacial. Most probably, they retreated south during the final cold phase, and spread again northwards during the climatic amelioration at the end of the Younger Dryas cold snap, ~11.8 kya, which is when *C. c. italicus* appeared. There is evidence (Markova *et al*., 1995) suggesting that, in Asia, populations of *C. pygargus* behaved similarly to the European *C. capreolus*, i.e. moving southwards during cold episodes to return northwards during interglacials. These repeated movements must have considerably affected the genetic structure of populations.

Within the area of distribution of *C. pygargus* sampled for this study we found no evidence attesting to the existence of genetically distinct populations, not even in those of the putative subspecies *C. p. pygargus* and *C. p. tianschanicus*. According to our results (see also Xiao *et al*., 2007; Vorobieva *et al*., 2011), roe deer in Asia are genetically heterogeneous, but no geographical or subspecific patterns in either modern or ancient samples can be detected. Vorobieva *et al*. (2011) have suggested that the lack of natural barriers may have favoured gene flow and the mixture of different populations of roe deer in their Asian range. In contrast, our results confirm the existence of genetically different populations for *C. capreolus*, i.e. subspecies, in Central-southern Italy (*C. c. italicus*) and, in particular, Southern Spain. In the Iberian peninsula, the correspondence of the southern subspecies to *C. c. garganta* deserves confirmation. Unlike Central Asia, Southern Europe contains ecological barriers, e.g. mountain chains and peninsulas, which may explain why several populations of roe deer could develop independent genetic characteristics, as a result of isolation and genetic drift.

Our data show that *C. pygargus* matrilines are distributed farther west than is currently assumed, reaching East Poland and the Baltic coast. It might be a human-introduced species, but this seems to be ruled out by both mitochondrial and nuclear evidence. None of the Siberian haplotypes observed in Poland and Lithuania was recovered from Eastern European and Central Asian populations of *C. pygargus* (Xiao *et al*., 2007; Vorobieva *et al*., 2011; this study). If the introduction of Siberian genotypes into the European gene pool was relatively recent (e.g. 150–200 years), signature of admixture should be detectable also at microsatellite loci.

Relatively cold and dry climates characterize the present distribution of *C. pygargus*. It would make sense that *C. pygargus* had a more western distribution during the Late Pleistocene, as this was a period with cold and dry environments in Europe. The steppe biome (which *C. pygargus* inhabits today; Danilkin, 1996) stretched much farther to the west during the Late Pleistocene and many species, adapted to a cold and dry environment, had a more western distribution during this period (Stewart *et al*., 2010). Siberian roe deer may have colonized parts of north-eastern Europe, where they met *C. capreolus* populations that were arriving from the south. Being closely related and morphologically similar (*C. pygargus* is a third to a quarter larger and bears slightly larger antlers), its presence may simply have gone undetected until today, possibly also in the fossil records.

Introgression of *C. pygargus* haplotypes into *C. capreolus* through F1 females cannot be ruled out either, as indicated by the lack of differentiation at the nuclear level. This event would be consistent with Haldane's (1922) rule, which states that female hybrids are generally less infertile than male hybrids, although further sampling in the overlap area(s) of these species and the use of different types of genetic markers are needed to support our hypothesis.

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