Molecular Ecology (2004) **13**, 3071–3083 doi: 10.1111/j.1365-294X.2004.02279.x

Phylogeography of roe deer (*Capreolus capreolus*) populations: the effects of historical genetic subdivisions and recent nonequilibrium dynamics

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Abstract

We sequenced 704 mitochondrial DNA (mtDNA) control-region nucleotides and genotyped 11 autosomal microsatellites (STR) in 617 European roe deer (*Capreolus capreolus*) samples, aiming to infer the species' phylogeographical structure. The mtDNA sequences were split in three distinct haplogroups, respectively, named: Clade West, sampled mainly in Iberia; Clade East, sampled mainly in Greece and in the Balkans; and Clade Central, which was widespread throughout Europe, including the eastern countries and Iberia, but not Greece. These clades might have originated in distinct Iberian and Balkanic refuges during the penultimate or the last glaciations. Clades East and West contributed little to the current postglacial mtDNA diversity in central Europe, which apparently was recolonized mainly by haplotypes belonging to Clade Central. A unique subclade within Clade Central grouped all the haplotypes sampled from populations of the Italian subspecies *C. c. italicus*. In contrast, haplotypes sampled in central and southern Spain joined both Clade Central and Clade West, suggesting that subspecies *C. c. garganta* has admixed origin. STR data support a genetic distinction of peripheral populations in north Iberia and southern Italy, and show the effects of anthropogenic disturbance in fragmented populations, which were recently reintroduced or restocked and not may be in mutation–drift equilibrium. Roe deer in central Europe are mainly admixed, while peripheral populations in north Portugal, the southern Italian Apennines and Greece represent the remains of refugial populations and should be managed accordingly.

Keywords: *Capreolus capreolus*, European roe deer, glacial refuges, microsatellites, mitochondrial DNA control region, phylogeography, postglacial recolonization

Received 13 February 2004; revision received 4 June 2004; accepted 4 June 2004

Introduction

Natural factors have shaped the main genetic subdivisions in animal species with widespread distributions in Europe during Quaternary climate changes (Hewitt 2000). More recently, deforestation, the spread of agriculture, hunting and other kinds of human-induced disturbances have deeply affected the size, structure and dynamics of natural populations (Maehr *et al*. 2001; Harris *et al*. 2002). This is especially apparent in species of large mammals, which experienced dramatic fluctuations during the last few centuries, and that are either fragmented in threatened declining populations (i.e. many large carnivores; Breitenmoser 1998), or are very common, but intensively managed for hunting purposes (i.e. ungulates; Rhodes & Smith 1992). Genetic equilibrium cannot be attained over short time scales in fluctuating populations, and nonequilibrium factors could affect the estimates of genetic diversity, gene flow, or

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divergence (Hey & Machado 2003). Understanding the dynamics of geographical structuring in perturbed populations is important for evolutionary and conservation biology, but it is challenging, because equilibrium and nonequilibrium factors are not easily disentangled.

The roe deer (*Capreolus*, Artiodactyla, Cervidae) includes two species, the smaller European *C. capreolus* (distributed in western Europe), and the larger Siberian *C. pygargus* (distributed in Asia and eastern Europe) roe deer (Danilkin 1996; Randi *et al*. 1998). The European roe deer is widespread across the continent (Fig. 1), with populations distributed in former Mediterranean glacial refuges (Sierra de Cadiz in southern Spain, southern Apennines in Italy, southern Balkans), and in northern regions (Scandinavia) or mountain ranges (the Alps and Pyrenees) that were glaciated until the Holocene (*c*. 10 000 years ago). Roe deer are highly adaptable, living in broad-leaved forests, ecotonal strips and agricultural areas, in mountains or in lowland regions (Danilkin 1996). However, in Scandinavia the roe deer is absent beyond the Arctic circle, suggesting that habitats where there is permafrost could limit its northward distribution. Hence, predictably, cyclic expansion of permafrost and the concomitant contraction of forests in central Europe, could have forced roe deer populations to retreat to southern refuges, while deglaciations and the expansion of forests during the Holocene have certainly fostered roe deer's colonization of the Alps, Pyrenees, central Europe and Scandinavia.

Deforestation and over-hunting led to decline and eradication of local roe deer populations, particularly in central and south Iberia (Boutin 1990; Aragón *et al*. 1995a), in the western Italian Alps and Apennines (Perco & Calò 1994), and in Greece (Adamakopoulos *et al*. 1991). After World War II some populations began to expand naturally, following the expansion of forests in mountains, or colonizing agricultural lowlands (Tellería & Virgós 1997; Albanis *et al*. 2000). Population decline was successfully hindered by reintroductions, which were often carried out using nonindigenous roe deer. All extant populations in the western Italian Alps and many populations in the northern Apennines were reintroduced or restocked in the last few decades (Perco & Calò 1994).

The roe deer is massively hunted and managed in all European countries, except in Greece and Portugal. A prerequisite for adequate conservation and management plans is the correct definition of the taxonomic rank of populations (Moritz 1994). However, subspecies distinction in roe deer is uncertain. Nowadays *C. capreolus* is considered a monotypic species (Danilkin 1996), although two subspecies were described in the past: the Italian roe deer *C. c. italicus* (Festa 1925), distributed in southern Italy, and the Spanish roe deer *C. c. garganta* (Meunier 1983), from central–southern Spain. The identity of the Spanish subspecies is doubtful (Aragón *et al*. 1995b), but Lorenzini *et al*. (2003) suggested a genetic differentiation between Andalusian and northern roe deer populations in Spain. Populations of *C. c. italicus* showed unique mitochondrial DNA (mtDNA) haplotypes (Randi *et al*. 1998; Vernesi *et al*. 2002), distinctive microsatellite genotypes (Randi & Mucci 2001; Lorenzini *et al*. 2002), and skull morphometry (Montanaro *et al*. 2003).

Patterns of geographical diversification in roe deer are poorly known. Published research used limited geographical sampling, and maternally inherited mtDNA sequences (Randi *et al*. 1998; Wiehler & Tiedemann 1998; Vernesi *et al*. 2002). In this study, we have investigated mtDNA and simple tandem repeats (STR) genetic diversity in roe deer populations across the species distribution range, including the nominate species and both subspecies. We aimed to: (i) assess whether maternal mtDNA and biparental STR markers allow description of concordant population subdivisions and processes, and identification of extant genetic units; and (ii) infer the consequences of natural and anthropogenic factors in shaping the observed population structure.

Materials and methods

Sample collection

A total of 773 tissue samples were collected and stored at −20 °C in 95% ethanol. They were taken from 44 localities in Europe (Fig. 1a), including natural, reintroduced or restocked populations (Table 1). All of these samples were used in phylogenetic analyses. Smaller sample sizes in population genetic analyses were avoided by pooling samples (which were not significantly differentiated) in three distinct groups: north Spain (Galicia and Asturias), Germany (Westerwald and Bayreuth) and south Italy (Castelporziano, Gargano and Orsomarso). Samples from central (Madrid region, $n = 6$) and south Spain (Cadiz; $n = 5$), from Novi Bečej — Novo Miloševo (Serbia; $n = 6$), and a population that was reintroduced from Slovenia in Val Susa (Italy, Torino; $n = 3$) were not used. Thus, in population genetic analyses there were 36 distinct groups, each one with $n \geq 10$ (Table 2). Sample no. 42 was collected from the original location of the Italian roe deer subspecies *Capreolus capreolus italicus* (Castelporziano, a Mediterranean forest close to Rome). Samples no. 43 and no. 44 were collected from the other two Italian roe deer populations surviving in protected areas in southern Italy (Parco Nazionale del Gargano, Monti dell'Orsomarso; Fig. 1). Throughout this paper this group will be known as '*C. c. italicus*'. Samples no. 4 and no. 5 were collected within the distribution range of *C. c. garganta* in central (Madrid region) and southern (Cadiz) Spain.

Laboratory methods

Total DNA was extracted following Gerloff *et al*. (1995). The entire mtDNA control-region was successfully amplified

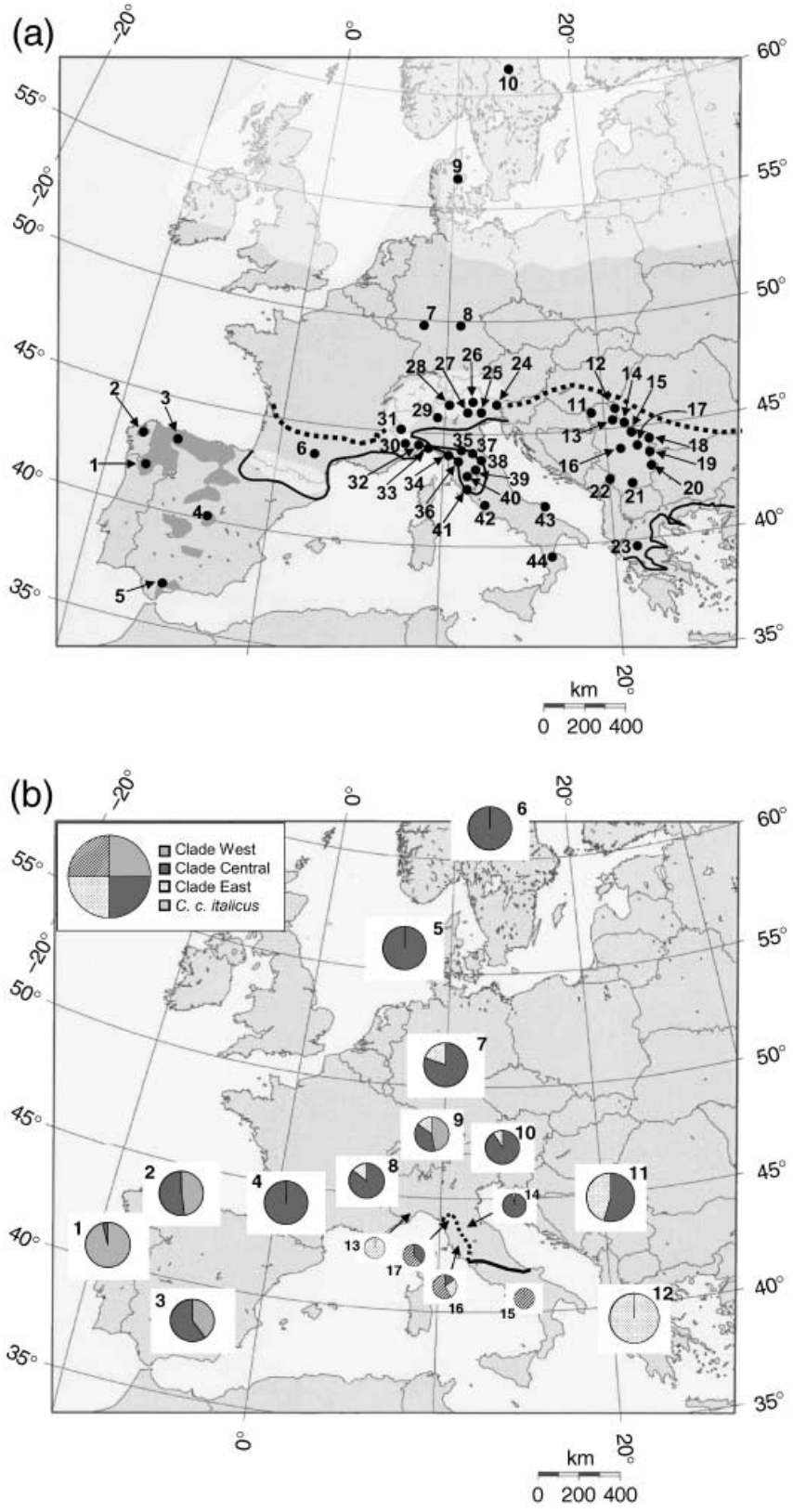


Fig. 1 (a) Distribution of roe deer (*Capreolus capreolus*) in Europe and sampling locations. The continuous black lines indicate the approximate southern limits of the main distribution range of roe deer. Fragmented populations in Iberia and southern Italy are indicated by dark areas and sampling locations. The interrupted lines indicate the southernmost limit of permafrost at the last glacial maximum. The white areas indicate the Fennoscandian, Pyreneean and Alpine ice caps at the last glacial maximum. The locations of the sampled populations are indicated by dots and arrows, and numbered as in Table 1. (b) Proportion of mtDNA clades in regional groups of sampled roe deer populations. The diagrams indicate the proportions of mtDNA control region haplotypes belonging to Clade West, Central, East and '*C. c. italicus*' in groups: 1 = Portugal; 2 = north Spain (Galicia, Asturias); 3 = central and south Spain (Segovia, Toledo, Cadiz); 4 = France; 5 = Denmark; 6 = Sweden; 7 = Germany; 8 = western Italian Alps (Cuneo, Torino); 9 = central Italian Alps (Sondrio, Brescia, Lecco); 10 = eastern Italian Alps (Belluno, Treviso, Asiago, Vicenza); 11 = Serbia, Montenegro, Kosovo; 12 = Greece; 13 = northwestern Apennines (Savona); 14 = northeastern Apennines (Bologna, Firenze, Forlì, Cesena); 15 = populations of *C. c. italicus* (Castelporziano, Gargano, Orsomarso); 16 = south Tuscany (Grosseto, Siena); 17 = north Tuscany (Arezzo, Pistoia, Massa Carrara) and Modena.

Table 1 Sampling locations of roe deer (*Capreolus capreolus*) used in this study

Region	Locality*	Sample size	Origin of populations†
Portugal	1 — Northern Portugal	27	Natural
Spain	2 — Galicia	19	Natural
	3 — Asturias (Oviedo, Leon)	30	Natural
	4 — Madrid (Segovia, Toledo)	6	Natural
	5 — Cadiz	5	Natural
France	6 — Tolouse	18	Natural
Germany	7 — Westerwald	10	Natural
	8 — Bayreuth	10	Natural
Denmark	9 — Kaloe	12	Restocked
Sweden	10 — Uppsala	10	Natural
Serbia	11 — Bački Monoštor	10	Natural
	12 — Novi Kneževac	20	Natural
	13 — Ada Bečej	27	Natural
	14 — Novi Bečej, Novo Miloševo	6	Natural
	15 — Deliblatska Peščara	14	Natural
	16 — Stragari (Kragujevac)	30	Natural
	17 — Petrovac na Mlavi, Svilajnac	25	Natural
	18 — Severni Kučaj	17	Natural
	19 — Negotin	15	Natural
	20 — Stara planina	27	Natural
Kosovo	21 — Kosovo (Priština)	13	Natural
Montenegro	22 — Montenegro (Berane)	11	Natural
Greece	23 — Epirus (Zagori)	16	Natural
Italy Alps	24 — Belluno	12	Natural
	25 — Treviso	14	Natural
	26 — Asiago	22	Natural
	27 — Vicenza	20	Natural
	28 — Sondrio, Brescia	15	Natural, restocked
	29 — Lecco	19	Natural, restocked
	30 — Cuneo	19	Natural, restocked
	31 — Torino (Val di Susa)	3	Reintroduced
Italy Apennines	32 — Alessandria	14	Reintroduced
	33 — Savona	43	Reintroduced
	34 — Massa Carrara	42	Natural, restocked
	35 — Modena	15	Natural, restocked
	36 — Pistoia	20	Natural, restocked
	37 — Bologna, Firenze	27	Natural, restocked
	38 — Forlì, Cesena	14	Natural, restocked
	39 — Arezzo	19	Natural, restocked
	40 — Siena	43	Natural
	41 — Grosseto	25	Natural
Italy *C. c. italicus*	42 — Castelporziano	15	Natural
	43 — Gargano	8	Natural
	44 — Orsomarso	3	Natural

*Geographic locations of the sampled populations are mapped in Fig. 1(a).
†Populations are ranked as: 'natural' for populations that were never officially restocked (undocumented restocking cannot be excluded); 'restocked' for populations that were officially restocked; 'reintroduced' for populations that were completely reintroduced in an area where indigenous roe deer were recently extirpated.

by polymerase chain reaction (PCR) in 728 roe deer samples using the external primers LcapPro and HcapPhe (Randi *et al*. 1998). Automatic sequencing of 704 nucleotides from the left and right sides of the mtDNA control region was performed, using the two PCR primers and the internal primers Lcap362 and Hcap493 (Randi *et al*. 1998). Sequences were aligned with a control region sequence of European roe deer (access no. Z70318; Douzery & Randi 1997), and unique haplotypes were identified using COLLAPSE 1.0 (D. Posada; http://bioag.byu.edu/zoology/crandall_lab/programs.htm). From each of the 36 sampled groups 617 roe deer were randomly selected; these were genotyped by

Table 2 Estimates of gene diversity at mitochondrial DNA (mtDNA) control region and 11 microsatellite loci (STR) in roe deer

Group*	Region and population	mtDNA†				STR†			
		(n_i/n_h)	h	k	$\theta_{(s)}$	n_i	H_O	H_E	A
1	N. Portugal	27/10	0.83 (0.04)	5.4 (2.7)	5.3 (2.4–11.2)	16	0.48	0.57	4.7
2	N. Spain	46/10	0.82 (0.04)	6.9 (3.3)	3.5 (1.7–7.2)	21	0.60	0.65	6.5
3	France	18/11	0.94 (0.04)	4.1 (2.1)	11.0 (4.6–26.8)	13	0.61	0.79*	6.4
4	Germany	20/13	0.91 (0.05)	4.5 (2.3)	15.0 (6.5–35.5)	10	0.67	0.72	5.6
5	Denmark	12/6	0.80 (0.09)	4.8 (2.5)	4.1 (1.4–11.5)	10	0.55	0.69	4.4
6	Sweden	10/4	0.71 (0.12)	2.7 (1.6)	1.9 (0.6–6.1)	10	0.60	0.64	4.1
7	Serbia 11	10/8	0.96 (0.06)	7.5 (3.8)	16.4 (4.9–59.0)	10	0.75	0.72	5.0
8	Serbia 12	20/9	0.80 (0.09)	4.5 (2.3)	5.7 (2.4–13.2)	11	0.58	0.66	5.0
9	Serbia 13	27/14	0.94 (0.02)	6.1 (3.0)	11.0 (5.3–22.8)	12	0.72	0.71	5.9
10	Serbia 15	14/7	0.85 (0.07)	6.3 (3.2)	4.9 (1.8–12.9)	12	0.64	0.59	4.4
11	Serbia 16	30/20	0.96 (0.02)	7.6 (3.6)	25.1 (12.3–51.9)	14	0.70	0.71	6.0
12	Serbia 17	25/18	0.97 (0.02)	7.0 (3.4)	27.4 (12.5–62.3)	10	0.59	0.68	5.4
13	Serbia 18	17/13	0.96 (0.03)	8.2 (4.0)	23.4 (9.0–64.6)	12	0.64	0.70	5.6
14	Serbia 19	15/13	0.97 (0.04)	7.0 (3.5)	43.1 (13.7–149.0)	13	0.64	0.67	5.8
15	Serbia 20	27/17	0.94 (0.03)	6.9 (3.4)	18.7 (9.0–39.5)	11	0.54	0.60	5.3
16	Kosovo 21	13/10	0.92 (0.07)	5.1 (2.6)	18.0 (6.2–55.7)	10	0.69	0.60	3.4
17	Montenegro 22	11/9	0.96 (0.05)	7.0 (3.6)	20.7 (6.3–73.8)	10	0.74	0.69	4.8
18	Greece 23	16/4	0.44 (0.14)	3.0 (1.6)	1.4 (0.4–3.9)	24	0.68	0.53*	4.4
19	Italy Alps 24	12/6	0.76 (0.12)	5.0 (2.6)	4.1 (1.4–11.5)	11	0.67	0.74	5.5
20	Italy Alps 25	14/4	0.39 (0.16)	2.4 (1.4)	1.5 (0.4–4.4)	12	0.70	0.70	5.0
21	Italy Alps 26	16/4	0.35 (0.15)	1.2 (0.8)	1.4 (0.4–3.9)	22	0.65	0.58	4.7
22	Italy Alps 27	20/4	0.28 (0.13)	2.3 (1.3)	1.2 (0.4–3.4)	19	0.67	0.66	4.9
23	Italy Alps 28	15/10	0.94 (0.04)	7.0 (3.5)	12.0 (4.6–31.9)	15	0.71	0.75	6.4
24	Italy Alps 29	19/5	0.62 (0.10)	4.5 (2.3)	1.9 (0.7–4.9)	18	0.68	0.73	5.9
25	Italy Alps 30	17/4	0.65 (0.09)	4.0 (2.1)	1.3 (0.4–3.7)	19	0.69	0.72	6.2
26	Apennines 32	14/4	0.49 (0.15)	2.4 (1.4)	1.5 (0.5–4.4)	11	0.67	0.70	5.1
27	Apennines 33	27/4	0.62 (0.07)	2.8 (1.5)	1.0 (0.3–2.8)	43	0.61	0.65	6.6
28	Apennines 34	23/3	0.42 (0.10)	2.3 (1.3)	0.7 (0.2–2.1)	42	0.52	0.53	4.7
29	Apennines 35	15/4	0.60 (0.11)	5.1 (2.6)	1.4 (0.5–4.1)	15	0.65	0.67	5.1
30	Apennines 36	10/3	0.30 (0.18)	3.3 (1.9)	1.0 (0.3–3.6)	20	0.70	0.68	5.1
31	Apennines 37	26/4	0.62 (0.06)	3.3 (1.7)	1.1 (0.3–2.9)	27	0.56	0.59	4.4
32	Apennines 38	13/3	0.59 (0.12)	2.5 (1.4)	0.9 (0.2–2.9)	14	0.67	0.60	3.7
33	Apennines 39	18/3	0.31 (0.13)	0.5 (0.4)	0.7 (0.2–1.4)	19	0.56	0.58	4.1
34	Apennines 40	43/5	0.49 (0.08)	1.5 (0.9)	1.2 (0.5–3.0)	33	0.57	0.66	6.5
35	Apennines 41	24/4	0.71 (0.04)	3.2 (1.7)	1.1 (0.4–3.0)	25	0.69	0.73	6.0
36	*C. c. italicus*	24/4	0.56 (0.09)	1.6 (1.0)	1.1 (0.4–3.0)	23	0.62	0.63	4.4
	Average	19.7	0.97	7.9	63.7	17.2	0.63	0.77	14.1
	(SD)	(8.4)	(0.01)	(3.7)	(52.7–76.9)	(8.8)	(0.11)	(0.11)	14.1

For group composition, see: Table 1 and Materials and Methods.

n_i = number of genotyped individuals; n_h = number of observed mtDNA haplotypes; h = haplotype diversity; k = average pairwise sequence divergence; $\theta_{(s)} = 2N_e m$ (N_e = effective population size; m = mutation rate of the haplotype), computed from the number of segregating sites (Tajima 1983); H_O = observed heterozygosity; H_E = unbiased expected heterozygosity; A = mean number of alleles per locus. Standard deviations (SD) are in brackets.

*Significant ($P < 0.001$) departures from Hardy–Weinberg equilibrium, as estimated using F_{IS}. Critical levels in simultaneous tests of significance were adjusted using Hochberg's procedure (Legendre & Legendre 1998: p. 18), as implemented in ADJUSTED-P-VALUES (P. Legendre; http://www.fas.umontreal.ca/biol/legendre).

PCR amplifications (annealing temperature 55 °C) of 11 microsatellites: NVHRT16, NVHRT21, NVHRT24, NVHRT71 (Roed & Midthjell 1998), BMC1009, OarFCB304 (Talbot *et al*. 1996), ILSTS058, ILSTSO11 (Kemp *et al*. 1995), mcM505 (Hulme *et al*. 1995), OarH51 (Pierson *et al*. 1994), and RT1 (Wilson *et al*. 1997). The PCR products were analysed automatically using an ABI 3100 sequencer and the programs GENESCAN3.7 and GENOTYPER2.1 for microsatellites, or SEQUENCING ANALYSIS 3.7 and SEQSCAPE 1.1 for sequences. Details of laboratory protocols are available upon request.

Analyses of the mtDNA sequences

Phylogenetic trees were reconstructed using MEGA 2.1 (Kumar *et al*. 2001; http://www.megasoftware.net/), with the neighbour-joining procedure (Saitou & Nei 1987) and Tamura and Nei's TN93 genetic distance model (Tamura & Nei 1993), which is appropriate to describe the evolution of control region sequences. Trees were rooted using homologous control region sequences of Siberian roe deer (Douzery & Randi 1997), and support for the internodes was assessed after 10 000 bootstrap resampling steps (BP; Felsenstein 1985). The complete alignment ($n = 161$ haplotypes) was analysed and a reduced data set ($n = 81$ haplotypes) was obtained by excluding all singletons (i.e. the 80 haplotypes that were sampled only once).

Networks are better suited than phylogenetic methods to infer haplotype genealogies at the population level because they explicitly allow for extant ancestral sequences and alternative connections (Bandelt *et al*. 1999). We used both the complete and reduced alignments with the median-joining network procedure (Bandelt *et al*. 1999), implemented in NETWORK 3.1.1.1 (http://www.fluxus-technology.com/). We used the 'star contraction algorithm' in NETWORK to collapse star-like clusters to a smaller number of representative single sequences, with the aims: (i) to display the inner structure of the network; and (ii) to identify those star-like clusters that represent events of demographic expansion (Forster *et al*. 2001).

Haplotype diversity (h), average pairwise nucleotide substitutions (k), nucleotide diversity (π) and analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) with Φ-analogues of Wright's (1965) F-statistics, were computed using ARLEQUIN (Schneider *et al*. 2002; http://anthropologie.unige.ch/arlequin). AMOVA was performed first using only haplotype frequency differences among populations, then including sequence divergence among haplotypes (estimated by TN93 distances). Mismatch distributions were analysed using the sudden expansion model (Rogers & Harpending 1992), and goodness-of-fit tests (sum of squared deviations, *SSD*; Harpending's raggedness index, R; Schneider & Excoffier 1999) of the observed to the estimated mismatch distributions were computed. Assumption of neutrality of mutations was tested by Tajima's D (Tajima 1989) as implemented in DNASP 3.99 (Rozas *et al*. 2003). DNASP was used also to estimate $\theta_{(s)} = 2N_e m$ (N_e = effective population size; m = mutation rate of the haplotype), computed from the number of segregating sites (Tajima 1983).

Microsatellite diversity within and among populations

Commonly used estimates of genetic diversity (heterozygosity H, and number of alleles A) were computed for each locus and population using GENETIX 4.03 (Belkhir *et al*. 2001; http://www.University-montp2.fr/~genetix/genetix.htm). Deviations from Hardy–Weinberg equilibrium for each locus and each population, and across loci and populations, were assessed using GENEPOP 3.2a (Raymond & Rousset 1995). ARLEQUIN was used to compute AMOVA with F_{ST} analogues. Patterns of differentiation were visualized by factorial correspondence analysis (CA; Benzecri 1973) of population multilocus scores computed using GENETIX.

Results

Mitochondrial DNA sequences

The mtDNA alignment (728 individuals, 704 nucleotides, outgroups excluded), showed 161 haplotypes (GenBank accession nos AY625732–AY625892), defined by 70 polymorphic sites including 69 substitutions (62 transitions and seven transversions) and two insertions/deletions. Mitochondrial DNA diversity was high in roe deer (Table 2), which showed on average one distinct haplotype over 4.5 individuals (728/161 = 4.5). Haplotypes were distributed evenly, each one having a frequency lower than 9% in the total sample. Haplotype diversity was high ($h = 0.971 \pm 0.002$, standard deviation), but nucleotide diversity ($\pi = 0.011 \pm 0.006$) and pairwise divergence ($k = 7.89 \pm 3.67$) were small, suggesting that roe deer populations had historically large effective size (N_e), but that extant mtDNA lineages originated recently. Tajima's neutrality test, computed from the number of segregating sites, was negative ($D = -0.52$) and not significant ($P > 0.10$).

Mismatch analyses supported a pattern of demographic expansion. The goodness-of-fit tests were not significant (*SSD* = 0.0041, $P = 0.20$; $R = 0.0051$, $P = 0.66$). The main expansion event occurred at $\tau = 8.6$ (lower bound $\tau = 4.3$, upper bound $\tau = 15.3$; a level = 0.05), and involved a population change from an initial $\theta = 1.06$ (0.00–7.16), to a final $\theta = 139.06$ (19.69–535.66). However, the mismatch plot was bimodal (not shown), with peaks at $\tau = 7$ and 11, suggesting the expansion of two distinct mtDNA clades.

Mitochondrial DNA diversity was higher in roe deer sampled from eastern countries (Serbia, Montenegro and Kosovo), than elsewhere in Europe (Table 2). Values of $\theta_{(s)}$ in samples from central–northern Europe, eastern Europe, Italian Alps and Apennines were significantly different ($P = 0.0036$; Friedman's test). In particular, $\theta_{(s)}$ was significantly higher in samples from Serbia, than in samples from the Italian Alps ($P = 0.0063$) and Apennines ($P = 0.0007$; paired t-test). Eastern European roe deer also showed the highest values of haplotype diversity, which was significantly different from h in the Alpine ($P = 0.0037$; paired t-test) and Apennine samples ($P = 0.0001$), but not significantly different from the other sampled populations in Europe ($P = 0.1834$). Genetic variation was significantly partitioned among the 36 groups, with $\Phi_{ST} = 0.44$ (estimated

using AMOVA and TN93 distances; $P = 0.00000$), or $F_{ST} = 0.26$ (using *F*-statistics computed from haplotype frequencies; $P = 0.00000$). The contribution of genetic distances increased the *F*-value, suggesting that genetically distinct mtDNA haplotypes are distributed in different geographical locations, and that, on average, haplotypes sampled within the same location are more similar to each other than to those found in other locations.

Phylogenetic relationships among the mtDNA haplotypes

Neighbour-joining trees, computed with the complete or the reduced alignments and TN93 distances, grouped all the European roe deer haplotypes in a clade (supported by BP = 100%), which was split into two main haplogroups (BP < 50%; Fig. 2 shows the neighbour-joining tree computed with the reduced data set of 81 haplotypes). A first haplogroup, thereafter named Clade East, was composed mainly by haplotypes sampled from Serbia, Montenegro and Kosovo, and included all the haplotypes sampled in Greece, plus six haplotypes collected in the Italian Alps and northern Apennines, and in Germany. These haplotypes did not form distinct subclades, but were nested within clusters of Serbian haplotypes.

A second haplogroup was split into two distinct lineages, respectively, named Clade West and Clade Central (Fig. 2). Clade West included sequences that were sampled only in Portugal and Spain, except for a subclade with three haplotypes that were collected in the central Italian Alps (sampling locations no. 28 and no. 29 in Fig. 1). Haplotypes in Clade Central were widespread in central and north Europe and Italy, as well as in east Europe (including Serbia, but not Greece) and in west Europe (including Spain and Portugal). A distinct subclade joined all the haplotypes from populations of *Capreolus capreolus italicus* (Castelporziano, Gargano and Orsomarso), as well as other samples collected from neighbouring central and southern Apennine localities.

Average interhaplotype sequence divergence was small (1.1% TN93 distance), the number of haplotypes (161) was greater than parsimony informative sites (41), and strong bootstrap support was not expected in neighbour-joining trees (Smouse 1998). Nevertheless the mtDNA alignment contained a significant phylogenetic signal, as indicated by the analysis of 100 000 random trees (generated by PAUP*; Swofford 2002), which showed a skewed distribution of tree length, and a significant value of the statistics $g_1 = -0.16$ ($P = 0.01$; see Table 2 in Hillis & Huelsenbeck 1992).

Network analyses and geographical distribution of the mtDNA haplotypes

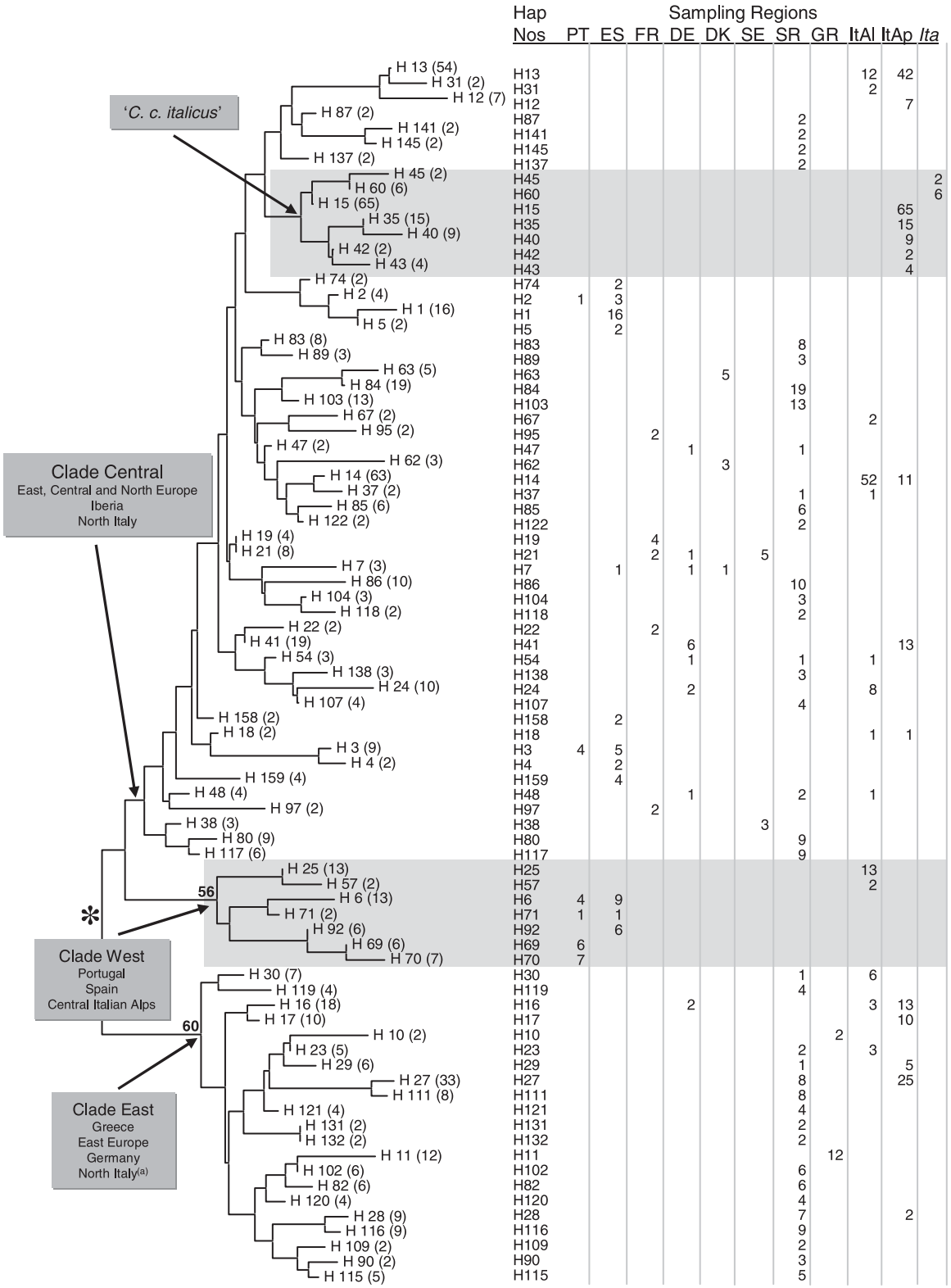
Networks computed using the complete or reduced data set were very reticulated, but concordantly split the haplotypes in three main groups, corresponding to Clade East, West and Central. Three rounds of star contraction with the star contraction algorithm in NETWORK, performed using the entire data set and a mutational distance radius $\delta = 5$, allowed the initial 161 sequences to be collapsed into a final set of 96 sequences, which were used to construct a median-joining network. The star-contracted network (Fig. 3) supported the distinction of the three main clades, collapsed the Alpine haplotypes in Clade West in a single sequence (H56), collapsed the nine haplotypes of '*C. c. italicus*' in three sequences (H35, H42 and H15), and supported the existence of a distinct mtDNA clade in Iberian roe deer joining Clade Central, which included the other Iberian mtDNA haplotypes.

The geographical distribution of the haplotypes is synthesized in Fig. 1(b). Pie diagrams showed that populations sampled in Europe have different compositions, and that control-region clades have restricted geographical distributions. Haplotypes of '*C. c. italicus*' were sampled only in neighbouring locations in central Apennines (locations 34, 35, 36, 40 and 41 in Tuscany and Emilia-Romagna; see Fig. 1), however, well beyond (stippled line in Fig. 1b) the putative southern distribution of *C. c. italicus* (continuous line in Fig. 1b).

Microsatellite diversity and geographical population structure

All the microsatellite loci were polymorphic, showing an average of 14.1 alleles per locus in the total sample ($n = 617$), and from 3.4 alleles (in roe deer from Kosovo) to 6.6 alleles (in a reintroduced population sampled from location 33) in the local samples. The difference in the average number of alleles per locus in the total or in the local samples indicates that distinct alleles are differentially distributed in the sampled populations. The total sample was not in Hardy–Weinberg equilibrium ($P < 0.000$; multilocus multipopulation test), confirming that populations are genetically distinct. The local populations were in Hardy–Weinberg equilibrium, excluding samples from France and Greece ($P < 0.001$). Heterozygosity and the average number of alleles per locus did not show any detectable geographical trend (Table 2). However, microsatellite variability was significantly partitioned among the 36 groups ($F_{ST} = 0.16$; $P < 0.0000$; AMOVA).

The roe deer sampled from the different regions were distributed in distinct areas of the CA plot (Fig. 4), suggesting geographical subdivisions. Roe deer sampled from eastern Europe and from the Italian Alps were partially admixed in the central part of the CA plot, while samples collected from the geographical peripheries (central and southern Apennines, north Iberia) were distinct, plotting, respectively, towards the left and lower sides of the distribution. This plot shows that the central cluster of samples



Hap Nos	PT	ES	FR	DE	DK	SE	SR	GR	ItAl	ItAp	*Ita*
H13									12	42	
H31									2		
H12										7	
H87							2				
H141							2				
H145							2				
H137							2				
H45											2
H60											6
H15										65	
H35										15	
H40										9	
H42										2	
H43										4	
H74		2									
H2	1	3									
H1		16									
H5		2									
H83							8				
H89							3				
H63					5						
H84							19				
H103							13				
H67									2		
H95			2								
H47				1			1				
H62					3						
H14									52	11	
H37							1		1		
H85							6				
H122							2				
H19			4								
H21			2	1		5					
H7		1		1	1						
H86							10				
H104							3				
H118							2				
H22			2								
H41				6						13	
H54				1			1		1		
H138							3				
H24				2					8		
H107							4				
H158		2									
H18									1	1	
H3	4	5									
H4		2									
H159		4									
H48				1			2		1		
H97			2								
H38						3					
H80							9				
H117							9				
H25									13		
H57									2		
H6	4	9									
H71	1	1									
H92		6									
H69	6										
H70	7										
H30							1		6		
H119							4				
H16				2					3	13	
H17										10	
H10								2			
H23							2		3		
H29							1			5	
H27							8			25	
H111							8				
H121							4				
H131							2				
H132							2				
H11								12			
H102							6				
H82							6				
H120							4				
H28							7			2	
H116							9				
H109							2				
H90							3				
H115							5				

Fig. 2 Neighbour-joining tree of roe deer mtDNA control region haplotypes, computed using TN93 genetic distances and a reduced alignment of 80 haplotypes (singletons excluded). The trees were rooted using homologous Siberian roe deer sequences (position of the root indicated by an asterisk). The main clades are labelled and bootstrap percentages are indicated at the main internodes. The table on the right shows the geographical distribution of these haplotypes (PT = Portugal; ES = Spain; FR = France; DE = Germany; DK = Denmark; SE = Sweeden; SR = Serbia, Montenegro, Kosovo; GR = Greece; ItAl = Italian Alps; ItAp = Italian Apennines; *Ita* = *C. c. italicus*).

from Serbia, Kosovo and Montenegro includes also the roe deer from Greece and the samples collected from the two populations that were reintroduced in the western Italian Alps (Alessandria and Savona) using roe deer from eastern Europe or eastern Alps. Roe deer from central and south Apennines plot towards the left side of the CA, differentiating mainly along FC-I (which explains 13.01% of the total genetic variation), while roe deer sampled from the northern Apennines (Forlì, Cesena, Bologna, Firenze, Arezzo) plot towards the upper right part of CA. Samples collected from *C. c. italicus* and neighbouring areas also plot towards the left side of the CA. Samples collected from western (Iberia, France) and northern Europe (Sweden) plot towards the lower right part of the CA, differentiating along FC-II (which explains 11.38% of the total genetic variation.

Discussion

Global phylogeographical patterns in roe deer

Phylogenetic trees and networks identify three main mtDNA haplogroups, which could have originated in Iberia (Clade West and perhaps Central) or in the Balkans (Clade East). Clade West apparently contributed little to the current mtDNA diversity in central Europe, which is mainly because of the widespread distributions of Clade East and Central. Some Clade West haplotypes were sampled in the Italian Alps, suggesting a postglacial colonization route from Iberia towards the Mediterranean coasts, or the presence of ancestral haplotypes in the Alps. The restricted distribution of Clade East haplotypes supports the existence of an eastern glacial refuge, from which, however, roe deer did not disperse extensively westward. In contrast, the widespread distribution of Clade Central haplotypes in the Balkans, central and north Europe, Apennines, Alps (although some haplotypes could have been translocated in the western Alps), and Iberia, supports the existence of distinct refugial populations that contributed extensively to the recolonization of Europe. These findings indicate that roe deer dispersed in Europe from multiple refuges, not from a single western Mediterranean refuge, as previously suggested (Wiehler & Tiedemann 1998).

Phylogenetic trees (Fig. 2) and networks (Fig. 3), and the mismatch distributions showed instances of two main expansions, respectively, at $\tau = 11$ and $\tau = 7$ mutations. Assuming, in a very simple way, that divergence time is $T = \tau/2\mu$, with $\mu = \lambda g$ (i.e. the rate of annual substitution per haplotype $\lambda = 0.04–0.08 \times 10^{-6} \times 750$ nucleotides, per generation time $g = 3$ years; Randi *et al*. 1998), coalescent times can be estimated to range from 122 000 to 244 000 years for the oldest, and from 78 000 to 156 000 years for the most

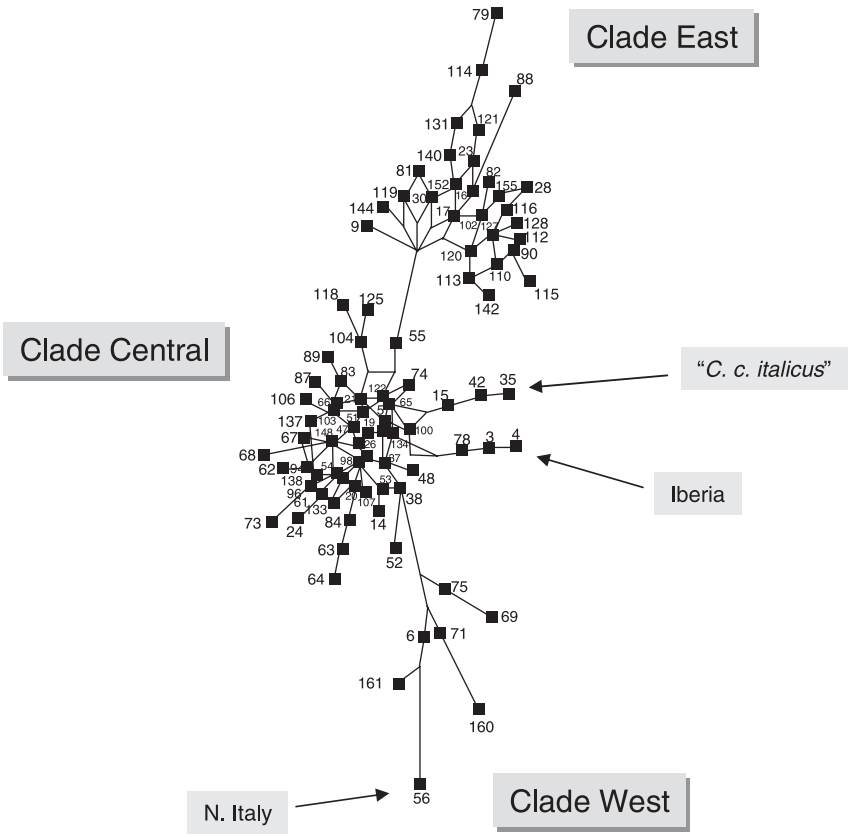


Fig. 3 Star-contracted median-joining network computed using NETWORK (with default options and all sites equally weighted), and the entire data set of 161 haplotypes. Haplotypes are indicated by numbers, and circles are not proportional to frequencies. The main clades preserved after star-contraction are labelled.

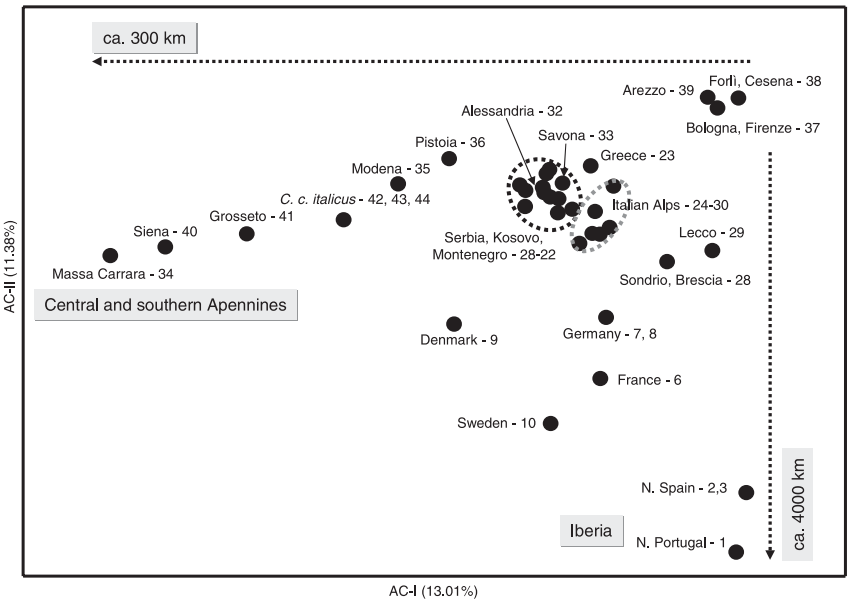


Fig. 4 Factorial correspondence analysis (CA) of population multilocus scores computed using GENETIX. Multilocus scores are plotted in the bivariate space defined by the first two factorial components (CA-I and CA-II). Locations of individuals sampled from the main geographical regions are labelled. Sampled groups are numbered as in Fig. 1 and Table 1. The dotted ellipses were drawn by hand to indicate populations sampled in the Balkans and in the Italian Alps. The arrows indicate the approximate linear geographical distances between populations plotting, respectively, towards the upper left or lower right corners of the CA space.

recent expansions, respectively (in agreement with Randi *et al*. 1998; Vernesi *et al*. 2002). These dates, which might be underestimated because of recurrent mutations at hypervariable control region sites, could identify two waves of population expansion, respectively, during the penultimate (*c*. 200 000 years ago), and the last (*c*. 130 000 years ago) interglacials. Extensive mtDNA and STR genetic diversity suggests that roe deer populations did not suffer any recent global strong bottleneck. The negative value of Tajima's neutrality test, which might reflect the effects of population histories, can result from postglacial population expansions.

During glacial periods, permafrost and Arctic tundra ecosystems were widespread in central Europe down to a latitude of 45° (Andersen & Borns 1997). Temperate animal populations were forced to survive in fragmented southern refuges, following the range shifts of broad-leaved and Mediterranean forests. A prevalent eastward roe deer colonization of central Europe correlates with patterns of broad-leaf forest expansion from the east, as inferred both from fossil pollen and molecular data sets (Petit *et al*. 2003). Alternatively, divergent mtDNA lineages could have evolved in Iberia, thereafter spreading towards central Europe and Italy following the postglacial colonization routes that were documented in other species (Taberlet *et al*. 1998). Additional sampling in France and Germany is needed to assess the distribution of Iberian haplotypes in central Europe, and locate the geographical origin of Clade Central in Iberia or in the Balkans. The original populations in western Alps and Apennines have been eradicated and were recently reintroduced using roe deer from eastern Alps and east Europe. Hence, only museum samples could provide support to the existence of southwestern or eastern colonization routes.

Local diversification and subspeciation in peripheral populations

Phylogenetic trees and networks identify a clade of haplotypes with restricted geographical distribution in the central and southern Apennines, within the range of *Capreolus capreolus italicus*. The star-contraction analysis preserved this clade, thus identifying an event of demographic expansion of phylogenetically related populations that are currently distributed in the central and southern Italian Apennines. The average mtDNA sequence divergence among haplotypes in clade '*C. c. italicus*' is low ($d = 0.36\%$), indicating a recent coalescence. The origin of this clade is the likely consequence of population isolation/expansion in a southern Italian refuge during the last glacial maximum/early Holocene. Mitochondrial, STR and morphometric data (Randi *et al*. 1998; Randi & Mucci 2001; Lorenzini *et al*. 2002; Vernesi *et al*. 2002; Montanaro *et al*. 2003) indicate that these vicariant populations evolved distinctive diagnostic traits, and support the validity of subspecies *C. c. italicus* Festa (1925). However, mtDNA haplotypes of clade '*C. c. italicus*' were detected also in roe deer sampled in north Tuscany and Emilia-Romagna, north of the putative distribution limits of the Italian subspecies, where they are admixed with roe deer bearing haplotypes belonging to Clade Central (Fig. 1b).

The original distribution of *C. c. italicus* is unknown, because roe deer in the Apennines were already largely eradicated before World War II. Molecular and morphometric data now suggest that populations of *C. c. italicus* persisted not only in the protected areas of Castelporziano, Gargano and Orsomarso (Fig. 1a), but also in southern Tuscany, and probably in small remnant populations

along the ridge of the Apennines, in Tuscany and Emilia-Romagna (locations nos 34, 35, 36, 40 and 41; Fig. 1a). Alternatively, or concomitantly, roe deer bearing *'C. c. italicus'* mtDNA haplotypes might have recently expanded their range towards northern Tuscany and Emilia-Romagna. In these areas, roe deer populations are currently admixing, as a result of reintroduction and restocking operations, thus threatening the integrity of *C. c. italicus*.

Roe deer in central and southern Spain showed haplotypes belonging to two distinct and distantly related clades (Figs 2 and 3), which originated either in Clade West or Central. In the southern Spanish population from Cádiz we found three mtDNA haplotypes: no. 158, no. 159 (linked to Clade Central), and no. 160 (belonging to Clade West). Moreover, haplotype no. 158 was shared with populations in central Spain (Segovia, Burgos). If Clade Central originated in eastern Europe, roe deer in Spain could have admixed origins, and monophyly of subspecies *garganta* would be not supported by molecular data.

Recent population changes, disturbance and nonequilibrium effects

Deforestation and over-hunting led to the eradication of roe deer populations in Spain, Italy and Greece. After World War II, natural expansion or local reintroductions led to the reconstitution of extant populations, which are often expanding and admixing, sometimes after prolonged isolation in fenced or protected areas. The roe deer is subject to strong hunting pressure, which affects abundance and distribution and may modify population structure and reproductive behaviour (Milošević-Zlatanović *et al*. 2003). Thus, the demographic structure of some roe deer populations in Europe fluctuated recently, and probably these populations are not in genetic equilibrium.

Roe deer sampled from different areas of the Apennines, spanning a range of *c*. 300 km, are genetically more distinct than roe deer sampled from across all Europe, which span a range of more than 4000 km (Fig. 4). These results suggest that genetic drift as a result of recent fragmentation, isolation and bottleneck greatly inflated the observed genetic distances among roe deer populations distributed in the Apennines. Microsatellites support the phylogeographical patterns described by the mtDNA sequences, that is differentiation of peripheral roe deer populations in north Iberia and south Italy *vs*. greater admixture of populations sampled in central Europe and in the Balkans. However, nonequilibrium features warn against the use of pairwise population distances to infer mtDNA or microsatellite population trees (Lorenzini *et al*. 2002; Vernesi *et al*. 2002). Bottlenecks and founder events may result in the loss of genetic variability, but they can also result in a rapid differentiation between populations, through random sampling of alleles at polymorphic loci.

Expectations from the southern refuges phylogeographical model (Hewitt 2000) suggest that genetic diversity should be greater in refugial populations. However, roe deer in Italy could have lost genetic diversity because of population decline and drift, and are the less variable among the studied samples. Quite surprisingly, population no. 33 that was reintroduced in Liguria showed the highest number of alleles ($A = 6.6$). However, this population originated from roe deer from three different regions that were released in 1952 (two males and one female from Tuscany), in 1959 (two males, four females and three juveniles from Slovenia), and in 1974 (six roe deer from Trentino, eastern Alps). Admixed populations can be variable, particularly if they expand rapidly and retain most of their original genetic diversity. Moreover, recently founded populations can show high values of allele number and heterozygosity, which result from the sampling process (Tarr *et al*. 1998).

Conclusions

Roe deer in Europe showed a complex pattern of population structuring, which probably results from historical vicariance in southern glacial refuges (as described mainly by mtDNA findings), and subsequent population admixture as a result of natural secondary contacts or of anthropogenic disturbance (as described mainly by STR data). Maternal and biparental markers used in this study described concordant patterns of population structure, that is population admixture in the central Europe *vs*. greater diversification towards the peripheries. As expected, the STR markers were more informative in describing the consequences of the most recent population events (isolation, expansion, translocations, current dispersal rates). In accordance with current taxonomy (Danilkin 1996), roe deer in most of Europe should belong to a single panmictic population. In contrast, peripheral roe deer populations in north Portugal, southern Italian Apennines (and perhaps Greece) may represent the remains of late glacial refugial populations that should be preserved and not artificially admixed with other geographical populations.

Acknowledgements

We deeply appreciated the collaboration of everybody who helped in sampling collection: M. Hadži-Pavlovič (National Park 'Đerdap'), T. Radosavljevič (Hunters Association 'Stragari') and K. Laslo (Hunters Association 'Bečej') in Serbia; A. Giannakopoulos and H. Goumas in Greece; M. Paganin and G. Stefani (Asiago), E. Bonavetti (Val Camonica), P. Gatti (Lecco), G. Meneguz (Torino and Alessandria), S. Mattioli, V. Guberti and E. Raganella (Bologna), C. Matteucci (Forlì), M. Ferri (Modena), M. Apollonio (Arezzo), L. Orlandi, S. Nicoloso and DREAM (Tuscany), L. Bonfigli (Massa Carrara), A. Scappi (Grosseto, Siena), I. Farronato (Vicenza),

V. Gabardo (Treviso), B. Morriconi and Hunters Association of Alta Valtellina (Sondrio), G. Benvenuti (Firenze), A Drovandi (Pistoia), M. Cattani (Marradi), M. Meacci (Arezzo), S. Spanò and A. Marsan (Savona), B. Audino (Cuneo), L. Pedrotti (Bormio) in Italy; W. d'Oleire-Oltmanns in Germany. Most of the Portuguese samples were provided by BTVS-ICN (Wild Animal Tissue Bank, Portuguese Conservation Institute). V. Piorno and G. Pajares provided Galician and Asturian samples, respectively. We thank the officials of the Serbian Hunters Association for field and data acquisition assistance. We thank J.G. Martínez and J.L. Fernández for comments to the manuscript. Funding were provided to E.R. by INFS, and Ministero dell'Ambiente, Direzione Conservazione della Natura.

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