Evolutionary Ecology Research, 2012, **14**: 897–920

Conservation of threatened local gene pools: landscape genetics of the Italian roe deer (*Capreolus c. italicus*) populations

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ABSTRACT

Background: The endemic Italian roe deer (*Capreolus c. italicus*) is threatened by introgressive hybridization with the introduced and expanding European subspecies *Capreolus c. capreolus*. Population genetic surveys show that some populations in central Italy are not yet admixed with the introduced subspecies.

Question: Is it possible to identify and map the distributions of native and admixed roe deer populations?

Methods: We obtained and analysed diagnostic mitochondrial DNA control-region sequences and individual genotypes at 11 autosomal microsatellite loci in 1051 roe deer samples collected from the entire distribution of Italian roe deer and from reference populations of European roe deer. We used classical and Bayesian statistical approaches to describe the genetic substructure of roe deer populations in Italy. We used admixture analyses and landscape genetic tools to map the fine-scale distributions of Italian roe deer populations and locate their admixture zones.

Results: A very few fragmented patches of the Italian roe deer do survive in central Italy. Although these populations are seriously threatened by hybridization with expanding European roe deer, they can be genetically identified and, by means of translocations, saved from genetic extinction.

Discussion: Italian roe deer populations exist and are still viable, but their survival is threatened by the expansion of reintroduced European roe deer. The rapid identification of suitable and pristine areas to which pure individuals from remaining patches could be translocated appears the best way to preserve the Italian roe deer genetic pool.

Keywords: Bayesian clustering, *Capreolus capreolus italicus*, conservation genetics, genetic admixture and introgression, Italian roe deer, landscape genetics.

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INTRODUCTION

The complex dynamics of fragmentation and isolation in glacial refuges during Pleistocene climate and landscape changes in the Northern Hemisphere fostered the evolution of genetically distinct populations, which expanded northwards during the temperate interglacial periods (Hewitt, 2004; Stewart *et al.*, 2010). Some of these populations are identified as subspecies or 'evolutionarily significant units' (Moritz, 1994; Pennock and Dimmick, 1997; Waples, 1998; Hey *et al.*, 2003; Latta, 2008). Several secondary contacts with hybridization and introgression among post-glacial expanding populations eventually occurred in Europe and North America (Taberlet *et al.*, 1998; Randi, 2007; Hickerson *et al.*, 2010). However, some populations did not commingle, despite their potential for dispersal across the contact zones. For instance, populations of highly mobile species like the wolf [*Canis lupus* (Musiani *et al.*, 2007)] and the caribou (*Rangifer tarandus*) are subdivided into genetically distinct groups [or 'ecotypes' (Cronin *et al.*, 2005; Koblmüller *et al.*, 2009; McDevitt *et al.*, 2009b)] that do not interbreed if in close geographical proximity. Historical factors, prey specialization or habitat preferences, besides obvious geographic barriers to dispersal, might limit genetic exchanges, forcing potentially interbreeding populations to remain reproductively isolated and genetically distinct (Coulon *et al.*, 2006; Fontaine *et al.*, 2007). Genetic isolation might eventually result in specialized local adaptations, which should be identified and preserved, especially if threatened by the consequences of natural or anthropogenic ecological changes (Harrison *et al.*, 2006; Thuiller *et al.*, 2006).

The distribution ranges of many populations have been strongly impacted by anthropogenic events worldwide. In particular, during the last few centuries, populations of large vertebrates (carnivores and ungulates) declined and at times disappeared completely in many European countries due to over-hunting, deforestation, the spread of modern agriculture, and urbanization (Breitenmoser, 1998; Thuiller *et al.*, 2006). After the Second World War, however, ecological changes in mountain areas and improved wildlife management helped to reverse some of these negative trends. The recovery of ungulates has been favoured by the spread of forests, as well as by translocation programmes and restocking, which, however, have raised the risk of genetic admixtures between local and alien genotypes (Scandura *et al.*, 2008; Senn and Pemberton, 2009; McDevitt *et al.*, 2009a).

The European roe deer (*Capreolus capreolus*) is the most common deer in Europe and is widespread across a variety of ecosystems, but there are fragmented populations living in typical Mediterranean habitats, in southern Italy and Spain (Gortázar *et al.*, 2000; Focardi *et al.*, 2009). Phylogeographic studies have identified three main roe deer genetic assemblages, which had their origins in southern glacial refuges, and that colonized central and northern Europe most probably during the Holocene (Randi *et al.*, 2004). Two subspecies, the Spanish roe deer [*C. c. garganta* (Cabrera, 1916)] and the Italian roe deer [*C. c. italicus* (Festa, 1925)] evolved in isolation in the Iberian and Italian peninsulas (Lorenzini *et al.*, 2003; Randi *et al.*, 2004; Royo *et al.*, 2007). The endemic Italian roe deer shows distinct skull morphometry (Montanaro *et al.*, 2003), a diagnostic clade of unique mtDNA haplotypes (Randi *et al.*, 1998; Vernesi *et al.*, 2002), and distinct multilocus microsatellite genotypes (Lorenzini *et al.*, 2002; Randi *et al.*, 2004). In the past, the Italian subspecies was probably widespread in the Mediterranean region in Italy, but it was almost completely eradicated before the Second World War, surviving in isolation in just three protected areas: Castelporziano Preserve (near Rome; Lazio region), Gargano National Park (Puglia region), and Orsomarso massif (within the Pollino National Park; Calabria region; see Fig. 1). These populations, numbering just a few hundred individuals, are now endangered (Focardi *et al.*, 2005, 2009). Recently, however, Italian roe deer mtDNA haplotypes

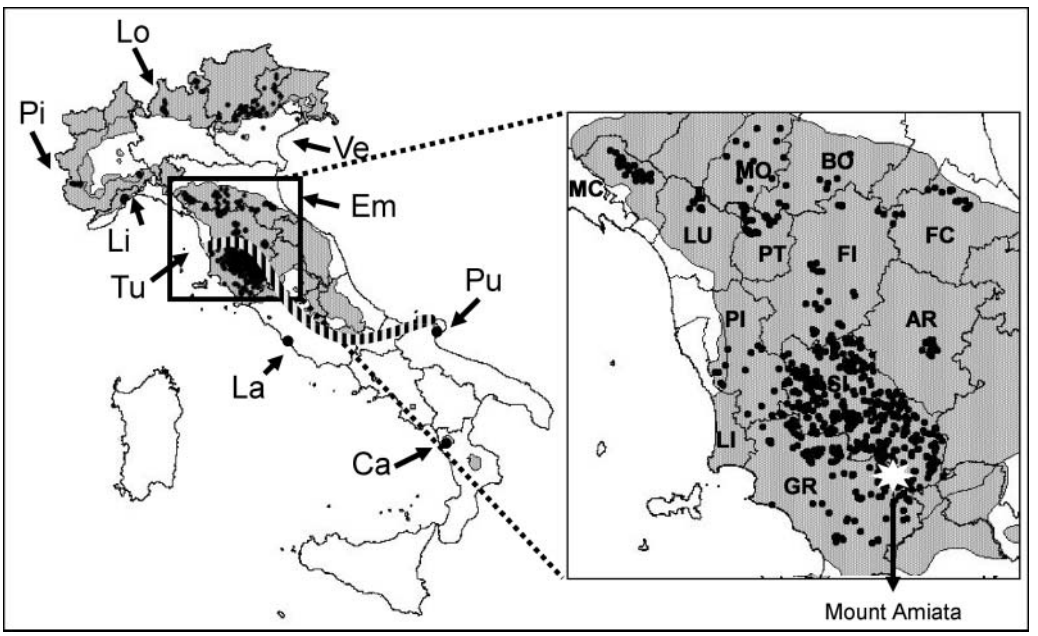


Fig. 1. Distribution of the roe deer (*Capreolus capreolus*) samples used in this study. Grey areas show the approximate species' distribution. The presumed northern limit of the endemic Italian roe deer subspecies, *C. c. italicus*, is indicated by the interrupted line. Acronyms indicate Italian regions: Ve = Veneto; Lo = Lombardia; Pi = Piemonte; Li = Liguria; Em = Emilia Romagna; Tu = Toscana; La = Lazio; Ca = Calabria; Pu = Puglia. The expanded insert shows details of the sampling areas and sample locations in Emilia-Romagna and Toscana. Acronyms indicate provinces: MC = Massa Carrara; LU = Lucca; MO = Modena; BO = Bologna; PT = Pistoia; PI = Pisa; FI = Firenze; FC = Forlì-Cesena; AR = Arezzo; SI = Siena; LI = Livorno; GR = Grosseto.

were discovered in roe deer populations in southern Toscana and across the Apennine ridge in Emilia-Romagna, suggesting a northward expansion, or waves of genetic introgression, beyond the suspected edge of the historical distribution of *C. c. italicus* (Randi and Mucci, 2001; Vernesi *et al.*, 2002; Randi *et al.*, 2004). In most of these areas, the Italian roe deer populations are surrounded by expanding introduced European roe deer populations, and are thus threatened by hybridization and genetic extinction (Gentile *et al.*, 2009).

The identification of Italian roe deer mtDNA haplotypes is straightforward (Randi *et al.*, 2004). However, the assignment of populations and individuals cannot be based on maternally inherited mtDNA alone. Hybridization with the European roe deer might be widespread, and maternal markers might severely underestimate admixture and introgression rates, particularly in species with sex-biased dispersal (Lawson Handley and Perrin, 2007; Lovari *et al.*, 2008). We still lack explicit procedures to identify pure or admixed Italian roe deer individuals and populations using autosomal markers. In this study, geographic distributions of mtDNA haplotypes and multilocus autosomal microsatellite genotypes were analysed with Bayesian clustering and landscape genetics models and used to: (1) describe patterns of the genetic variation in European and Italian roe deer; (2) identify and map the remnant Italian roe deer populations; and (3) locate the admixture zones and identify admixed individuals. Results show that the simultaneous assessment of the geographical distribution of mtDNA

haplotypes and microsatellite genotypes is necessary to identify Italian or European roe deer populations. Bayesian clustering and spatial genetic models further provided detailed distributions of non-hybridizing versus hybridizing populations, thus pinpointing those areas that should receive prioritization for the conservation of the endemic Italian roe deer.

METHODS AND MATERIALS

Tissue collection and history of the sampled populations

From 2000 to 2006 we collected a total of 1051 roe deer tissue samples, stored in 90% ethanol, including deer from natural, restocked, and reintroduced populations in Italy (Table 1; Fig. 1). First, European roe deer samples were obtained from four regions in the Alps and western Apennines (Veneto, Lombardia, Piemonte, and Liguria; $n = 170$). Local roe deer populations in the central and western Alps (Lombardia and Piemonte) and western Apennines (Liguria) had become completely eradicated due to over-hunting. These populations were reconstructed during the last few decades through the reintroduction of

Table 1. Details on the origin (region and province) and number of roe deer samples used in this study

			mtDNA		
Region	Province	Samples	343 bp	704 bp	Microsatellites
Veneto	Belluno	11	–	10	11
	Treviso	12	–	12	12
	Vicenza	41	–	32	41
Lombardia	Sondrio	8	–	7	8
	Brescia	7	–	6	7
	Lecco	18	–	18	18
Piemonte	Cuneo	19	–	16	19
	Alessandria	11	–	10	11
Liguria	Savona	43	–	27	43
Emilia-Romagna	Modena	15	–	14	15
	Bologna	8	–	3	8
	Forlì-Cesena	25	–	24	25
Toscana	Massa Carrara	41	19	21	41
	Lucca	30	30		14
	Pistoia	66	56	10	34
	Pisa	45	35	10	33
	Firenze	40	33	7	31
	Arezzo	19	–	18	19
	Siena	407	324	71	407
	Grosseto	155	104	51	139
Lazio	Roma (Castelporziano)	21	–	15	21
Puglia	Foggia (Gargano)	8	–	8	2
Calabria	Cosenza (Orsomarso)	1	–	1	–
Total		1051	601	391	959

Note: Number of samples sequenced for a short (343 bp) or a long (704 bp) fragment of the mtDNA control-region and genotyped at 11 autosomal microsatellite loci.

roe deer from Europe (Slovenia, Austria, Hungary) and eastern Alps (Perco and Calò, 1994). Roe deer in the eastern Italian Alps (Veneto) originated through the expansion of remnant local populations after the Second World War (Masseti, 2003). Second, the roe deer populations sampled in Emilia-Romagna and Toscana ($n = 851$) originated in part from reintroductions of European roe deer from the eastern Italian Alps or other European countries. Remnant patches of local European roe deer could have survived along the Apennine ridge in Emilia-Romagna and northern Toscana (Randi, 2005). The survival of remnant Italian roe deer populations in southern Toscana has been suggested by Vernesi *et al.* (2002) and Randi *et al.* (2004). Thus, the samples collected in Emilia-Romagna and Toscana could belong to the European or Italian roe deer subspecies, or could be hybrids. The genetic identification of these samples was the main aim of this study. Finally, additional samples ($n = 30$) from the historical range of *C. c. italicus* were collected in southern Italy (Castelporziano, Gargano, and Orsomarso).

Laboratory methods

Total DNA was extracted using the Qiagen Tissue Kit (Qiagen) and a Multiprobe II EX liquid handling workstation (Perkin Elmer). The entire mtDNA control-region was PCR-amplified in 391 samples using primers *LcapPro* and *HcapPhe* (Randi *et al.*, 1998), and sequences of 704 bp were obtained using the two PCR primers and two internal primers, *Lcap362* and *Hcap493* (Randi *et al.*, 1998). Moreover, the first part of the mtDNA control-region (343 bp), which contains a single nucleotide deletion that is diagnostic to identify the Italian roe deer haplotypes (Randi *et al.*, 2004), was amplified in 601 samples from Toscana using primers *LcapPro* and *Hcap493*, and sequenced using primer *Hcap493*. Sequences were obtained in an ABI 3130XL automated sequencer, and analysed with the software SEQUENCING ANALYSIS 5.3 and SEQSCAPE 2.5 (Applied Biosystems). Long (704 bp) and short (343 bp) alignments were constructed using BIOEDIT 7.1.3 (Hall, 1999), and unique haplotypes were identified with COLLAPSE 1.2 (D. Posada; http://darwin.uvigo.es/software/collapse.html). The long alignment includes the sequences analysed by Randi *et al.* (2004) (GenBank accession nos. AY625732–AY625892). Multilocus genotypes of 959 samples were obtained by PCR amplifications of 11 autosomal microsatellites (Randi *et al.*, 2004). Alleles and genotypes were identified in an ABI 3130XL sequencer with the software GENEMAPPER 4.0 (Life Technologies). Details of the laboratory procedures are available on request (see also Randi *et al.*, 2004).

Analyses of the mtDNA sequences and microsatellite markers

Unrooted phylogenetic trees were constructed with the long mtDNA alignment using MEGA 5.05 (Tamura *et al.*, 2011; http://megasoftware.net/), the neighbour-joining procedure (Saitou and Nei, 1987), and a Tamura-Nei's genetic distance matrix (Tamura and Nei, 1993), which is appropriate to describe the evolution of control-region sequences. Networks of the short mtDNA alignment, obtained with the median-joining network procedure (Bandelt *et al.*, 1999) implemented in NETWORK 4.610 (http://www.fluxus-technology.com/sharenet.htm), were used to identify Italian or European roe deer haplotypes. The Italian roe deer haplotypes are defined by: (1) their connection to the Italian roe deer mtDNA clade; and (2) the presence of a single nucleotide deletion at position 103 of the roe deer mtDNA alignment (GenBank AY625732–AY625892). Haplotype diversity (*Hd*) and nucleotide diversity (*Pi*) were computed using DNASP 5 (Librado and Rozas, 2009; http://www.ub.edu/dnasp/). Commonly used estimates of genetic

diversity at microsatellite loci – average number of alleles (*Na*), average number of effective alleles (*Ne*), expected (*He*) and observed (*Ho*) heterozygosity – and a test for departure from Hardy-Weinberg equilibrium (HWE) were computed using GENALEX v. 6.41 (Peakall and Smouse, 2006; http://www.anu.edu.au/BoZo/GenAlEx/new_version.php). Bottleneck effects were assessed using Wilcoxon's signed-rank test and the mode-shift test (Cornuet and Luikart, 1996; Luikart *et al.*, 1998) in BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996; http://www.ensam.inra.fr/URLB/bottleneck/pub.html). BOTTLENECK was run with 1000 replicates and the two-phase mutation model (TPM; proportion of stepwise mutations in TPM = 70%).

Bayesian population clustering

The genetic structure of the sampled populations was assessed using the microsatellite genotypes and the Bayesian clustering procedures as in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003). STRUCTURE was designed to identify the K populations (genetic clusters) and assign individuals to these populations. Individuals are assigned probabilistically to one cluster (the population of origin), or to more than one cluster if their genotypes are admixed. Assignments are based on threshold values of individual coefficients of membership (q_i values), which are estimates of genotype admixture (Vähä and Primmer, 2006; Barilani *et al.*, 2007). Exploratory analyses were performed with $K = 1$–12, including the samples collected in the Alps ($n = 959$), or using only the samples from the Apennines and Toscana ($n = 766$). A burn-in period of 20,000 steps followed by 200,000 iterations ensured convergence of the MCMC. All simulations were independently replicated four times for each value of K, using the admixture and the independent allele frequency models (Pritchard *et al.*, 2000). Individuals were assigned to the clusters using only genetic information, and regardless of sampling location (options *usepopinfo* = 0, *popflag* = 0). The optimal K was chosen as the value that maximized the increase in the posterior probability of the data [ΔK (Garnier *et al.*, 2004; Evanno *et al.*, 2005)]. Coefficients of membership (corresponding to estimates of population or individual admixture) averaged across four replicates were obtained by CLUMPP v.1.1.2 (Jakobsson and Rosenberg, 2007; http://www.stanford.edu/group/rosenberglab/software.html). The software DISTRUCT 1.1 (Rosenberg, 2004; http://www.stanford.edu/group/rosenberglab/software.html) was used to plot the graphical coloured representations of population (Q_I values) and individual (q_i values) admixtures. Box plot graphs of the individual q_i values, split by their mtDNA haplotypes, were computed in STATVIEW 5.0.1 (SAS Institute, Inc., 1992) and were used to identify, for each K, the threshold q_i values to be used for the identification of Italian roe deer individuals.

The robustness of the clusters obtained with STRUCTURE was tested using BAYESASS 1.3 (Wilson and Rannala, 2003). Unlike other assignment tests or Bayesian clustering, BAYESASS does not assume Hardy-Weinberg and linkage equilibrium and is appropriate when the sampled populations might deviate from equilibrium. BAYESASS was run for a total of 3 million iterations, with a burn-in period of 999,999 steps and default parameters.

Landscape genetic analyses and assignment testing

Spatial distributions of individual haplotypes or genotypes of the samples collected from the Apennines and Toscana were mapped with ARCVIEW GIS 3.1 (ESRI, 1999). The software GENELAND 4.0.3 (http://www2.imm.dtu.dk/~gigu/Geneland/) was used to reconstruct the posterior geographical distribution of the genetic clusters in a Poisson-Voronoi tessellation of the sampling space. This procedure simultaneously uses information on genotypes and

geographic locations to infer the spatial population structure of the samples. The spatial distributions of the sampled q_i values are interpolated, thus mapping, as precisely as possible, the location of the genotypes (in this case, Italian vs. European roe deer clusters) and their eventual admixture zones. We ran five GENELAND replicates of 100,000 MCMC iterations (with thinning = 100), using the independent allele frequency model and allowing K to vary from 1 to 10. Uncertainty on spatial coordinates was set either to 0 or 1 (±1° of latitude and longitude), in line with information on roe deer home range (less than 100 ha) in a fragmented landscape (Cargnelutti *et al.*, 2002) and maximum juvenile dispersal range (Coulon *et al.*, 2006).

GENELAND outputs were compared with results obtained from TESS 2.3.1 (http://membres-timc.imag.fr/Olivier.Francois/tess.html) and BAPS 5.4 (http://web.abo.fi/fak/mnf//mate/jc/software/baps.html). These programs implement different Bayesian clustering approaches, thus allowing comparison of results obtained based on different assumptions.

BAPS can perform admixture analyses at both the individual and group level. Spatial results are displayed using a coloured Vonoroi tessellation based on a discrete sampling site. BAPS was used with the admixture model, K varying from 1 to 14. In total, 100 iterations were run and four replicates for each run were performed. Population clustering and individual assignments were performed also in TESS without assuming predefined populations. Analyses were performed with the 'Without Admixture' and 'With Admixture' models, using 20,000 sweeps, 2000 sweeps discarded as burn-in. The value of the spatial interaction parameter (ψ), representing the strength of the spatial autocorrelation, was set at the default value of 0.6. The number of populations (K) was allowed to vary from 2 to 14. The deviance information criterion [DIC (Spiegelhalter *et al.*, 2002)] was used to measure the prediction power of the model, and the lowest value was chosen to identify the optimal K. The individual membership values from both TESS and BAPS were plotted using the package Fields in R (http://cran.r-project.org/web/packages/fields/index.html). The identification of admixture zones at fine-scale was obtained by mapping the individual membership values (q_i) from STRUCTURE using ARCVIEW GIS.

RESULTS

Genetic diversity in roe deer populations

Both mtDNA sequences and microsatellite genotypes were variable in all the studied populations, with the exception of monomorphic mtDNA sequences in isolated small Italian roe deer populations in Castelporziano and Gargano (Table 2). The alignment of the short mtDNA sequences showed 28 haplotypes defined by 23 polymorphic sites. Haplotype diversity ranged from $Hd = 0.48$ to 0.80; nucleotide diversity was always lower than $Pi = 0.01$. The highest Hd values were detected in the Alps, in reintroduction areas (Lombardia, Piemonte; $Hd = 0.73$) where roe deer of different geographical origins are admixing, and across the Apennine ridge in Emilia-Romagna ($Hd = 0.80$), where genetically distinct populations are expanding and admixing. The isolated Italian roe deer populations in Castelporziano and Gargano showed their own, distinct mtDNA haplotypes. The roe deer populations in Lombardia, Piemonte, and Toscana showed the highest number of observed ($Na = 7.4$–10.2) and effective ($Ne = 4.0$–4.2) alleles per microsatellite locus, a sign of admixture in areas where the Italian and the introduced European roe deer are expanding and interbreeding (see below). Again, the Italian roe deer populations in

Table 2. Genetic diversity at the short mtDNA control-region (343 bp) and 11 autosomal microsatellite loci in roe deer populations sampled from the species distribution range in Italy (see distribution map in Fig. 1)

Sampling region	*Hd*	*Pi*	*Na*	*Ne*	*Ho*	*He*	F_{IS}	*P*
Veneto	0.48	0.0039	7.3	3.4	0.66	0.68	0.02	0.14
Lombardia	0.73	0.0114	7.4	4.2	0.69	0.73	0.07	0.03
Piemonte	0.73	0.0119	7.8	4.5	0.68	0.73	0.08	0.03
Liguria	0.62	0.0069	5.8	3.2	0.61	0.62	0.02	0.19
Emilia-Romagna	0.80	0.0068	4.5	2.7	0.62	0.60	–0.01	0.34
Toscana	0.62	0.0054	10.2	4.0	0.62	0.72	0.13	0.00*
Lazio (Castelporziano)	0.00	0.0000	3.7	2.6	0.64	0.59	–0.07	0.08
Puglia (Gargano)	0.00	0.0000	1.5	1.4	0.36	0.22	–0.45	0.00*

Note: *Hd* = mtDNA haplotype diversity; *Pi* = mtDNA nucleotide diversity; *Na* = average number of alleles per microsatellite locus; *Ne* = average effective number of alleles per locus; *Ho* = observed heterozygosity; *He* = expected heterozygosity; F_{IS} = deviation from Hardy-Weinberg equilibrium; *P* = significance of the F_{IS} values. *Significant departures from HW equilibrium. The single sample genotyped from Orsomarso was not analysed.

Castelporziano and Gargano showed the lowest allelic diversity. A sign test and Wilcoxon tests, performed in BOTTLENECK, showed significant excess heterozygosity ($P < 0.05$), and the mode-shift test resulted in a multimodal curve, confirming the occurrence of a recent bottleneck in the Castelporziano population. Microsatellite loci were usually in HWE (at threshold $P < 0.01$), except in the roe deer populations sampled in Toscana, further suggesting that these populations are currently admixing, and in Gargano (likely as a consequence of the small sample size, $n = 8$; Table 2).

Geographical distribution of the Italian roe deer mtDNA haplotypes

The alignment of long mtDNA sequences included 47 new haplotypes obtained from the 391 samples typed in this study, and 119 haplotypes previously published (Randi *et al.*, 2004). The neighbour-joining tree (Fig. 2) and the median-joining network (not shown) together split these 166 haplotypes into three main groups, corresponding to Clades East, West, and Central, as described in Randi *et al.* (2004; see their figure 2). A distinct sub-clade, nesting within the Central Clade and containing the haplotypes of *C. c. italicus*, was confirmed also using the new sequences (Fig. 2). A synapomorphic diagnostic deletion mapping at position 103 of the roe deer mtDNA alignment was used to assign the 601 new short sequences either to the *C. c. italicus* sub-clade (hereafter mtDNA IT), or to the other *C. europaeus* haplogroups (hereafter mtDNA EU). Analysis of the long and short sequences showed that all roe deer collected from the Alps (Veneto, Lombardia, and Piemonte; $n = 111$), from Liguria, and from the provinces of Bologna (BO), Forlì Cesena (FC), and Arezzo (AR) (Fig. 1 and Fig. 3A; $n = 72$) exhibited exclusively the mtDNA EU haplotypes. In contrast, all the roe deer sampled within the historical range of *C. c. italicus* (Lazio, Puglia, and Calabria in Fig. 1; $n = 24$) showed exclusively mtDNA IT haplotypes. Finally, both mtDNA IT ($n = 480$) and mtDNA EU ($n = 240$) haplotypes were detected in the other provinces of central Italy (in Emilia-Romagna and Toscana; Fig. 1 and Fig. 3A). Identification of mtDNA haplotype was unsuccessful in 46 samples.

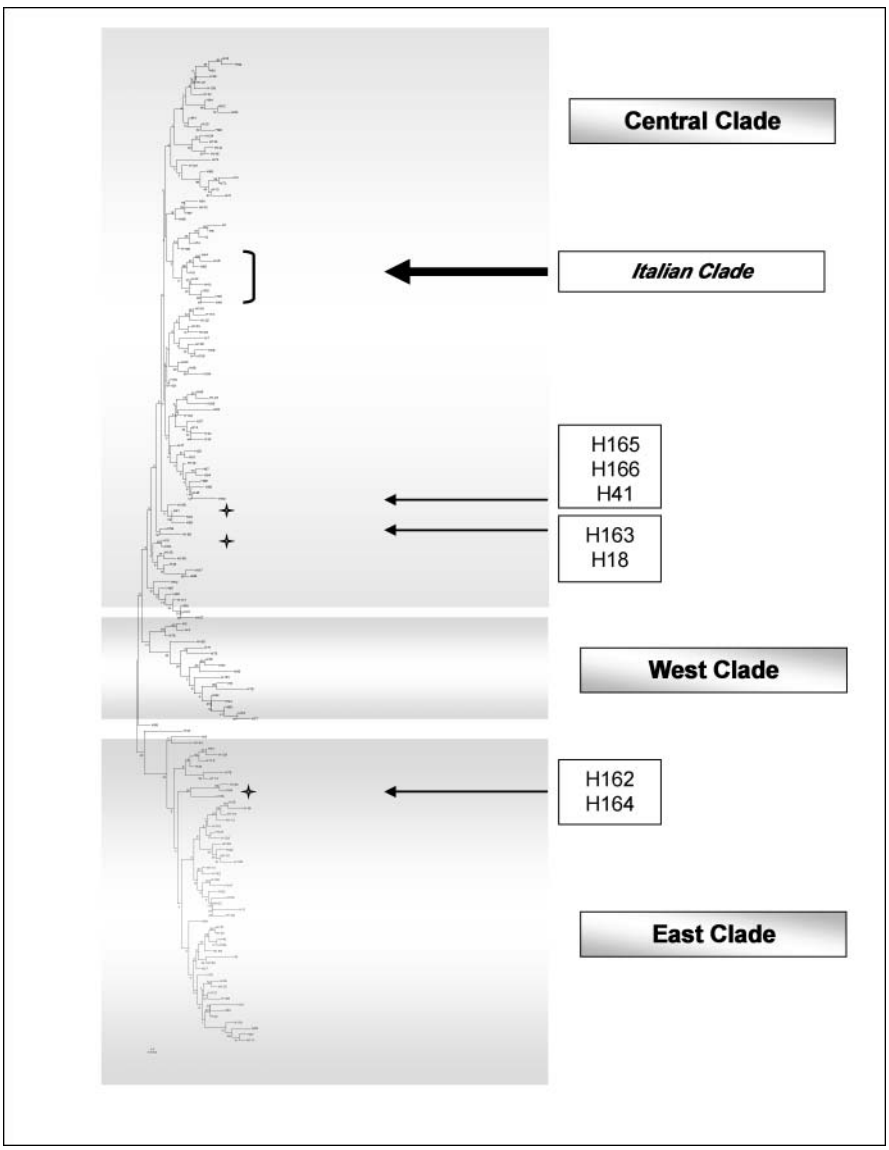


Fig. 2. Neighbour-joining tree of roe deer mtDNA control-region haplotypes (704 bp) computed using a Tamura-Nei genetic distance matrix (Tamura and Nei, 1993). The three main mtDNA clades (West, Central, and East) are indicated on the right side of the tree. The thick arrow shows the position of the Italian clade. The numbers refer to non-Italian haplotypes found in the Italian Appenines, while the thin arrows indicate the position inside the clades.

The highest frequencies of mtDNA IT haplotypes (Fig. 3A) were detected in Modena (MO; 53.33%), Massa Carrara (MC; 82.92%), Siena (SI; 75.18%), and Grosseto (GR; 69.06%), well beyond the historical range of *C. c. italicus* (delimited as shown in Fig. 1). The mtDNA IT haplotypes in Toscana were prevalent only in the central sector of the Province of Siena (SI), and in the northern part of the Province of Grosseto (GR; Fig. 3A).

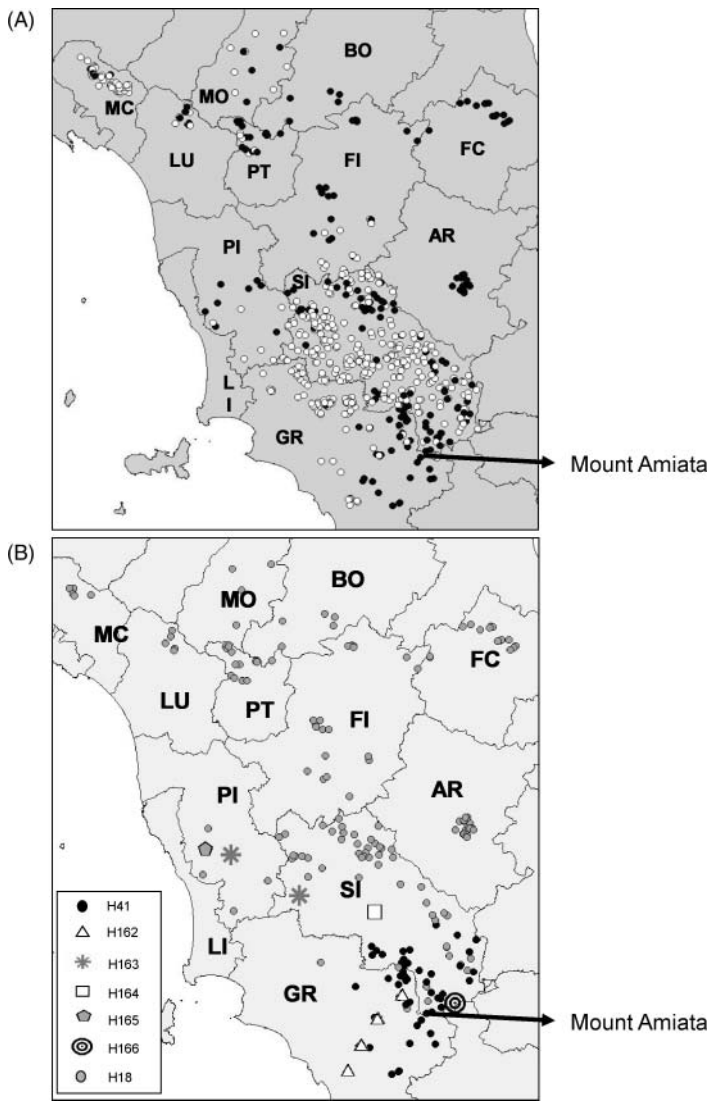


Fig. 3. Distribution of the roe deer mtDNA haplotypes sampled in Emilia-Romagna and Toscana. (A) Distribution of the Italian roe deer mtDNA (white dots) and European roe deer mtDNA haplotypes (black dots). (B) Distribution of the five European roe deer mtDNA haplotypes sampled in Toscana, and which were not found anywhere else in Italy (see text for descriptions).

Moreover, five different mtDNA EU haplotypes, which do not belong to the mtDNA IT clade, were found in southern Toscana [Siena (SI) and Grosseto (GR); Fig. 3B]. They are closely related to other haplotypes in the Central or East Clade (Fig. 2), which were found in the European roe deer populations sampled outside Italy only. In particular, haplotype H166 (GenBank accession no. KC178714), which is closely linked to haplotype H41, sampled in Germany [black dots in Fig. 3B; GenBank accession no. AY625772 (Randi *et al.*, 2004)], was found around Mount Amiata where European roe deer from the former

Czechoslovakia were released about 60 years ago (Mazzoni della Stella, 1990). Haplotypes H162 and H164 (GenBank accession nos. KC178710 and KC178712), closely linked to haplotypes clustering in Clade East, have been sampled only in the Balkan region to date (Randi *et al.*, 2004; this study). Haplotypes H165 and H163 (GenBank accession nos. KC178713 and KC178711), related to the European roe deer haplotypes clustering in the Central Clade, were found in Pisa (PI) and in the northern sector of Siena province (SI).

Population genetic structure

The optimal genetic clustering of the total sample ($n = 959$, including all the genotypes from the Alps, Apennines, and southern Italy) was obtained using STRUCTURE with K ranging from 4 to 6 (ΔK 3–4 = 3.9; ΔK 4–5 = 7.8; ΔK 5–6 = 54.0), after which the K values reached a plateau (not shown). These K values were used to assess the hierarchical pattern of population clustering (Fig. 4A). At $K = 4$, the samples split into a first cluster (cluster I in Fig. 4A) grouping all the Alpine populations (roe deer sampled in Veneto, Lombardia, and Piemonte) and the western Apennine populations (the reintroduced roe deer in Liguria) with an average $Q_I = 0.88$. This cluster shows little sign of admixture. A second cluster (cluster II in Fig. 4A) includes the roe deer sampled across the eastern sector of the Apennine ridge in Emilia-Romagna and Toscana [Arezzo (AR), Forlì-Cesena (FC), Firenze (FI), and Bologna (BO) provinces; see Figs. 1 and 3], which again shows little sign of admixture ($Q_I = 0.92$). In contrast, cluster III, which groups the populations sampled across the western sector of the Apennine ridge in Emilia-Romagna and Toscana [Pistoia (PT), Lucca (LU), Modena (MO), and Massa Carrara (MC) provinces; see Figs. 1 and 3] shows stronger signs of admixture ($Q_I = 0.52$). The last cluster (cluster IV) includes the populations sampled from Toscana [mainly the provinces of Pisa (PI), Siena (SI), and Grosseto (GR)], and shows strong signs of genetic admixture ($Q_I = 0.53$). At $K = 6$ (Fig. 4A), the remnant eastern and central Alpine populations, and the populations reintroduced in the western Alps were split into two clusters (with $Q_I = 0.83$ and 0.95, respectively). The subdivision between roe deer sampled in Emilia-Romagna and north Toscana was confirmed, as there was strong admixture of roe deer sampled in Toscana. STRUCTURE at $K = 8$ (Fig. 4A) confirmed the population clustering obtained with $K = 4$ or 6, clearly highlighting the admixed structure of roe deer sampled in Toscana [Grosseto (GR) and Siena (SI)]. The two small populations of Italian roe deer living in Castelporziano and Gargano were not consistently assigned to any cluster, likely due to the consequences of population bottleneck and isolation.

In a second session of Bayesian clustering analyses, all the samples from the Alps, western Apennines, and southern Italy were removed, and the admixture pattern was assessed using STRUCTURE with only the samples from Emilia-Romagna and Toscana ($n = 766$; Fig. 4B). The optimal genetic clustering ($K = 4$–6) confirms the patterns described using the complete data set (Fig. 4A), and indicates a sharp contrast between poorly admixed populations sampled from the provinces of Bologna (BO), Arezzo (AR), and Massa Carrara (MC), and strongly admixed populations sampled from the other provinces, in particular from Pisa (PI), Siena (SI), and Grosseto (GR) (Toscana). However, it is noteworthy that also within the most admixed clusters there were distinct subgroups showing small individual proportions of admixture (Fig. 4C). The six clusters obtained by STRUCTURE were tested in BAYESASS, which does not assume HWE in the sampled populations. All the individuals assigned to the six clusters by STRUCTURE were also assigned to the same clusters by

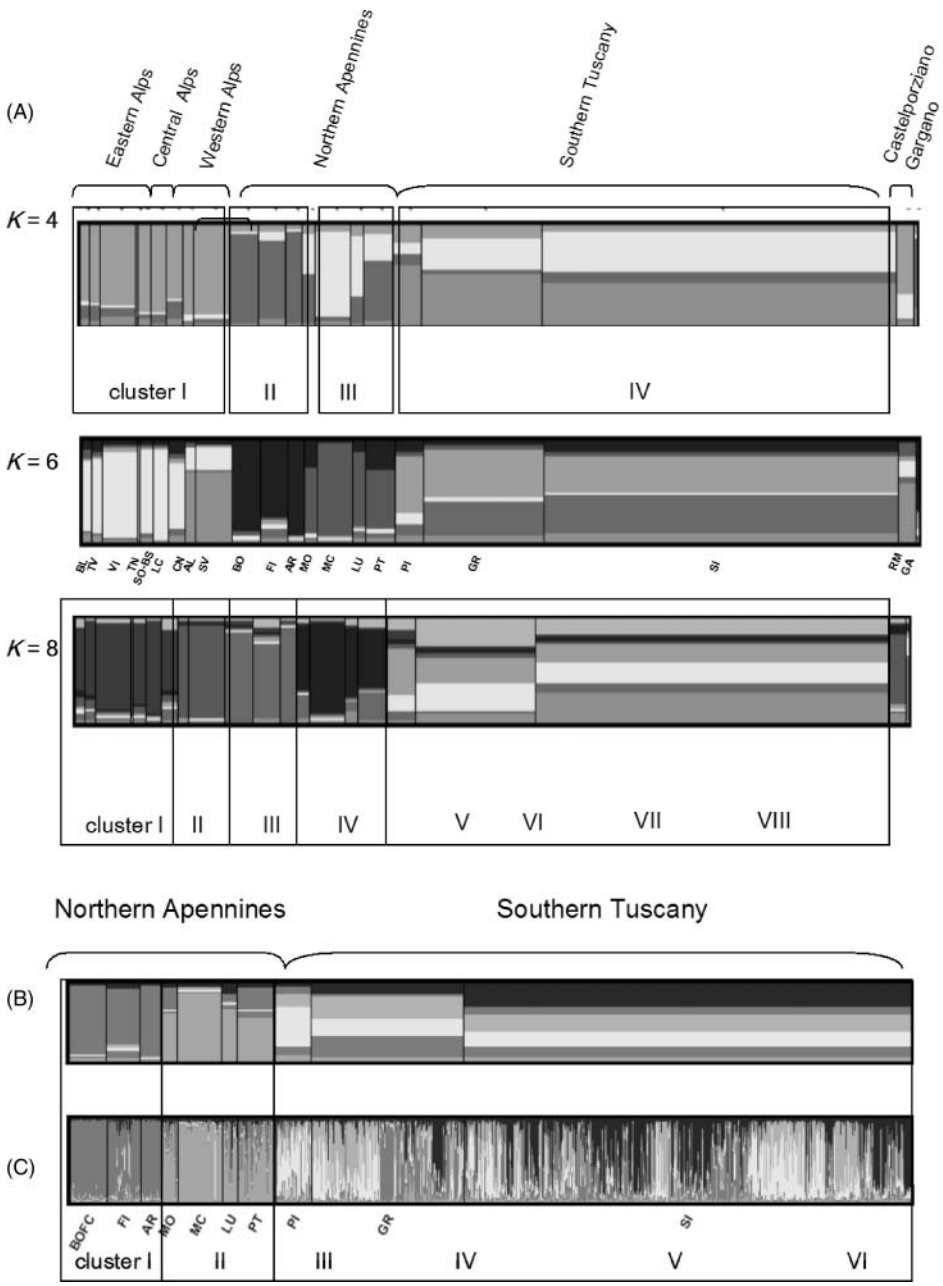


Fig. 4. (A) Results of STRUCTURE analyses obtained using the entire roe deer sample set. Plots of the averaged coefficients of membership (Q values), corresponding to estimates of population admixture, across four STRUCTURE replicates as obtained by CLUMPP (Jakobsson and Rosenberg, 2007). Results obtained at $K = 4$, $K = 6$, and $K = 8$ are shown. The origin of the sampled populations is indicated at the top of the upper plot (regions) and at the bottom of the middle plot (provinces). Vertical black lines separate individuals from different populations. Population structuring (B) and individual admixture proportions (C) of roe deer samples collected from the *C. c. italicus* distribution areas in Emilia-Romagna and Toscana obtained with STRUCTURE at $K = 6$.

BayesAss, with probability scores from 0.95 to 1.00. Only two individuals assigned to cluster I and VI had probability values lower than this (0.88 and 0.94, respectively).

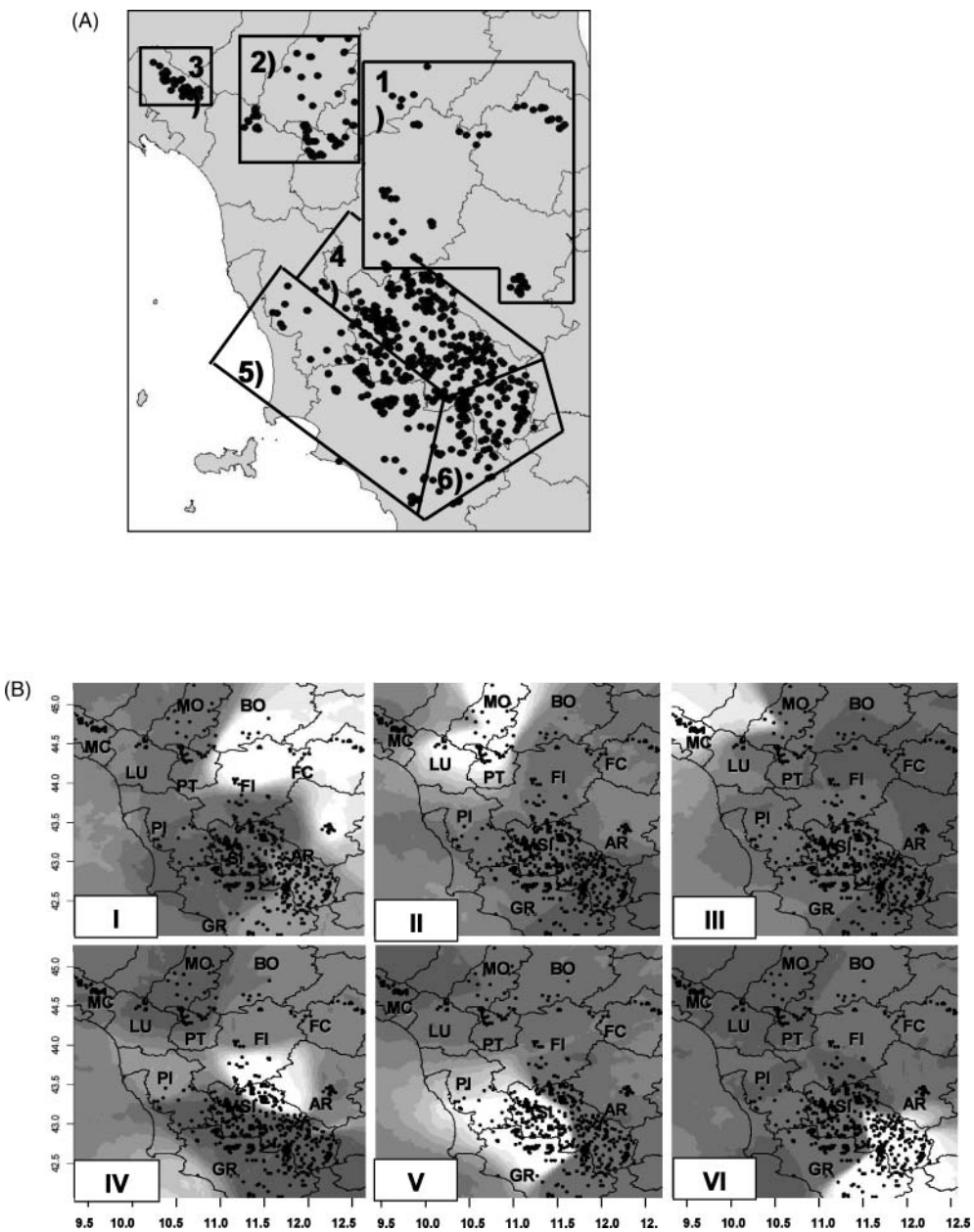
Landscape genetic analyses

The geographical locations of the genetic clusters were determined with Geneland, Tess, and Baps. An optimal number of six distinct spatial clusters was obtained by Geneland using both genetic (multilocus genotypes) and geographical (sampling locations) information (Fig. 5B). Three clusters grouped the roe deer distributed across the Apennine ridge: (1) cluster I includes samples from Arezzo (AR), Forlì Cesena (FC), Bologna (BO), and north Firenze (FI); these samples were assigned to cluster I also by Structure (see Figs. 4B, 4C, 5A) and show mainly mtDNA EU haplotypes (see. Fig. 3A); (2) cluster II includes samples from Pistoia (PT), Modena (MO), and Lucca (LU), as with Structure (Figs. 4B, 4C), and shows both mtDNA EU and mtDNA IT haplotypes; (3) cluster III groups almost all the highly admixed samples collected from Massa Carrara (MC). The other three clusters group roe deer mainly from southern Toscana: (4) cluster IV includes samples from south Firenze (FI) and north Siena (SI), which have admixed mtDNA haplotypes; (5) cluster V includes samples from north Grosseto (GR) and eastern Siena (SI), which have almost exclusively mtDNA IT haplotypes; and (6) cluster VI is located in south Siena (SI) and Grosseto (GR), the areas where mtDNA EU is largely prevalent (Fig. 3A). Therefore, Geneland results, in line with Structure, allowed mapping of clusters IV and V that include the core areas of the distribution of *C. c. italicus* mtDNA haplotypes in Toscana (Fig. 5A and 5B).

The DIC values in both the 'With Admixture' and 'Without Admixture' models in Tess let us identify six clusters largely coincident with the Geneland results (data not shown), with one exception. In Geneland, cluster IV grouped samples collected towards the borders of southern Firenze, western Arezzo, and northern Siena provinces, while in Tess it grouped almost exclusively samples from southern Siena province. Baps identified nine clusters, roughly corresponding to Geneland and Tess, except for three further subdivisions (Fig. 5C): (1) Geneland clusters V and VI were split into sub-clusters; (2) seven individuals from south Grosseto showing Italian mtDNA and included in cluster VI by Geneland, were placed in a distinct cluster (Cluster VI c; Fig. 5c); (3) samples from Pisa were grouped outside Cluster V, nine of them showing mtDNA EU and two mtDNA IT (Cluster V b; Fig. 5c).

Identification of the admixed roe deer genotypes and location of the admixture zones

Individual genotypes assigned to each of the six clusters obtained by Structure analyses were split into two subgroups according to their mtDNA haplotypes: mtDNA IT or mtDNA EU. The box-plot graphs of the individual q_i values, split by their mtDNA haplotypes (Fig. 5D), showed that all genotypes joining clusters IV and V, with an individual membership value $q_i > 0.80$, bore exclusively mtDNA IT, except for a few samples in admixed areas [four samples in Pisa (PI) province and eleven samples in Siena (SI)]. In contrast, genotypes joining clusters I, II, III, and VI showed a prevalence of mtDNA EU (clusters I and II) or were highly admixed with both mtDNA IT and mtDNA EU (clusters III and VI). Consequently, we assumed that individual genotypes could be assigned to the Italian roe deer subspecies at a threshold $q_i > 80\%$ in clusters IV and V, in association with



mtDNA IT haplotypes. Samples within the core area of the Italian roe deer distribution in Toscana [provinces of Grosseto (GR) and Siena (SI)] were identified as: (1) Italian roe deer, if they showed mtDNA IT and $q_i > 0.80$; (2) European roe deer, if they showed mtDNA EU and $q_i < 0.20$; and (3) admixed, if they showed any mtDNA and $0.20 < q_i < 0.80$.

A distribution map (Fig. 6A), summarizing the results, shows that: (1) There was no admixture in Bologna (BO), Forlì Cesena (FC), Arezzo (AR), and Massa Carrara (MC); almost all the samples were assigned to a single cluster with $q_i > 0.80$ [Bologna (BO), Forlì

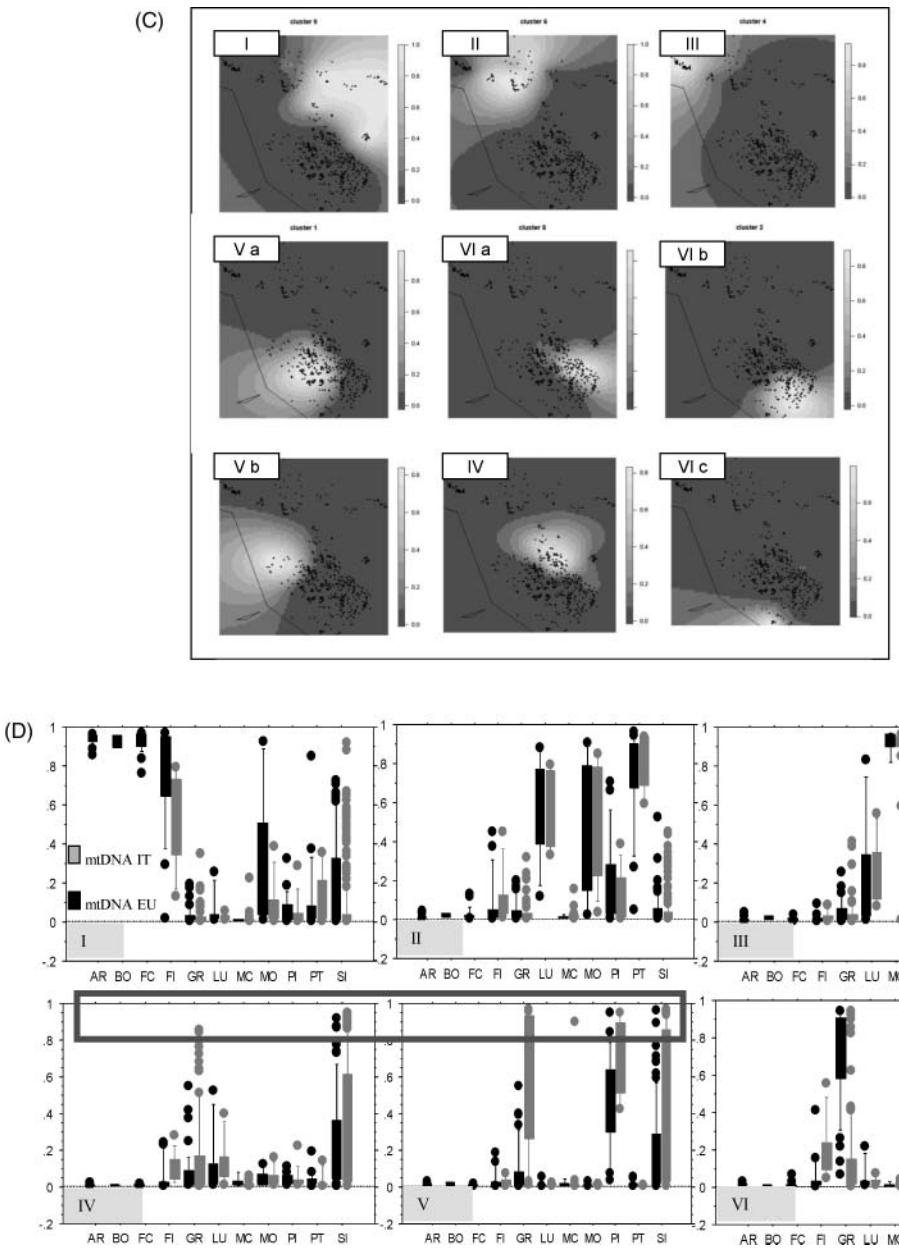


Fig. 5. (A) Geographical distribution of the clusters obtained with STRUCTURE. (B) Interpolated posterior probability of individual roe deer genotypes to belong to clusters I–VI as obtained with GENELAND. White areas represent the maximum posterior probability of individuals to belong to a distinct genetic cluster. (C) Interpolated posterior probability of individual roe deer genotypes to belong to clusters I–VI as obtained with TESS. Further regrouping for clusters V and VI is shown using lower-case letters. White areas represent the maximum posterior probability of individuals to belong to a distinct genetic cluster. (D) Box-plots of individual roe deer q_i values obtained with STRUCTURE at $K = 6$ and split by their mtDNA haplotypes (Italian in grey; European in black). In the plots, each haplotype is associated with its q_i value. In clusters IV and V, individuals with q_i values > 80% are highlighted.

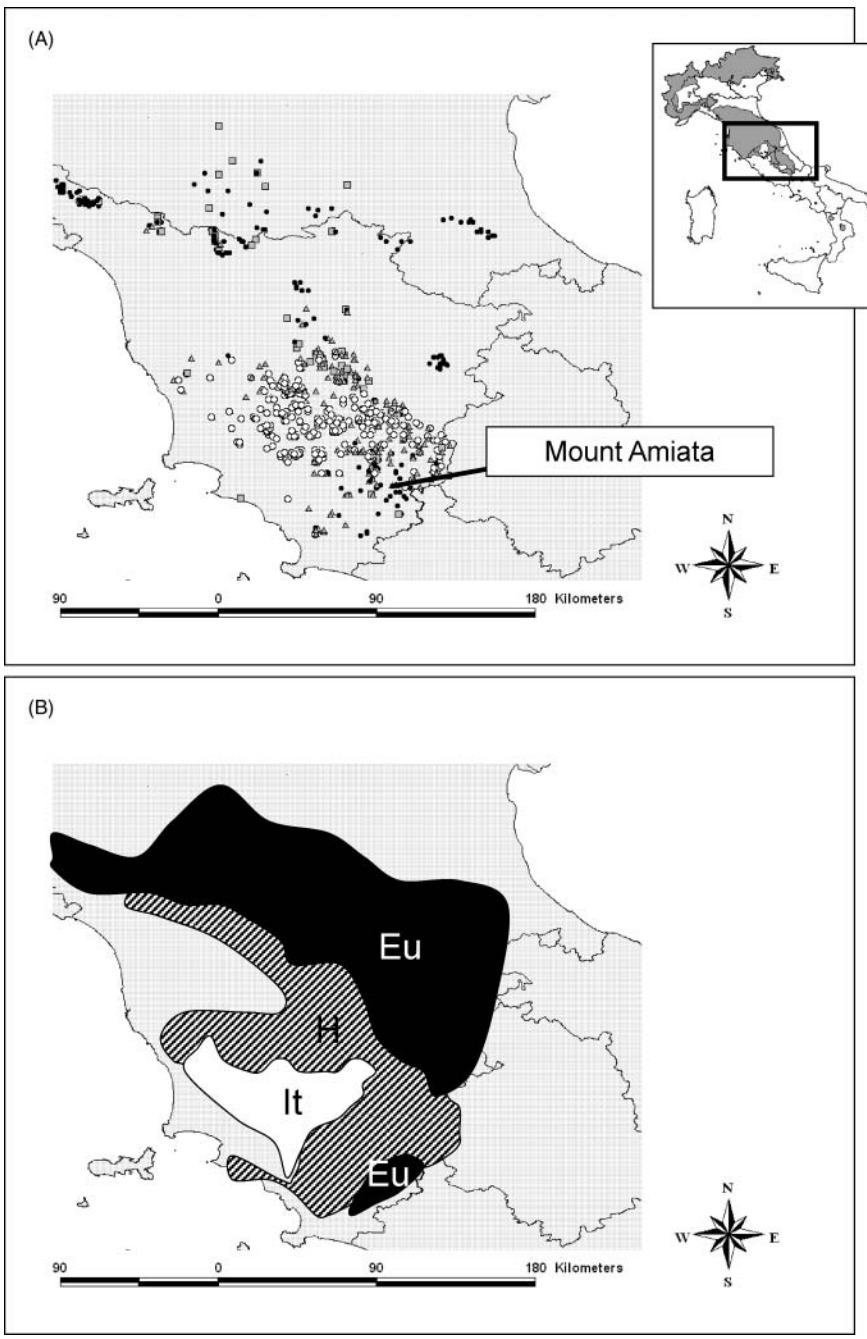


Fig. 6. (A) Distribution of individual membership values (q_i). European and Italian roe deer genotypes are represented respectively by black and white circles if they had an individual membership value > 80%. Individuals showing an individual membership value 20% < q < 80% and an Italian component > 20% are mapped with grey triangles. Grey squares are used to identify only the European admixed individuals. (B) Distribution of remnant populations of *C. c. italicus* (white area) and European roe deer (black areas). Striped areas represent admixture zones between Italian and European roe deer.

Cesena (FC) = 96.96%; Arezzo (AR) = 100%; Massa Carrara (MC) = 95.12%], although in Massa Carrara (MC) a prevalence of mtDNA IT was detected (82.92%). (2) Roe deer from Firenze (FI) were admixed, showing both Italian and European mtDNA; 64.51% of the samples were assigned to a single European roe deer group (Cluster I), 19.35% were split into two or more European roe deer clusters, and 16.12% were admixed. (3) In Grosseto (GR), 40.28% of the samples were assigned to Italian cluster V, all of them showing Italian mtDNA sequences, while 7.19% were associated with both the Italian clusters VI and V. In total, 23.7% were assigned to European cluster II, while the remaining 28.77% of genotypes were admixed, with 60% of them showing Italian mtDNA. (4) In Siena (SI), 47.66% of the samples were assigned to Italian clusters IV and V, but 5.67% of them showed European mtDNA. Around 6.0% of individuals were unequivocally assigned to European clusters I, II or III. Altogether, 35.13% of the samples were admixed and both European and Italian mtDNA lineages were present (62.5% of mtDNA IT in admixed). (5) Admixture was detected in Pisa (PI): around 27% of the samples were assigned to Italian cluster V, but only 56% of them showed mtDNA IT. (6) Genotypes partially assigned to clusters IV and V with $q > 0.80$ and mtDNA IT were considered Italian roe deer. The admixture zones were mapped on ArcView GIS. The main contact areas between the Italian and the European roe deer, as described by the high proportion of admixed individuals, are located in south and southeast Toscana, between the provinces of Siena and Grosseto.

DISCUSSION

In this study, we describe the genetic structure and the geographical distributions of the surviving populations of Italian roe deer, an endemic subspecies endangered by past over-hunting, habitat fragmentation, and by current hybridization with introduced European roe deer. The rapid expansion of the reintroduced alien European roe deer within the historical range of the Italian roe deer, threatens the extinction of the endemic subspecies by genetic admixture and introgression. Active conservation initiatives are, therefore, needed. The Italian roe deer action plan (Focardi *et al.*, 2009) recommended that: (1) pure Italian roe deer individuals and populations should be genetically identified and mapped; (2) hybridization and introgression with alien European roe deer should be constantly monitored; and (3) new pure Italian roe deer populations should be established immediately in suitable Mediterranean areas, located as far as possible from the introduced European roe deer populations. In this study, we implemented a molecular genetic identification protocol and landscape genetic procedure providing the opportunity for the careful identification of roe deer subspecies, populations, and individuals, either pure or admixed, thus offering a useful tool to implement conservation initiatives.

The low mtDNA sequence divergence between the Italian and the European roe deer mtDNA haplotypes [average TN93 $D = 1.1\%$ (Randi *et al.*, 2004)] indicates a recent origin of the Italian subspecies. The Italian roe deer might be considered a southern isolate, which evolved in the Mediterranean regions during the last glacial maximum. Rivers or other geographic barriers (Vernesi *et al.*, 2002), or the ecological and behavioural consequences of local adaptations, might have limited the northward expansion of the Italian roe deer, preventing its admixture with the European roe deer populations distributed in the western Apennines and Alps. Other mammalian species show similar, albeit in some cases relict, phylo-geographic patterns: the Italian hare [*Lepus corsicanus* (Pierpaoli *et al.*, 1999)], the Apennine chamois [*Rupicapra r. ornata* (Rodriguez *et al.*, 2009)], the Italian wolf [*Canis lupus italicus* (Lucchini

et al., 2004)], and otter populations in southern Italy [*Lutra lutra* (Mucci *et al.*, 2010)], confirming the role of southern Italy as a Pleistocene glacial refuge. The reconstruction of late Pleistocene evolutionary events [using the methods of phylogeography (Hofreiter *et al.*, 2004; Kholodova, 2009; Rodriguez *et al.*, 2009)], together with the assessment of recent anthropogenic impacts [through a variety of population and landscape genetic approaches (Manel *et al.*, 2003)], has led to the identification of local populations that are either evolutionarily significant units [ESU (Moritz, 1994)] or conservation significant units [CSU (Whitehead *et al.*, 2004)], which need to be actively preserved.

The Italian roe deer (*Capreolus capreolus italicus*) was described by Festa (1925) as an endemic subspecies distributed in the Mediterranean regions of Italy (see also von Lehmann, 1973). Recent genetic analyses have confirmed the distinctiveness of the Italian roe deer populations (Lorenzini *et al.*, 2002; Vernesi *et al.*, 2002; Randi *et al.*, 2004). Historically, the Italian roe deer populations are likely to have ranged from southern Toscana (Siena and Grosseto) to along the entire western side of the central and southern Apennines. The distribution range on the eastern side of the Apennines was probably much narrower (Boitani *et al.*, 2003) (Fig. 1). The remaining isolated small Italian roe deer populations are confined within two protected areas in southern Italy: the Castelporziano Reserve (close to Rome) and the Gargano National Park (in the Puglia region; Fig. 1). These populations number no more than a few hundred individuals and, although they are not currently threatened by hybridization, their conservation status is not without risks. Both populations have monomorphic mtDNA (Vernesi *et al.*, 2002; Randi *et al.*, 2004; this study) and strongly reduced genetic diversity at microsatellite loci, compared with all the other studied Italian or European roe deer populations (Randi *et al.*, 2004; Lorenzini and Lovari, 2006) (see Table 2). The lack of genetic diversity is a consequence of long-lasting fragmentation and isolation at low effective population size. Both populations are restricted to islands of suitable habitat within a matrix of urbanized or agricultural unsuitable habitats, which makes any future expansion highly improbable, raising the risks of erosion of genetic variability, inbreeding and inbreeding depression. The Italian roe deer in Castelporziano is part of a complex community of large ungulates, including wild boar (*Sus scrofa*), fallow deer (*Dama dama*), and red deer (*Cervus elaphus*) populations. Deep interspecific competitive interactions for habitat and food resources caused a recent dramatic decline of the roe deer population (Focardi *et al.*, 2006). Introduced European roe deer from central and western Europe are already in contact with the Italian roe deer population surviving in the Pollino National Park, which is strongly threatened by hybridization (Gentile *et al.*, 2009). All roe deer populations in the northern Apennine hills suffered long-lasting declines and fragmentation. It is likely these populations were never completely eradicated, and instead small isolates survived in southern Toscana and scattered across the Apennine ridge (Masseti, 2003) (Fig. 6B). All these populations are now expanding, recolonizing most of the species' historical range, leading to zones of admixture between local Italian and introduced European roe deer (Lorenzini *et al.*, 2002; Vernesi *et al.*, 2002; Randi *et al.*, 2004).

The diagnostic haplotypes allowed a straightforward assessment of the distributions of Italian roe deer mtDNAs, which were present in southern Toscana and, surprisingly, also across the Apennine ridge in Emilia-Romagna, north of the putative historical range of *C. c. italicus*. We cannot exclude the presence of undescribed remnant Italian roe deer populations in the north of Toscana. Alternatively, the northern presence of mtDNA IT haplotypes could be due to the recent expansion of natural populations and mtDNA introgression. The presence in southern Toscana of mtDNA EU haplotypes, which have never been detected anywhere else in Italy, witness the genetic consequences of documented

or unofficial introductions of European roe deer (Lorenzini *et al.*, 1996; Masseti, 2003). The presence of haplotypes previously detected only in Germany (H166) and in Eastern Europe (H162, H164) indicates that introduced European roe deer survived, reproduced, and expanded north out of the introduction areas, thus threatening the genetic integrity of the local Italian roe deer. Roe deer males are at least partially territorial during the reproductive period (Bideau *et al.*, 1983). Although the behavioural ecology of the roe deer is poorly understood, it seems that dispersal is male-biased, and females are more philopatric (San José and Lovari, 1998). Consequently, the population structuring described by mtDNA should be stronger than that described by microsatellites. However, most of the populations in the central Apennines are certainly not in demographic and genetic equilibrium, and the observed structuring might have been largely determined by ongoing population expansion and colonization processes. Both Bayesian clustering and landscape genetic analyses of microsatellite genotypes identified populations that are either poorly or strongly admixed. The geographical locations of these populations are concordant with the distribution of the mtDNA IT or EU haplotypes, indicating that pure or hybridizing European and Italian roe deer populations can be identified using the association of both maternal and autosomal DNA markers. In this study, we implemented the following assignment procedure: (1) multilocus microsatellite genotypes were clustered using STRUCTURE and only genetic information; the optimal K number was identified (in this case $K = 6$); (2) individuals assigned to each cluster were split into two groups, according to their mtDNA type, identified as mtDNA IT (Italian roe deer haplotypes) or mtDNA EU (European roe deer haplotypes); (3) the clusters grouping individuals with $q_i > 0.80$ and with mtDNA IT only were identified (that is, clusters IV and V); (4) the robustness of these clusters was tested using BAYESASS, which did not assume HWE; (5) the spatial locations of the genetic clusters were assessed using landscape genetics programs (GENELAND, TESS, and BAPS). This approach could also be used to identify the distributions of local gene pools and admixture areas of recent origin in other species (see, for example, Nielsen *et al.*, 2001; Pearse and Crandall, 2004; Mucci *et al.*, 2010). Although it was possible to outline the existence and the location of hybrid areas, accurate identification of the admixed individuals was more problematic. A few genetic markers might efficiently cluster genetically differentiated populations also at small F_{ST} values (Nielsen *et al.*, 2001; Falush *et al.*, 2003; Latch *et al.*, 2006), but show limited resolution power to identify individual admixed ancestries (Vähä and Primmer, 2006). The use of at least 50–100 microsatellite loci was suggested by Rosenberg *et al.* (2003). Simulations by Vähä and Primmer (2006) showed that detection of hybrid individuals in the first generation was achieved using 12–24 markers, while at least 48 microsatellite loci were necessary to detect backcrosses. In this study, we used eleven microsatellite loci, thus underestimating the occurrence of introgressed individuals in the populations.

The results consistently showed that those individuals showing mtDNA IT haplotypes and $q_i > 0.80$ are distributed not randomly in the sampled space, but they cluster in a geographic area in southern Toscana. This area is surrounded by admixed populations, composed of individuals that show mtDNA IT or mtDNA EU haplotypes and $q_i < 0.80$; this area is identified as the contact and admixture zone between local Italian and introduced European roe deer. Clusters mapping further away from the contact zones included only admixed individuals with introgressed mtDNA IT, or European roe deer genotypes (Figs. 3A, 6A). The current range of the Italian roe deer distribution in Toscana is very restricted: genetically pure Italian roe deer survive only in a core area located in the southern Province of Siena and in the northern Province of Grosseto (~2000 km^2; Fig. 6B).

These populations are surrounded by admixed roe deer populations (Fig. 6B), living in territory that does not present any obvious barrier to further range expansion. The present distribution of released European haplotypes and the location of admixed areas lead us to hypothesize that the main expansion is northward. The main threat is from the released populations inhabiting the southern areas of Tuscany rather than European individuals living in the north and along the edge of the Apennines.

Conservation genetics of the Italian roe deer

Our results indicate that the introduction of alien roe deer strongly affected the genetic composition of native Italian roe deer populations, which are threatened by genetic extinction. The restricted distribution of Italian roe deer shown in Fig. 6B will probably contract further in the near future. In contrast to other species in which it was demonstrated that exogenous individuals possess lower fitness than indigenous individuals (Nielsen *et al.*, 2001), there is no reason to hypothesize that ecological conditions could differentiate the fitness of Italian and European individuals, which have similar ecology and behaviour (Focardi *et al.*, 2009; Gentile *et al.*, 2009). Roe deer released in Mediterranean areas survived and expanded rapidly (Mattioli, 1994; Gentile *et al.*, 2009). The only way to save the Italian population is to identify a large uncontaminated and isolated region in which pure Italian animals should be released. Southern regions of Italy might offer a sanctuary for the maintenance of the subspecies. Reliable genetic identifications are needed to implement conservation actions, which could be based either on the protection of existing Italian roe deer populations (which should be precisely identified and mapped), or on the founding of new Italian roe deer populations by translocation in suitable areas within the subspecies' historical range in southern Italy.

ACKNOWLEDGEMENTS

This study has been supported by the Provinces of Grosseto and Siena. We wish to thank everyone who helped facilitate collection of samples, especially DREAM Italia and UCZNA, and the ATCs of Lombardia, Veneto, Piemonte, Liguria, Emilia Romagna, and Toscana. We want to thank Giampiero Sammuri, Giorgia Romeo, Maddalena Mattii, Sandro Nicoloso, Lilia Orlandi, and Stefano Focardi for their support in the implementation of this study

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