Published by Associazione Teriologica Italiana
Hystrix, the Italian Journal of Mammalogy
Volume 34 (1): 8–13, 2023
OPEN ACCESS
Available online at:
http://www.italian-journal-of-mammalogy.it
doi:10.4404/hystrix-00585-2022

Research Article

Contribution of a native roe deer lineage to the recolonisation of the northern Apennines, Italy

Roberta Lecis[1,*], Paolo Bongi[2], Claudia Russo[3], Francesca Cecchi[3], Marco Del Frate[1], Roberta Chirichella[4], Marco Apollonio[1], Massimo Scandura[1]

[1]*Department of Veterinary Medicine, University of Sassari, Via Vienna 2, Sassari, Italy*
[2]*Ambito Territoriale di Caccia Massa, Largo Bonfigli 3, Aulla, Italy*
[3]*Department of Veterinary Science, University of Pisa, Viale delle Piagge 2, Pisa, Italy*
[4]*Department of Humanities and Social Sciences, University of Sassari, Via Roma 151, Sassari, Italy*

Keywords:
Capreolus capreolus italicus
mtDNA
microsatellites
genetic make-up
admixture
gene flow

Article history:
Received: 12 October 2022
Accepted: 16 January 2023

Acknowledgements
We are grateful to M. Corradi (Parco Regionale Boschi di Carrega), E. Mori, S. Luccarini and L. Mattioli for sample collection, and to N. Mucci (ISPRA) for sharing information on previously published data. We also thank A. Canu and G. Floridia for their assistance in laboratory analyses. The study was supported by the Ambito Territoriale di Caccia Massa, and we thank its president F Merlini, for his collaboration. This study was supported by a grant of the University of Pisa (Ateneo funding 2015/2016 Prof.ssa F. Cecchi and Ateneo funding 2015 Prof.ssa C. Russo) and one of the University of Sassari ("Fondo di Ateneo per la Ricerca 2019" - Prof. M. Scandura).

Abstract

After facing a great decline all over Europe during the past centuries, starting from the second half of the XX century the roe deer (*Capreolus capreolus*) was reintroduced and strongly managed throughout its range, as other ungulate species. Overhunting and habitat change were the main factors threatening roe deer populations in Italy, where small remnant populations of putatively native roe deer survived in a few localities of eastern Alps and central-southern Italy. We investigated the genetic variation of a roe deer population inhabiting the northern Apennines in the province of Massa-Carrara (Tuscany, Italy), analysing both mitochondrial DNA control region and a total of 11 autosomal microsatellite loci, to identify possible sources and recolonisation patterns, as well as the local prevalence of native *Capreolus capreolus italicus* gene pool. Analyses revealed an admixed nature of roe deer in this area, merging both native and non-native lineages, with a dominance of *italicus* haplotypes in the matriline and a majority of non-native genetic component in the autosomal markers. The high similarity with roe deer from neighbouring areas suggests a natural population origin by immigration. Two scenarios may explain the observed pattern of genetic variation: a colonisation by a limited number of immigrants from a single admixed source (either north or south-east), or a two-step recolonisation, firstly from the south, where the *italicus* ancestry was prevalent, and then from the north, mostly by individuals carrying *C. c. capreolus* genes. This study shows the genetic consequences of translocations even in populations not directly targeted by human interventions and highlight how investigating genetic variation might be essential in species management.

Introduction

In the last decades, various ungulate species have been restocked and reintroduced across Europe for conservation and hunting purposes, to reverse the decline they had experienced during the previous century (Apollonio et al., 2014, 2010). Due to these management actions, native and exotic gene pools might have come into contact, leading to hybridisation, introgression and possible decline of local biodiversity (Linnel and Zachos, 2011). Many recent studies on ungulate species such as red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) pointed out that the current population genetic structure is affected by past human manipulations (Hoglund et al., 2013; Mucci et al., 2012; Scandura et al., 2011). Therefore, especially in heavily managed species, maintaining native genetic diversity would be an essential part in the design of management policies.

The roe deer is the most widespread ungulate species in Europe, inhabiting various ecosystems across many countries. After facing a great decline all over Europe during the last century, mainly due to overhunting and habitat loss, this species has been recently reintroduced and strongly managed throughout its range, as other ungulates (Apollonio et al., 2014, 2010). Some fragmented relict populations are inhabiting Mediterranean habitats, in southern Spain and central/southern Italy (Gentile et al., 2008). Italian roe deer populations sharply declined over the XIX century, only surviving in three protected areas in central and southern Italy — Castelporziano (Lazio), Gargano (Puglia) and Orsomarso (Calabria) — as well as in Maremma (Tuscany) (Loy et al., 2019). These nuclei represent remnants of an endemic Italian lineage, classified as a distinct subspecies (*Capreolus capreolus italicus* Festa, 1925, hereafter *Cci*), which is characterised by different body size and coat colour when compared to other roe deer (Boitani et al., 2003; Focardi et al., 2009). It was also reported to carry unique genetic variants in both nuclear and mitochondrial DNA (Biosa et al., 2015; Mucci et al., 2012; Gentile et al., 2008; Lorenzini et al., 2002). On top of this, roe deer belonging to the European subspecies (*Capreolus capreolus capreolus*, hereafter *Ccc*) were repeatedly introduced over the Italian western Alps and Apennines, using stocks originating from the Eastern Alps, Central Europe, and the Balkans (Vernesi et al., 2002). Therefore, different lineages came into contact and concerns were raised about the genetic integrity of the relict Italian subspecies (Mucci et al., 2012). In some areas inhabited by the Italian subspecies, a mixture of individuals with Cci and Ccc ancestries were identified (Biosa et al., 2015; Gentile et al., 2008).

Based on previous studies, Italian roe deer were initially thought to have the Arno River in Tuscany as a natural northern limit (Vernesi et al., 2002). Recent genetic studies identified Italian mtDNA haplotypes beyond this boundary, in Northern Tuscany and Emilia-Romagna (Mucci et al., 2012; Gentile et al., 2008). On the other hand, microsatellite data gathered in some of these areas suggested different degrees of admixture with *Ccc*, resulting from the encounter between individuals descending from non-native introduced stocks and expanding native nuclei (Biosa et al., 2015). The recent spread of the species across

*Corresponding author
Email address: rlecis@uniss.it (Roberta Lecis)

doi:10.4404/hystrix-00585-2022

central Italy leads one to wonder to what extent native roe deer have contributed to the origin of present-day populations.

The Tyrrhenian side of the northern Apennines was recolonised by roe deer only lately and was not directly interested by reintroductions. Roe deer recovery took place in a few decades as a result of natural expansion Carnevali et al. (2009). Two possible routes of recolonisation could have contributed to this process: from the north-east (Parma province) and from the south-east (Lucca province). In a previous study, these sources revealed a different genetic make-up, the former having a prevailing non-native while the latter a prevailing native origin (Biosa et al., 2015). Discovering the origin of expanding population in the northern Apennines is essential to comprehend i) the admixture extent between the two subspecies in this part of Italy and ii) the native roe deer's ability to colonize the northern edge of the Mediterranean biogeographic region, due to its presumed adaptive advantage over non-native individuals. In this study, we investigated the genetic composition of local roe deer populations in the province of Massa-Carrara (Italy), to identify possible sources and recolonisation patterns, as well as the relative abundance of native *Cci* gene pool in this area. As in previous works (Biosa et al., 2015; Mucci et al., 2012), we simultaneously investigated mtDNA and nuclear markers, to successfully identify the different genetic components associated to the *Cci* or *Ccc* ancestry, and compared roe deer from the investigated area with individuals from central-northern Italy, and from areas of southern Tuscany which host the source *Cci* population.

Material and methods

Sampling and study area

A total of 167 new roe deer samples, including muscles and ear tissues, were collected and stored in absolute ethanol or frozen until DNA extraction. Sampling was conducted in Massa-Carrara province (hereafter MAS), the northernmost area in the region of Tuscany (Italy), between the northern Apennines and the Tyrrhenian coast (Fig. 1). A total of 110 roe deer tissue samples were collected between 2010 and 2011 during the regular hunting seasons in four hunting districts. As a reference for the analysis, specimens were also sampled in two areas of central and northern Italy: Casentino (CAS, 21 samples), in the central Apennines, and Trentino (TRE, 36 samples) in the central-eastern Alps (Fig. 1). A total of 167 new roe deer samples, including muscles and ear tissues, were stored in absolute ethanol or frozen until DNA extraction.

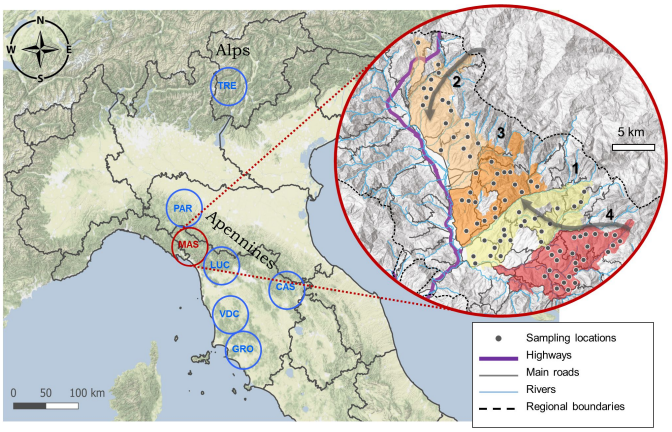

Figure 1 – Geographic representation of the roe deer sampling areas (MAS, CAS, TRE) and reference areas (LUC, PAR, VDC, GRO) in central/northern Italy. Coloured areas in MAS (circled map) refer to the hunting districts for roe deer (numbered from 1 to 4). Arrows indicate the possible routes from neighbouring areas. MAS=Massa; CAS=Arezzo-Casentino; TRE=Trentino; LUC=Lucca; PAR=Parma; VDC=Val di Cecina; GRO=Grosseto.

The roe deer hunting districts in MAS are located on the eastern side of the Magra river running across the province. The hunting area, which spans 150 km^2 and lies between 44°8′ and 44°27′ N, 10°4′ and 9°56′ and 10°4′ E, is characterised by woodland dominated by turkey oak (*Quercus cerris*), chestnut (*Castanea sativa*), and beech (*Fagus sylvatica*). Elevation ranges from 162 to 1238 m a.s.l. The climate is temperate, sub-oceanic, with a mean annual temperature ranging from 10 to 13 °Cand a mean annual precipitation of about 1500 mm (Farina, 1980). Other ungulate species living in the area are red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*). Predators such as wolves (*Canis lupus*) and red foxes (*Vulpes vulpes*) can also be found. In Massa-Carrara province, roe deer population monitoring and selective hunting began in 1997 and has been ongoing ever since (Orlandi et al., 2006). In the last 25 years, roe deer population density fluctuated, with average values ranging from 13.4 head/km^2 in 2017 to 27.0 head/km^2 in 2003 (Bongi, 2018).

During the first half of the XX century, the roe deer was absent in the northern Apennines, with a possible exception in the Casentinesi Forests (Ghigi, 1950, 1917). At that time a nucleus was created in an isolated residual lowland forest of Parma province - north to MAS - with deer originating from the Balkans (Boschi di Carrega Regional Park, pers. comm.). Between 1968 and 1973, 16 *italicus* roe deer coming from Capalbio (Maremma), and subsequently 23 Alpine roe deer from Trento province were released in upper Garfagnana (Lucca province), east to MAS, in the natural reserves of Lamarossa, Orecchiella, and Pania di Corfino (Masseti, 2003; Mattioli, 1994). At the end of the 1970s, roe deer were reported to be present with low numbers in MAS, representing the northernmost part of the Apennine population (Perco, 1981). The species reappeared in the study area apparently as a result of natural expansion from neighbouring areas (i.e. Parma and Lucca provinces), although the occurrence of local undocumented reintroductions cannot be fully ruled out. The population slowly increased in numbers and started to be legally hunted only in 1997, when the first two hunting districts were established (1 and 2 in Fig. 1). Two additional districts were created in the following years, between 2000 and 2003 (3 and 4 in Fig. 1).

Mitochondrial DNA amplification and analysis

DNA extraction was performed using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma), following the manufacturer's instructions for tissue samples. Mitochondrial control region (CR) sequences were amplified using primers LcapPro and HcapPhe, developed by Randi et al. (1998), in a randomly selected subsample (n=44) of the 110 roe deer tissue samples collected in Massa. PCR conditions and protocol were the same as reported by Biosa et al. (2015). PCR products were purified by Exo/SAP digestion and sequenced using the forward primer LcapPro and the BigDye Terminator kit version 3.1 (Applied Biosystems). In order to increase the MAS sample size, our novel sequences were pooled with 42 sequences obtained by Mucci et al. (2012) from roe deer inhabiting the same area. Similarly, homologous sequences representing roe deer inhabiting other geographic areas of central Italy, obtained from previous publications (n=106) were downloaded from GenBank to create a comprehensive alignment (704 bp) for phylogenetic analyses. A *Capreolus pygargus* sequence was used as outgroup to root the tree.

A multiple sequence alignment of 192 roe deer CR sequences was created in MEGA X (Kumar et al., 2018; Tamura et al., 2021), haplotypes were identified using Fabox DNA Collapser (Villesen, 2007), and then named following Biosa et al. (2015). To distinguish haplotypes belonging to the *Cci* (*italicus*) lineage, we followed Randi et al. (2004), differentiating them by a nucleotide deletion at position 103 in the sequence alignment. All other haplotypes not presenting the indel were assigned to a different *Ccc* (*europeus*) mtDNA lineage, either belonging to the Central (C), Western (W) or Eastern (E) clades (Randi et al., 2004). Starting from FASTA sequence alignments, Haplowebmaker (Spöri and Flot, 2020) was used to generate and visualise median-joining networks.

Phylogenetic analyses were performed using the Maximum Likelihood (ML) method implemented in MEGA X (Kumar et al., 2018; Tamura et al., 2021), after testing the best evolutionary model to fit the data. Tamura 3-parameter with discrete Gamma distribution and invariable sites (T92 + G + I) was selected as the best model to analyse our data and to infer roe deer evolutionary history. A discrete

Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter=0.1000). The rate variation model allowed for some sites to be evolutionarily invariable (+I, 45.60% sites). Bootstrap values and reliability of internal branches were calculated from 1000 replicates. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3-parameter model, and then selecting the topology with the highest log likelihood value.

Microsatellite genotyping and analysis

All roe deer DNA samples were genotyped with a panel of 11 polymorphic autosomal microsatellites: Roe01, Roe06, Roe08, Roe09, NV16, NV21, NV24, RT1, ILSTS011, FCB304, BMC1009 (as in Biosa et al., 2015). Loci were amplified in three multiplexed (multiplex A: Roe01, Roe08, Roe09; multiplex B: RT1, NV21, BMC1009; multiplex C: Roe06, NV16, ILSTS011) and single PCRs (NV24 and FCB304). Amplicons were analysed in an ABI PRISM 3730XL Avant automatic sequencer (Applied Biosystems) by BMR Genomics sequencing service (Padova, Italy). Electropherograms were scanned in Peak Scanner 1.0 software (Applied Biosystems).

Novel genotypes were combined with previous data we obtained from 95 individuals sampled in different areas of central Italy, close to the northern border of the Italian subspecies' range (Biosa et al., 2015): Parma (PAR, 29), Lucca (LUC, 23), Val di Cecina (VDC, 18), Grosseto (GRO, 15), Casentino (CAS, 10). These areas encompass a geographic range close to the study area where native or reintroduced *Cci* as well as exotic *Ccc* roe deer are present. VDC and GRO in southern Tuscany were used as reference *Cci* source areas and preferred over pure *Cci* populations from central-southern Italy (i.e. Castelporziano, Orsomarso, Gargano), which are small isolated populations affected by a strong genetic drift (Lorenzini et al., 2002). Therefore, roe deer population genetic make-up was assessed in the following areas: MAS, focus of the study; TRE, reference alpine *Ccc* population; CAS, reintroduced population with a prevailing *Ccc* ancestry; PAR, putative source population resulting from a reintroduction of *Ccc* individuals; LUC, putative source population resulting from mutiple reintroductions (from southern Maremma and from the eastern Alps), but which currently shows the *Cci* component as largely prevalent (PR-A in Biosa et al., 2015); VDC and GRO, reference native *Cci* populations (see Fig. 1).

GENALEX v. 6.5 (Peakall and Smouse, 2012) was used to compute observed heterozygosity (Ho), expected heterozygosity (He), mean number of different alleles per locus (NA), mean number of effective alleles per locus (NE), and F statistics. Deviations from Hardy–Weinberg equilibrium (HWE), per population and locus, and from linkage equilibrium (LE) were assessed in GENEPOP v. 4.7.5 (Raymond and Rousset, 1995). Markov chain parameters were: 1000 as dememorisation number, 100 batches and 1000 iterations per batch. Significance levels were adjusted using Bonferroni's correction. In order to assess levels of real-time gene flow among populations, GENALEX was implemented to perform an assignment test (following Paetkau et al., 2004), using the leave one out option. This calculates the expected genotype frequency at each locus, assuming random mating in the population, multiplies it across loci and log-transform it to obtain log-likelihood values. Each genotype is then assigned to the population with the highest (i.e. least negative) log-likelihood value.

Roe deer data were analysed by Bayesian clustering analysis in STRUCTURE v. 2.3.4 (Hubisz et al., 2009; Falush et al., 2007, 2003; Pritchard et al., 2000). The algorithm uses Bayesian clustering to identify the most likely number of genetic clusters within the dataset and calculates each individual proportional membership to each inferred genetic cluster. We performed 10 independent Markov chain Monte Carlo (MCMC) runs simulating a number of subpopulations (K) ranging from 1 to 10, with the following settings: admixture model, no population information, correlated allele frequencies, 200000 burn-in and 200000 iterations of data collection. The most likely value of K for each section was determined following the ΔK approach developed by Evanno et al. (2005) implemented in Structure Harvester (Earl and VonHoldt, 2012). Pophelper (Francis, 2017) was used to edit STRUCTURE results, visualise outputs and produce the final plots.

Results

Mitochondrial DNA

A total of six mtDNA CR haplotypes were identified in 86 roe deer originating from MAS (44 sequenced in this study and 42 from Mucci et al., 2012): HT13, HT15, HT16, HT43, which were previously detected (Randi et al., 2004), and two novel haplotypes (HT162, HT163), identified in this study for the first time. Among these, HT15 (corresponding to GenBank accession code AY625746) was the most frequent (74.4%) and had been previously found in other areas of central Italy, as well as HT13 (AY625744), which resulted the second most frequent haplotype in MAS (18.6%). All remaining haplotypes had frequencies lower than 3% (7% cumulatively). Between 1 and 14 nucleotide differences were observed among haplotypes, which clustered into three groups in the median-joining network (Fig. 2).

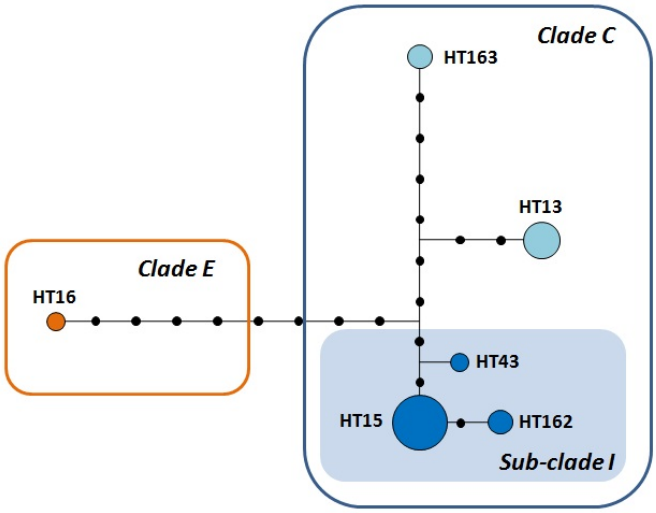

Figure 2 – Median-joining network of the mitochondrial control region haplotypes detected in roe deer from MAS (Massa-Carrara). Circle size is proportional to haplotype frequency in the population. Clade C = Central, Clade E = East, subClade I = *italicus*.

Out of a total of 192 CR-mtDNA sequences originating from roe deer in central Italy, we identified 43 unique haplotypes, which were subsequently used in MEGA X to track their evolutionary history. The Maximum Likelihood tree with the highest log likelihood (-1621.55) is shown in Fig. 3. Phylogenetic relationships among haplotypes sharply identify two main clades (C, Central and E, East). Clade C includes the I (*italicus*) subclade, represented by the *Cci* haplotypes, which however did not receive strong bootstrap support (Fig. 3).

According to the phylogenetic analysis (Fig. 3) and previous clade definition (Randi et al., 2004), three haplotypes (HT15, HT43 and HT162) belonged to the *Cci* lineage (subclade I) which reached an overall frequency of 77.9% in MAS. Two haplotypes (HT13 and HT163, in total 20.9%) were ascribed to clade C, and only HT16 (1.2%) belonged to clade E.

Microsatellites

All 11 microsatellite markers resulted polymorphic in the whole sample and in each population, with the only exception of Roe09, showing a fixed allele in VDC. A total of 103 alleles were found, with N_A ranging from 4 to 13 (mean number of alleles = 9.4 per locus). The most polymorphic loci were Roe08, RT1, and Roe06 (up to 9 alleles in single populations), while Roe01 and Roe09 were the less polymorphic ones across all populations. A low number of population-specific alleles was found. The highest average number of alleles was observed in MAS (N_A=6.36) and the lowest in VDC (N_A=3.73, Tab. 1). Considering N_E, which is not influenced by sample size, the highest allelic diversity was

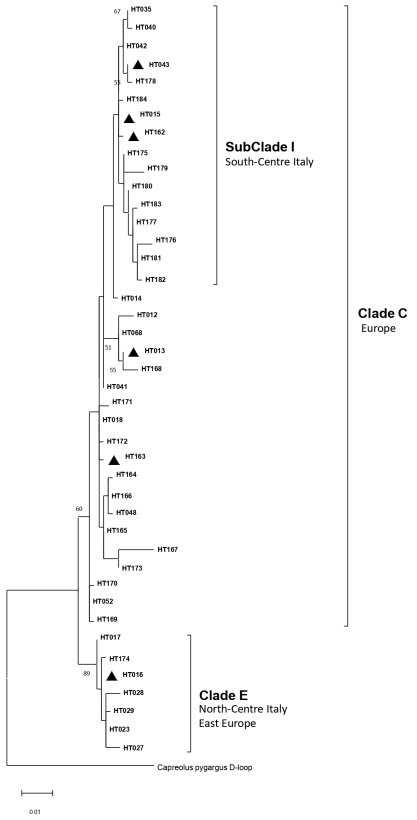

Figure 3 – Maximum likelihood tree of CR-mtDNA haplotypes so far observed in Italian roe deer. Black triangles represent haplotypes occurring in samples from MAS (Massa-Carrara). The tree is drawn to scale, with branch lengths measured in number of substitutions per site. Bootstrap values are shown at the nodes of the tree.

observed in LUC and TRE, which also showed highest heterozygosity levels.

MAS population deviated from Hardy Weinberg equilibrium at 4 out of 11 loci (two at α=0.01 and two at α=0.05) showing heterozygote deficiency. Only VDC and TRE showed similar deviations at just two loci (at α=0.01). The FIS value for MAS however was not particularly high (0.015). No locus pair deviated significantly from LE. Pairwise FST values calculated between MAS and all other populations ranged from 0.022 (MAS-PAR) to 0.116 (MAS-CAS). Levels of differentiation were lower for neighbouring areas (PAR and LUC, F_{ST}=0.022–0.036) and higher for medium to high distanced areas (F_{ST}=0.087–0.116). Consistently with F_{ST} estimations, based on the assignment test, possible real-time gene flow for the MAS population was almost exclusively limited to the nearby PAR and LUC populations (Tab. 2). Only one individual was assigned to a different population (CAS). Similarly, high gene flow was detected for the population pair VDC-GRO, while both most isolated populations (CAS and TRE) showed no individuals assigned to a different population.

When all 262 roe deer samples (originating from all localities) were included in STRUCTURE analysis, K=2 was obtained as the most likely clustering solution, which split MAS, PAR, LUC, VDC and GRO on one side, from CAS and TRE populations (Fig. 4a). A second Bayesian analysis included only the first group, totalling 195 roe deer, and produced again K=2 as the best output. Roe deer from VDC and GRO were assigned to a different genetic cluster than the remaining populations grouping MAS, PAR and LUC (Fig. 4b). A last Bayesian cluster analysis was restricted to this latter group of 162 roe deer and produced again 2 clusters (i.e. K=2) as the most likely partition. However, in this case, the partition was not as sharp as in the previous two analyses, and most MAS individuals were assigned to one of the two resulting clusters with q>0.8. Individuals from PAR showed a similar make-up, while those from LUC were pooled into a single cluster (Fig. 4c).

The stepwise Bayesian analysis clearly showed that: i) the three populations MAS-PAR-LUC are strictly related, ii) they are genetically closer to *Cci* reference populations (VDC-GRO) than to *Ccc* populations (CAS-TRE), iii) two genetic components are identified in the group MAS-PAR LUC. Nonetheless, these two clusters (Fig. 4c) cannot be directly associated with either *Cci* or *Ccc*.

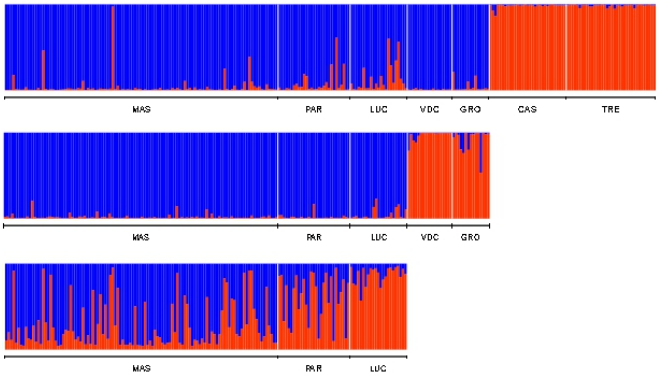

Figure 4 – Bar plots illustrating the genetic structure of analysed roe deer populations in Italy, inferred by Bayesian cluster analysis in STRUCTURE. K=2 was selected as the best clustering option in all three analyses according to the Evanno's method. a) Cluster analysis performed on 262 roe deer genotypes from 7 populations; b) cluster analysis performed on 195 roe deer genotypes from 5 populations; c) cluster analysis performed on 162 roe deer genotypes from 3 populations. Population codes are: MAS - Massa, PAR - Parma, LUC - Lucca, VDC - Val di Cecina, GRO - Grosseto, CAS - Casentino, TRE - Trentino.

Discussion

Natural and human-mediated dispersal processes influenced the recolonisation of the Italian peninsula by wild ungulates. We investigated here the dynamics potentially leading to the settlement of a flourishing roe deer population in central-northern Italy, encompassing the western side of northern Apennines and the hilly range of Massa-Carrara province. Our new genetic data for this area were compared and completed with data from different reference roe deer populations from central and northern Italy. These included two nuclei neighbouring the MAS population: one in the north (PAR) where *Ccc* (non-native) roe deer were introduced in the past century and showing a majority of *Ccc* lineage (nearly 60%, both at mtDNA and autosomal microsatellites, area PR-C in Biosa et al., 2015), and a neighbouring population in the south-east (LUC), where the species was reintroduced with *Cci* (native) individuals and that consistently showed a very high frequency (>70%) of *Cci* ancestry (area PR-A in Biosa et al., 2015). Other reference sampling areas were two native *Cci* populations (VDC and GRO, >80% *Cci* lineage in Biosa et al., 2015), and a native *Ccc* alpine population (TRE), as well as a recovered Apennine population (CAS) which resulted to have >90% *Ccc* ancestry (AR-C in Biosa et al., 2015).

According to mtDNA data, *Cci* haplotypes reach nearly 80% frequency in MAS roe deer, suggesting a prevailing contribution of *italicus* matriline to the origin of this newly established population. Similar results are found in the LUC population, where Italian roe deer were translocated from southern Tuscany in the 1960s–70s (Biosa et al., 2015). However, as previously observed in other studies combining uniparental (maternal) with biparental markers Olano-Marin et al. (2014); Mucci et al. (2012), in the present study autosomal microsatellites tell a different story. Pairwise FST values, assignment test and Bayesian analysis converge in showing a high similarity with both neighbouring areas (LUC and PAR) apparently connected by high levels of gene flow. Unlike mtDNA, however, nuclear markers do not lead to a sharp distinction between *Cci* and *Ccc* lineages, possibly because the used reference *Cci* populations (VDC and GRO) were not 100% pure. Notwithstanding, using purer southern populations (Castelporziano, Orsomarso Gargano) as a reference would not be a better option, as they have long been isolated, undergone a bottleneck and strong genetic drift, and show divergent allele frequencies at fast-

Table 1 – Diversity statistics in roe deer populations from central-northern Italy. N_A – mean number of alleles per locus, N_E – mean number of effective alleles per locus, H_O - observed heterozygosity, H_E - expected heterozygosity, F_{IS} – fixation index. For population codes see Fig. 1.

Population	N	N_A (±SE)	N_E (±SE)	H_O (±SE)	H_E (±SE)	F_{IS} (±SE)
MAS	110	6.36±0.68	2.97±0.36	0.577±0.055	0.602±0.059	0.015±0.060
PAR	29	5.45±0.53	3.28±0.36	0.605±0.049	0.653±0.053	0.039±0.049
LUC	23	5.64±0.69	3.68±0.42	0.718±0.051	0.688±0.056	-0.094±0.055
VDC	18	3.73±0.49	2.19±0.22	0.553±0.091	0.503±0.061	-0.111±0.120
GRO	15	4.64±0.62	2.83±0.35	0.508±0.074	0.593±0.067	0.104±0.077
CAS	31	4.82±0.38	2.71±0.39	0.548±0.055	0.576±0.050	0.031±0.048
TRE	36	6.09±0.64	3.68±0.35	0.690±0.028	0.703±0.040	-0.033±0.082
Overall	262	5.25±0.23	3.05±0.14	0.600±0.023	0.617±0.021	-0.006±0.028

Table 2 – Assignment test analysis performed in GENALEX (Paetkau's method, Paetkau et al., 2004). For each sampled population (in rows) the number of individuals assigned to each candidate population (in columns) is shown, together with the percentage (in brackets). The grey background indicates population groups showing non-negligible levels of gene flow. For population codes see Fig. 1.

		Assigned pop						
Real pop	N	MAS	PAR	LUC	VDC	GRO	CAS	TRE
MAS	110	89 (81%)	9 (8%)	11 (10%)	-	-	1 (1%)	-
PAR	29	6 (21%)	23 (79%)	-	-	-	-	
LUC	23	1 (4%)	1 (4%)	21 (91%)	-	-	-	-
VDC	18	-	-	-	16 (89%)	2 (11%)	-	-
GRO	15	-	-	-	3 (20%)	12 (80%)	-	-
CAS	31	-	-	-	-	-	31 (100%)	-
TRE	36	-	-	-	-	-	-	36 (100%)

evolving molecular markers (such as microsatellites, Lorenzini et al., 2002).

Altogether, the observed patterns can be explained by two different models of recolonisation. As first hypothesis, immigrants might have reached the area from a single source (PAR or LUC), carrying mitochondrial and autosomal alleles of both *Cci* and *Ccc*; but allele frequencies in MAS would have diverged due to the limited number of immigrants and a "founder effect". As an alternative explanation, a two-step recolonisation process might have taken place in MAS. In the first phase, roe deer gradually reached the MAS area from south-east (LUC), where the *Cci* ancestry was common both in maternal and biparental markers. In a second step, immigration mostly involved individuals from PAR, carrying *Ccc* alleles at high frequency. This could explain the higher similarity with PAR at autosomal loci, despite the dominance of *Cci* mtDNA haplotypes. The lower genetic diversity found in MAS roe deer, compared to the putative source populations suggests an origin from a limited number of immigrants.

The latter hypothesis is supported by local hunters' oral accounts, who report that roe deer reappeared first in the south-east of the province, near the border with LUC. Based on the area orography, roe deer migration from LUC might be actually simpler than from PAR. Thus a degree of gene flow from the north, increasing mitochondrial and autosomal *Ccc* alleles into the MAS population, is most likely to have occurred only afterwards. In both cases, one or two putative colonisation waves probably reached the MAS area from east (north-east or south-east), as the Magra river and the A15 highway to the west act as barriers crossing the territory from south to north. Such elements, together with orography (steep mountains, high elevations), may have constrained gene flow in this area leading to a sort of "cul-de-sac" that could explain a local increase of *Ccc* autosomal alleles and *Cci* mtDNA haplotypes by genetic drift. This hypothesis rules out a possible contribution from Ligurian populations (north-west of MAS area), confirmed by the low frequency of E clade haplotypes, that were shown instead to be very common in Liguria (Vernesi et al., 2002; Gentile et al., 2008). However, roe deer living on the western side of Magra river and A15 highway have never been genetically investigated, while some morphological differences from the roe deer living in MAS have been previously reported (Lazzini et al., 2016). Future studies to investigate this population genetic make-up will contribute to clarify the heterogeneous nature of roe deer inhabiting this region.

Observed haplotypes do not clarify the origin of the first immigrants that reached the MAS area, since the two most common ones (HT15 and HT13) were also found in PAR and LUC. Similarly, the only haplotype belonging to the East clade (HT16) was also present in the two neighbouring areas, with a high frequency in LUC (Biosa et al., 2015). Unlike HT15, belonging to the native lineage and occurring in central Italy only, both HT13 and HT16 were previously observed in other areas of central Italy and in the Alps (HT16 also in Germany). Thus, their presence is more likely due to natural short-range immigration than to undocumented release of roe deer from a distant source.

Our results support the idea that a natural expansion took place in the Northern Apennines, with a relevant contribution from the native *Cci* lineage. This recolonisation has brought the Italian subspecies, or its genes, far north of the Arno River, which was previously considered its northernmost geographical boundary (Gentile et al., 2008). Besides demographic and landscape factors, a possible role in roe deer colonisation dynamics might have been played by a putative adaptive advantage of *Cci* over *Ccc*. Recolonised areas lie at the northern edge of the Mediterranean biogeographic region, which represents the zone where the Italian subspecies diverged in allopatry. This should be investigated and would imply that the *Cci* lineage might be less likely to expand further northward.

Similar scenarios, where a native component and allochthonous strains have contributed to the recovery of a local population, were reported in central-south Portugal and north-east Spain (Barros et al., 2020), central Europe (Frosch et al., 2014), as well as in eastern Poland, involving in this case genetic introgression with another species, the Siberian roe deer *C. pygargus* (Olano-Marin et al., 2014).

Different lineages could have mixed in the past in several areas of the roe deer range in Europe as a combination of natural and human-mediated factors. In some cases, admixture jeopardised native gene pools, while in other cases it might have increased genetic diversity and produced new genetic settings contributing to the spread of this species. As previously observed (Mucci et al., 2012), populations in central Italy are basically the result of a secondary contact between native and introduced roe deer. This has apparently not prevented them from expanding to occupy almost all suitable ranges.The presence and prevalence of *Cci* (*italicus*) haplotypes in Massa (MAS) roe deer are indicative of the contribution that this native lineage gave to the northern Apennines recolonisation. Yet admixed, these populations are carri-

ers of endemic genetic diversity that deserves consideration in future informed species management and conservation.

No genetic investigation has compared yet modern roe deer with animals (e.g museum specimens) pre-dating last century translocations; similarly, no study has inspected the possible consequences of admixture on the morphology and ecology of the species. New research on these topics is therefore recommended.

References

Apollonio M., Andersen R., Putman R., 2010. European ungulates and their management in the 21st century. Cambridge University Press, Cambridge, United Kingdom.

Apollonio M., Scandura M., Sprem N., 2014. Reintroductions as a management tool for European Ungulates. In: Putman R., Apollonio M. (Eds.) Behaviour and management of European ungulates. Whittles Publishing, Dunbeath, Scotland. 46–77.

Barros T., Ferreira E., Rocha R.G., Brotas G., Carranza J., Fonseca C., Tinoco Torres R., 2020. The multiple origins of roe deer populations in Western Iberia and their relevance for conservation. Animals. 10: 2419.

Biosa D., Scandura M., Tagliavini J., Luccarini S., Mattioli L., Apollonio M., 2015. Patterns of genetic admixture between roe deer of different origin in central Italy. J. Mammal. 96(4): 827–838.

Boitani, L., Lovari, S., Vigna Taglianti, A., 2003. Mammalia III. Carnivora, Artiodactyla. Fauna d'Italia. Bologna: Calderini. Vol. 35.

Bongi P., 2018. Management plan of roe deer population in Massa-Carrara province. Technical report for Tuscany regional administration.

Carnevali L., Pedrotti L., Riga F., Toso S., 2009. Banca Dati Ungulati: status, distribuzione, consistenza, gestione e prelievo venatorio delle popolazioni di Ungulati in Italia. Report 2001-2005. Biol. Cons. Fauna. 117: 1–168. [in Italian]

Earl D.A., VonHoldt B.M., 2012. Structure Harvester: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4: 359–361.

Evanno G., Regnaut S., Goudet J., 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. Mol. Ecol. 14: 2611–2620.

Falush D., Stephens M., Pritchard J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164: 1567–1587.

Falush D., Stephens M., Pritchard J.K., 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol. Ecol. Notes 7: 574–578.

Farina A., 1980. Lunigiana: the environment and its characters. Itinerari Educativi, Centro Aullese di Ricerche e Studi Lunigianesi, Aulla.

Festa E., 1925. Il capriolo dell'Italia centrale. Bollettino del Museo di Zoologia e Anatomia Comparata dell'Università di Torino. 37: 1–2. [in Italian]

Focardi S., Montanaro P., La Morgia V., Riga F., 2009. Piano d'azione nazionale per il Capriolo italico (*Capreolus capreolus italicus*). Quad. Cons. Natura, Min. Ambiente – ISPRA. Vol. 31. [in Italian]

Francis R.M., 2017. Pophelper: an R package and web app to analyse and visualize population structure. Mol. Ecol. Resour. 17: 27–32.

Frosch C., Kraus R., Angst C., Allgower R., Michaux J., Teubner J., Nowak C., 2014. The genetic legacy of multiple beaver reintroductions in Central Europe. PloS ONE. 9(5): e97619.

Gentile G., Vernesi C., Vicario S., Pecchioli E., Caccone A., Bertorelle G., Sbordoni V., 2008. Mitochondrial DNA variation in roe deer (*Capreolus capreolus*) from Italy: evidence of admixture in one of the last *C. c. italicus* pure populations from central-southern Italy. Ital. J. Zool. 76: 16–27.

Ghigi A., 1917. I Mammiferi d'Italia considerati nei loro rapporti coll'agricoltura. Natura VIII, 85–137. [in Italian]

Ghigi A., 1950. La Vita degli Animali. I Mammiferi ed Uccelli sedentari delle terre continentali. U.T.E.T. Milano, Italy. [in Italian]

Hoglund J., Cortazar-Chinarro M., Jarnemo A., Thulin C.G., 2013. Genetic variation and structure in Scandinavian red deer (*Cervus elaphus*): influence of ancestry, past hunting, and restoration management. Biol. J. Linn. Soc. 109: 43–53

Hubisz M.J., Falush D., Stephens M., Pritchard J.K., 2009. Inferring weak population structure with the assistance of sample group information. Mol. Ecol. Resour. 9: 1322–1332.

Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.

Lazzini C., Del Frate M., Baruffetti M., Bongi P., 2016. Morphological traits in two roe deer populations hunted in north-western Apennine. Proceedings of the X Italian Congress of Theriology, Acquapendente, Italy.

Linnel, J., Zachos F., 2011. Status, distribution patterns of European ungulates: genetics, population history and conservation. In: Putman R., Apollonio M., Andersen R. (Eds.) Ungulate management in Europe. Cambridge University Press, Cambridge, UK. 12–53.

Lorenzini, R., Lovari, S., Masseti, M., 2002. The rediscovery of the Italian roe deer: Genetic differentiation and management implications. Ital. J. Zool. 69: 367–379.

Loy A., Aloise G., Ancillotto L., Angelici F.M., Bertolino S., Capizzi D., Castiglia R., Colangelo P., Contoli L., Cozzi B., Fontaneto D., Lapini L., Maio N., Monaco A., Mori E., Nappi A., Podestà M.A., Russo D., Sarà M., Scandura M., Amori G., 2019. Mammals of Italy: an annotated checklist. Hystrix It. J. Mamm. 30(2): 87–106. doi:10.4404/hystrix-00196-2019

Masseti M., 2003. Fauna Toscana. Galliformi non migratori, lagomorfi e artiodattili. Firenze. [in Italian]

Mattioli S., 1994. La reintroduzione dei Cervidi in Italia con particolare riferimento all'Appennino. La fauna appenninica e la sua conservazione. Airone, CFS. Caramanico (PE). 19–25.

Mucci, N., Mattucci F., Randi. E., 2012. Conservation of threatened local gene pools: Landscape genetics of the Italian roe deer (*Capreolus c. italicus*) populations. Evol. Ecol. Res. 14: 897–920.

Olano-Marin J., Plis K., Sönnichsen L., Borowik T., Niedziałkowska M., Jędrzejewska B., 2014. Weak population structure in European roe deer (*Capreolus capreolus*) and evidence of introgressive hybridization with Siberian roe deer (*C. pygargus*) in Northeastern Poland. PLoS ONE. 9(10): e109147.

Orlandi L., Leonessi L., Visani G., 2006. Roe deer management in Massa-Carrara province. Mori eds. Massa (MS).

Paetkau D., Slade R. Burden M., Estoup A., 2004. Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Mol. Ecol. 13: 55–65.

Peakall R., Smouse P.E., 2012. GenAlEx 6.5: Genetic Analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics. 28: 2537–2539.

Perco F., 1981. Capriolo. In: Pavan M., Boera B. ()Eds.) Distribuzione e biologia di 22 specie di Mammiferi in Italia. C.N.R. Roma, Italy. 147–152. [in Italian]

Pritchard J.K., Stephens M., Donnelly P., 2000. Inference of population structure using multilocus genotype data. Genetics. 155: 945–959.

Randi E., Pierpaoli M., Danilkin S.A., 1998. Mitochondrial DNA polymorphism in populations of Siberian and European roe deer (*Capreolus pygargus* and *C. capreolus*). Heredity. 80: 429–443.

Randi E., Alves P.C., Carranza J., Milosevic-Zlatanovic S., Sfougaris A., Mucci N., 2004. Phylogeography of roe deer (*Capreolus capreolus*) populations: the effects of historical genetic subdivisions and recent non-equilibrium dynamics. Mol. Ecol. 13: 3071–3083.

Raymond M, Rousset F., 1995. An exact test for population differentiation. Evolution. 49 (6): 1280–1283.

Scandura M., Iacolina L., Apollonio M., 2011. Genetic diversity in the European wild boar (*Sus scrofa*): phylogeography, population structure and wild x domestic hybridization. Mamm. Rev. 41: 125–137.

Spöri Y., Flot J., 2020. HaplowebMaker and CoMa: two web tools to delimit species using haplowebs and conspecificity matrices. Methods Ecol. Evol. 11(11): 1434–1438.

Tamura K., Stecher G., Kumar S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol. Biol. Evol. 38(7): 3022–3027.

Vernesi C., Pecchioli E., Caramelli D., Tiedemann R., Randi E., Bertorelle G., 2002. The genetic structure of natural and reintroduced roe deer (*Capreolus capreolus*) populations in the Alps and central Italy, with reference to the mitochondrial DNA phylogeography of Europe. Mol. Ecol. 11: 1285–1297.

Villesen P., 2007. FaBox: an online toolbox for fasta sequences. Mol. Ecol. Notes. 7(6): 965–968.

Associate Editor: R. Caniglia