

Assessing the distribution and genetic diversity of the broom hare, *Lepus castroviejo*, for improving conservation planning

José Diogo Ribeiro Rito da Costa

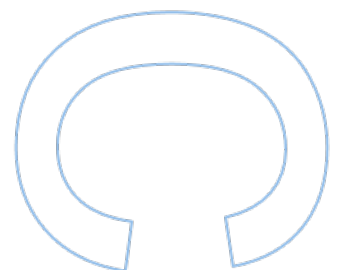
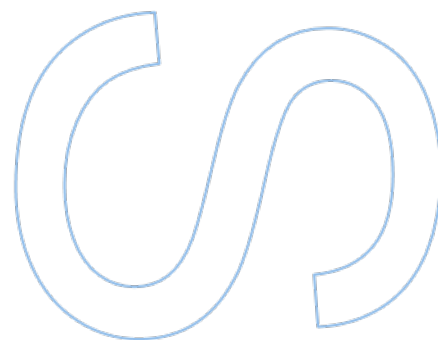
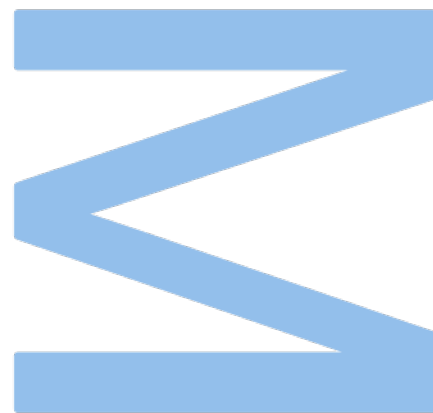
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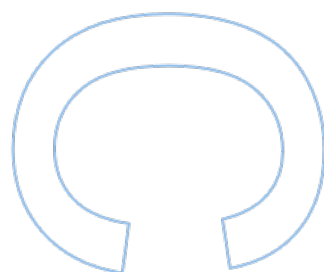
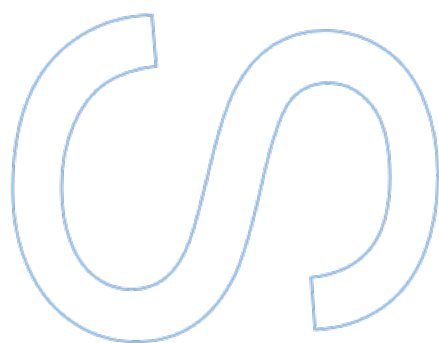
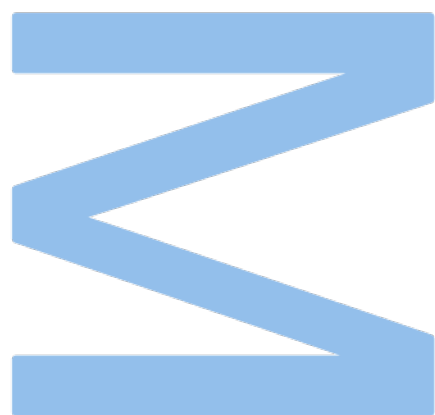
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Dedicated to my mother

Sworn Statement

I, José Diogo Ribeiro Rito da Costa, enrolled in the Master's Degree in Biodiversity, Genetics and Evolution at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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José Costa

30 September 2022

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Resumo

A lebre de piornal (*Lepus castroviejo*) é uma espécie ameaçada endémica da Península Ibérica, para a qual existe pouco conhecimento, principalmente em relação à sua história evolutiva, diversidade genética e demografia. Neste trabalho, utilizamos amostragem genética não invasiva (gNIS; n=185) em conjunto com recolha oportunista de espécimes (n=22) para estudar a diversidade genética, estrutura populacional, avaliar os limites da distribuição e inferir a possível hibridização com espécies de lebres vizinhas utilizando um painel de 15 microssatélites e um fragmento de 541 bp do gene mitocondrial citocromo-b (cytb). Amostras de várias populações da lebre Europeia (n=81, *L. europaeus*), da lebre Variável (n=77, *L. timidus*), da lebre Ibérica (n=68, *L. granatensis*) e da lebre Italiana (n=20, *L. corsicanus*) foram analisadas para comparar de forma precisa os padrões de diversidade genética e inferir hibridização. A identificação de espécies a partir das amostras gNIS foi feita utilizando polimorfismos localizado em pequenos fragmentos dos genes cytb (108bp) e transferrina (TF; 240bp), sendo a identificação de indivíduos obtida a partir de um painel de 11 microssatélites polimórficos para a espécie. A lebre de piornal (n=76) apresentou os níveis mais baixos de diversidade genética nuclear ($N_a=2.53$, $H_o=0.168$, $H_e=0.186$ e $F_{is}=0.101$), e níveis muito baixos de diversidade genética mitocondrial ($H_d=0.743$ e $\pi=0.01543$). Dois grupos foram inferidos a partir de análises Bayesianas segregando os indivíduos do Oeste e Este das montanhas Cantábricas. Não foram encontrados indícios de efeito *bottleneck* recentes para esta espécie. Indícios de hibridização desta espécie com a lebre Ibérica e a lebre Europeia também não foram detetados com os marcadores genéticos nucleares utilizados. No entanto, duas linhagens de DNA mitocondrial foram detetadas na lebre de piornal, o que de acordo com trabalhos anteriores corresponde a eventos de introgressão antigos de origem na Lebre variável. Este trabalho demonstra os benefícios na utilização de amostragem genética não-invasiva e enfatiza os níveis extremamente baixos de diversidade genética da lebre de piornal, reforçando a necessidade de definir medidas para proteger e continuar o estudo desta espécie ameaçada e endémica e da Península Ibérica.

Palavras-chave: Genética da Conservação, Genética Populacional, Estrutura Populacional, Amostragem Genética Não Invasiva (gNIS), Espécies Ameaçadas, Lebre de piornal, *Lepus castroviejo*, Diversidade Genética, Microssatélites.

Abstract

The broom hare (*Lepus castroviejo*) is a threatened Iberian endemism, restricted to Northwest Spain, for which there is very limited knowledge, particularly regarding its evolutionary history, population structure, genetic diversity, and demographic history. Genetic non-invasive sampling (gNIS; n=185) combined with opportunistic collection of specimens (n=22) was employed to unveil the genetic diversity, population structure and to assess the limits of the distribution range of this species as well as infer admixture with neighbouring hare species using a panel of 15 microsatellites and a 541bp fragment of the mitochondrial (mtDNA) cytochrome-b (cytB) gene. Additional samples from several populations of the European hare (n=81, *L. europaeus*), the mountain hare (n=77, *L. timidus*), the Iberian hare (n=68, *L. granatensis*) and the Italian hare (n=20, *L. corsicanus*) were analysed to accurately compare the genetic diversity patterns and infer admixture. Species ID from gNIS was inferred using polymorphisms located in small fragments of cytB (108bp) and Transferrin (TF;240bp) genes, while individual ID was obtained from a panel of 11 polymorphic loci for this species. The broom hare (n=76) showed the lowest levels of nuclear diversity ($N_a=2.53$, $H_o=0.168$, $H_e=0.186$ and $F_{is}=0.101$), and very low mitochondrial ($H_d=0.743$ and $\pi=0.01543$) diversity. Two shallow clusters were inferred from Bayesian analysis, segregating individuals from west and east of the Cantabrian Mountains. No recent population bottleneck for the species nor hybridization with the neighbouring European hare and Iberian hare were detected with the analysed nuclear genetic markers. However, two mitochondrial DNA lineages were detected in the broom hare, which according to previous work correspond to ancient events of introgression of mountain hare origin. This work emphasizes the extremely low levels of genetic diversity of the broom hare and highlights the need to underline measures to continue studying this endemic threatened species.

Keywords: Conservation genetics, Population Genetics, Population Structure, Genetic non-invasive sampling (gNIS), threatened species, Broom hare, *Lepus castroviejo*, Genetic diversity, Microsatellites.

Table of Contents

Acknowledgements.....	ii
Resumo	iv
Abstract.....	v
List of Tables	viii
List of Figures	ix
Supplementary Information.....	xi
List of Abbreviations	xv
1. Introduction	1
1.1. Species Conservation	1
1.2. The importance of genetic diversity	1
1.3. Genetic non-invasive sampling (gNIS).....	2
1.4. Molecular tools	3
1.5. The broom hare.....	5
1.6 Objectives	9
2. Materials and Methods	10
2.1. Study area.....	10
2.2. Sample collection	10
2.3. DNA extraction and species ID	11
2.4. Microsatellite genotyping and individual ID.	11
2.5. Amplification of the longer mitochondrial Cytochrome-b fragment.....	13
2.6. Data analysis.....	14
3. Results.....	18
3.1. Species and individual ID of the broom hare gNIS	18
3.2. Microsatellite markers and nuclear genetic diversity.....	19
3.3. Population differentiation and structure.....	24
3.4. Mitochondrial diversity and phylogeography	27
3.5. Demographic and evolutionary history	30

4. Discussion	32
5. Conclusion	36
6. Future Prospects	37
7. References	39
8. Supplementary Information.....	55

List of Tables

Table 1. Microsatellite markers and multiplex PCR conditions used in this study.	13
Table 2. Micro-checker outputs for the putative presence of null alleles and scoring errors by microsatellite marker and population analysed. Scoring errors marked by an x and null alleles marked with an asterisk.	20
Table 3. Nuclear genetic diversity parameters inferred from 15 microsatellite markers for all populations sampled in this study.	21
Table 4. Nuclear genetic diversity parameters inferred for all analysed populations using the 11 polymorphic microsatellite markers in the broom hare.	22
Table 5. Mitochondrial genetic diversity parameters inferred for the broom hare (<i>L. castroviejo</i>), the European hare (<i>L. europaeus</i>), the Iberian hare (<i>L. granatensis</i>), the mountain hare (<i>L. timidus</i>) and the Italian hare (<i>L. corsicanus</i>) utilizing the 541bp cytb haplotype sequences.	28
Table 6. Fu's and Tajima's D test statistics inferred for the broom hare using the 541bp fragment of cytochrome-b gene.	31

List of Figures

Figure 1. The broom hare (*Lepus castroviejo*). Credit: uploaded by Marc in: <https://www.biodiversity4all.org/photos/157176339>..... 5

Figure 2. Distribution range of the European hare (Hackländer and Schai-Braun 2019), Iberian hare (Soriguer and Carro 2019), mountain hare (Smith and Johnston 2019), Italian hare (Randi and Riga 2019) and broom hare from (Ballesteros and Smith 2019) and respective sampled populations: EUR1, EUR2, EUR3 and EUR4 for the European hare; GRA1, GRA2 and GRA3 for the Iberian hare; TIM1 and TIM2 for the mountain hare; and COR1 for the Italian hare (right). Broom hare distribution range (Ballesteros and Smith 2019) and location of the collected broom hare samples (left). Sampling locations for genetic non-invasive samples (gNIS) are shown as green circles, while tissue samples are shown as triangles: red for samples collected in 2019/21 and blue for samples collected in 2003/07. 6

Figure 3. Distribution map of broom hare individuals identified through microsatellite analysis utilizing the 15-microsatellite panel. Broom hare distribution outlined in black. Individuals detected through gNIS are shown as orange circles, while individuals collected by hunting or roadkill are shown as triangles: red for individuals collected in 2019/21 and blue for individuals collected 2003/2007 19

Figure 4. Values of number of alleles, allelic richness, observed and expected heterozygosity obtained for each one of the 15 microsatellite markers and populations analyzed in this study. Statistically significant differences between the broom hare (*L. castroviejo*) and the other hare species: European hare (*L. europaeus*), Iberian hare (*L. granatensis*), mountain hare (*L. timidus*) and Italian hare (*L. corsicanus*) - are represented by a red asterisk. 23

Figure 5. Values of mean number of alleles, allelic richness, observed and expected heterozygosity obtained from the literature (black dots) and values obtained for each one of the populations analyzed in this work using the 15 microsatellite markers (red dots). Values of statistically significant differences between the broom hare (*L. castroviejo*) and the other hare species: European hare (*L. europaeus*), Iberian hare (*L. granatensis*),

mountain hare (*L. timidus*) and Italian hare (*L. corsicanus*) are represented by a red asterisk. 24

Figure 6. Population differentiation and structure: **a)** Neighbor-Joining tree inferred from the pairwise Nei genetic distance among populations of each species (European hare - *L. europaeus*, EUR1, EUR2, EUR3, EUR4); Iberian hare - *L. granatensis*, GRA1, GRA2, GRA3; mountain hare - *L. timidus*, TIM1, TIM2; Italian hare - *L. corsicanus*, COR1; and broom hare - *L. castroviejo*, CAS1). Node labels represent bootstrap support for each node, while branch labels represent branch length according to Nei's standard genetic distance; **b)** Factorial correspondence analysis (FCA) plot obtained for the hare populations and using the 15 microsatellite markers ; **c)** Bayesian clustering results obtained in STRUCTURE for K=4 (the best DeltaK value) using all hare populations and 15 microsatellite markers. Bar color represents the membership proportion (qi) obtained for each cluster; **d)** Bayesian clustering results obtained in STRUCTURE for k =2 (the best DeltaK value) using only broom and Italian hare populations and 15 microsatellite markers; **e)** Bayesian clustering results obtained in STRUCTURE for K=2 (the best DeltaK value) using only the broom hare population and the 11 polymorphic markers for this species. Pie charts represents the individual membership proportion obtained for each cluster. 25

Figure 7. Factorial Correspondence Analysis (FCA) plot obtained for the 11 microsatellite loci using only the broom hare population (CAS1), divided by sampling periods (2003/2007 and 2019/2021). 26

Figure 8. Isolation-by-Distance. Mantel test performed utilizing GenAlEx to test the occurrence of a correlation between the genetic relatedness of individuals (QG Genetic Distance) and the logarithm of the geographic distance in Km. Each blue dot represents a pairwise comparison between two samples, trendline is displayed in orange and correlation coefficient (R2) displayed on the top right of the graph. The genetic and geographic distances had a negative significant correlation ($R_{xy} = -0.316$ $p = 0.001$). ... 27

Figure 9. Values of haplotype and nucleotide diversity obtained from the literature for the European hare (*L. europaeus*), the Iberian hare (*L. granatensis*), the mountain hare (*L. timidus*) and the Italian hare (*L. corsicanus*) (black dots) and same values

calculated for the broom hare (*L. castroviejo*) for the 541 cythochrome-b fragment analyzed (red dots). 28

Figure 10. Mitochondrial cytochrome-b haplotypes (541bp) found in this study for the broom hare. Median joining network estimated using PopART (Bandelt, Forster, and Röhl 1999; Leigh and Bryant 2015). Size of haplotypes is proportional to the number of samples; dashes represent number of mutations between each haplotype. The haplotypes belonging to the mountain hare (*L. timidus*) lineage introgressed in the Iberian (*L. granatensis*) and European (*L. euroapeus*) are outlined in red. 29

Figure 11. Distribution map of the broom hare haplotype lineages. Broom hare distribution outlined in black. Individuals carrying the identified haplotype lineages are represented by coloured dots. 29

Figure 12. Divergence time tree estimated with BEAST2 and calibrated with the *Lepus-Oryctolagus* and *L. corsicanus* – *L. castrovejoi* divergence times. Nodes labels represent divergence time in million years before the present and posterior probability, respectively. 30

Figure 13. Bayesian skyline plot (BSP) calculated using the 541 bp mitochondrial cytochrome-b fragment of the broom hare and calibrated with a mutation rate of 1% per Mya. 31

Supplementary Information

Table S1. Number of the broom hare samples collected at each Spanish Municipality. 55

Table S2. Summary of the nuclear genetic diversity parameters compiled in the literature for the European hare species..... 56

Table S3. Summary of the mitochondrial genetic diversity parameters compiled in the literature for the European hare species..... 57

Table S4. Maximum likelihood genotyping error rates for the polymorphic microsatellites of the broom hare individuals detected trough gNIS.....	57
Table S5. Probability of Identity (PI) and Probability of Identity considering siblings (PIsibs) of all analysed species utilising the 15 microsatellite panel.....	58
Table S6. Probability of Identity(PI) per locus for all analysed species.	58
Table S7. Probability of Identity considering siblings (PIsibs) per locus for all analysed species.....	58
Table S8. Statistical results of species pairwise comparisons of the nuclear genetic diversity parameters inferred for the studied populations using the 15 microsatellite markers.....	59
Table S9. Statistical results of species pairwise comparisons of the nuclear genetic diversity parameters inferred for the studied populations using the 15 microsatellite markers and all data available in the literature for the European hare species.	59
Table S10. Pairwise Nei's standard genetic distance matrix obtained for the studied populations using the 15 microsatellite markers.....	60
Table S11. Pairwise Fst matrix obtained for the studied populations using the 15 microsatellite markers.....	60
Table S12. Evanno table for the STRUCTURE analysis considering only broom hare individuals and the 11 polymorphic loci.	61
Table S13. Statistical results of species pairwise comparisons of the mitochondrial genetic diversity parameters inferred for the studied populations using the 541bp fragment of cytochrome-b gene and all data available in the literature for the European hare species.	61

Table S14. Statistical results of species pairwise comparisons of the nuclear genetic diversity parameters inferred for the studied populations using the 11 polymorphic microsatellite markers in the broom hare..... 62

Figure S1. Neighbor-joining tree inferred from the pairwise F_{st} distances among populations of each species: European hare - *L. europaeus*, EUR1, EUR2, EUR3, EUR4; Iberian hare - *L. granatensis*, GRA1, GRA2, GRA3; mountain hare - *L. timidus*, TIM1, TIM2; Italian hare - *L. corsicanus*, COR1; and broom hare - *L. castroviejo*, CAS1. Branch labels represent branch length according to the pairwise F_{st} distances 63

Figure S2. Bayesian clustering results obtained in STRUCTURE using all hare populations analyzed (European hare: EUR1, EUR2, EUR3 and EUR4; Iberian hare: GRA1, GRA2 and GRA3; mountain hare: TIM1 and TIM2; Italian hare: COR1 and broom hare: CAS1) and the 15 microsatellite markers: a) DeltaK vales for each value of K calculated trough the method of Evanno; b) Structure results for K equal 2, 4 and 7. Bar color represents the membership proportion (q_i) obtained for each cluster. 64

Figure S3. Bayesian clustering results obtained in STRUCTURE using the Italian (COR1) and broom hare (CAS1) populations and the 15 microsatellite markers: a) DeltaK vales for each value of K calculated trough the method of Evanno; b) Structure results for k equal 2, 3 and 4. Bar color represents the membership proportion (q_i) obtained for each cluster. Minor clusters represent the clusters that were not supported by the majority of replicates. 65

Figure S4. Bayesian clustering results obtained in STRUCTURE using the European hare (EUR1, EUR2, EUR3 and EUR4), the Iberian hare (GRA1, GRA2 and GRA3) and the mountain hare (TIM1 and TIM2) and the 15 microsatellite markers: a) DeltaK vales for each value of K calculated trough the method of Evanno; b) Structure results for the best Deltak value (K=3). Bar color represents the membership proportion (q_i) obtained for each cluster. 65

Figure S5. Bayesian clustering results obtained in STRUCTURE using the Broom hare population (CAS1) and the 11 microsatellites polymorphic for this species: a) DeltaK

values for each value of K calculated trough the method of Evanno; b) Structure results for k equal 2 and 4. Bar color represents the membership proportion (qi) obtained for each cluster. 65

Figure S6. Bayesian inference phylogenetic tree for all the available *Lepus* cytochrome-b haplotypes in NCBI (541bp). Nodes are labelled with Bayesian probability, Broom hare haplotypes are highlighted in red..... 65

Figure S7. Maximum likelihood phylogenetic tree for all the available *Lepus* cytochrome-b haplotypes in NCBI (541bp). Nodes are labelled with bootstrap support, Broom hare haplotypes are highlighted in red..... 65

List of Abbreviations

FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
UP	UNIVERSITY OF PORTO
GNIS	GENETIC NON-INVASIVE SAMPLING
CYTB	CYTHOCROME-B
TF	TRANSFERRIN
MTDNA	MITHOCONDRIAL DNA
BP	BASE PAIRS
NA	NUMBER OF ALLELES
HO	OBSERVED HETEROZYGOSITY
HE	EXPECTED HETEROZYGOSITY
AR	ALLELIC RICHNESS
FIS	INBREEDING COEFFICIENT
HP	HAPLOTYPE DIVERSITY
Π	NUCLEOTIDE DIVERSITY
ID	IDENTIFICATION
IUCN	INTERNATIONAL UNION FOR THE CONSERVATION OF NATURE
WWF	WORLDWIDE FUND FOR NATURE
MTDNA	MITHOCONDRIAL DNA
PCR	POLYMERASE CHAIN REACTION
RAPD	RANDOM AMPLIFIED POLYMORPHIC DNA
PI	PROBABILITY OF IDENTITY
PISIBS	PROBABILITY OF IDENTITY CONSIDERING SIBLINGS
FST	FIXATION INDEX
FCA	FACTORIAL CORRESPONDANCE ANALYSIS
NCBI	NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION
AIC	AKAIKE INFORMATION CRITERION
ML	MARGINAL LIKELIHOOD
BF	BAYES FACTOR
MRCA	MOST RECENT COMMON ANCESTOR
MCMC	MARKOV CHAIN MONTE CARLO
HWE	HARDY-WINBERG EQUILIBRIUM
LE	LINKAGE EQUILIBRIUM
NJT	NEIGHBOUR JOINING TREES

NE	EFFECTIVE POPULATION SIZE
YBP	YEARS BEFORE THE PRESENT
BSP	BAYESIAN SKYLINEPLOT
MYA	MILLION YEARS AGO
KYA	THOUSAND YEARS AGO
SNP	SINGLE NUCLEOTIDE POLYMORPHISM

1.Introduction

1.1. Species Conservation

The need for species conservation arises due to the rapid depletion of the planet's biological diversity as a direct or indirect consequence of human actions. Habitat destruction, deforestation, overexploitation, introduced species, poaching, culling, pollution, and climate change are some of the leading causes of species extinction (Shaffer 1981). According to the International Union for the Conservation of Nature (IUCN) 27% of mammals, 13% of birds, 37% of sharks and rays and 28% of reptiles are threatened with extinction (IUCN 2022). In total, more than 41,000 species are threatened with extinction worldwide (IUCN 2022). The current rate of extinction is at least 100 to 1,000 times higher than the background rate (Sukhdev et al. 2010; Ceballos et al. 2015), which is the natural rate of extinction in earth's history before human pressure became prominent.

The importance of species conservation and the preservation of biodiversity is rooted on the economic value of bioresources, ecosystem services, aesthetics, and the rights of living organisms to exist.

1.2. The importance of genetic diversity

The differences between DNA sequences of distinct individuals are what forms the genetic diversity of a species.

Genetic diversity is an important proxy for population fitness and adaptive potential, representing a key factor in the evolution of populations in response to environmental changes (Reed & Frankham 2003; Kardos et al. 2021). Due to its importance, the conservation of genetic diversity is recognized as one of the key aspects for biodiversity preservation. The conservation of intraspecific genetic diversity gains additional importance in the conservation and status assessment of endemic species. Due to their often limited distribution range, small population size and low genetic variability, endemic species are at increased risks of extinction (Işık 2011). Endemic species are also more likely to be negatively affected by genetic drift and inbreeding depression which increase the chances of offspring being affected by deleterious traits (Charlesworth & Willis 2009).

The importance of neutral genetic diversity as a proxy for assessing extinction risk is currently controversial with some authors pointing out its inflated significance in

conservation genetics (Teixeira & Huber 2021). Despite this controversy, empirical results support the correlation between levels of genetic variation and evolutionary response as well as risk of extinction (Markert et al. 2010; Ørsted et al. 2019). Lower levels of genetic diversity are, in general, correlated with lower evolutionary response, reduced fitness, and greater risk of extinction (Markert et al. 2010; Ørsted et al. 2019). Additionally, microsatellite markers have also been found to be accurate predictors of genome wide diversity (Mittell et al. 2015), and differences in genetic variability and effective population size were shown to predict rate of adaptation to novel environments (Santos et al. 2012). As such protecting genetic diversity is one of the fundamental steps in mitigating biodiversity loss (Leigh et al. 2019).

1.3. Genetic non-invasive sampling (gNIS)

Genetic non-invasive sampling (gNIS) highly potentiates genetic studies of animals without the need to capture or even observe them. This method is essential when studying endangered, elusive or rare species, as disturbing individuals of interest can be limited by both ethical and practical constraints (Ferreira et al. 2018).

However the usage of non-invasive samples also carries some difficulties and limitations, due to the low quantity and quality of the DNA found in non-invasive samples as well as the presence of PCR inhibitors (Waits & Paetkau 2005). This leads to a high cost of laboratory analysis and the need for experienced and skilled laboratory staff in order to provide high quality genetic data (Banks & Piggott 2022). Genetic non-invasive sampling requires the identification of genetic markers that can accurately distinguish individuals and diminish “false recaptures” (Banks & Piggott 2022) or “shadow effects” (Mills et al. 2000). The genetic markers need to be genotyped in samples with degraded DNA sources, and microsatellites or SNP's have shown to be the markers more prone to be successfully amplified in such poor quality samples (Sethi et al. 2014). Additionally, sample collection and storage are important to improve the success of this approach since it is important to collect fresh samples to obtain better amplification rates (Rehnus and Bollmann 2016) and avoid further DNA degradation by properly storing samples. DNA extraction and amplification of non-invasive samples also needs extra precautions since the low quantity of DNA makes it very susceptible to contamination. Genotyping errors due to allelic dropout are also common with these types of samples (Taberlet & Luikart 1999), these errors can be reduced dramatically by the usage of an adequate amount of replicates and the generation of consensus genotypes (Lampa et al. 2013).

Despite these limitations gNIS can be a powerful, ethical, and informative population monitoring tool for threatened, rare and elusive species (Ferreira et al. 2018). Genetic non-invasive sampling not only has the ethical advantage of minimizing risks to animal welfare over live trapping (Kleemann et al. 2022), but also it is usually easier and cheaper to collect samples than it is to trap live animals (Banks & Piggott 2022). This approach has been widely used for the study of natural populations (Beja-Pereira et al. 2009; Oliveira et al. 2010; Ahlering et al. 2011; Chaves et al. 2012; Arandjelovic & Vigilant 2018; Aristizábal Duque et al. 2018; Ferreira et al. 2018) and can provide information on population abundance, population structure, sex, diet, habitat occupancy, and population genetic diversity (Piggott et al. 2006; Lachish et al. 2011; Tende et al. 2014; Moßbrucker et al. 2015; Camp et al. 2020; Bannister et al. 2021).

1.4. Molecular tools

DNA based markers allow researchers to directly assess DNA variation instead of relying on the electrophoretic mobility of proteins as did previous methods (alloenzyme electrophoresis) (Schlötterer 2004). The invention of the polymerase chain reaction (PCR) allowed for the amplification of small segments DNA and negated the need to isolate large amounts of clean DNA to perform genetic studies.

1.4.1. Microsatellites

Microsatellites, sometimes called short tandem repeats (STR), or even simple sequence repeats (SSRs), are tandem iterations of short sequence DNA motifs and are extremely common in eukaryotic genomes (Richard et al. 2008). Microsatellite motifs can vary between one and up to six or ten repeats (Richard et al. 2008). The number of repeats of the motif varies, which leads to high polymorphism among individuals, (Bruford & Wayne 1993; Richard et al. 2008). The variation of motifs occurs due to DNA replication slippage and the mismatch repair system (Schlötterer 2000). Microsatellites tend to occur in DNA non-coding regions and are flanked by regions that consist of "unordered" conservative DNA. These flanking regions are critical because they allow the development of locus-specific primers, and subsequently the amplification of the desired microsatellites via Polymerase Chain Reaction (PCR).

Microsatellite markers offer multiple advantages for genetic studies; they are highly polymorphic, abundant, evenly distributed across eukaryotic genomes, locus specific (in

contrast with other multi-locus markers like RAPDs) and codominant which allows for heterozygotes to be distinguished from homozygotes. Furthermore, microsatellites are amplified through PCR methods making their amplification and genotyping relatively simple in addition to being able to be used with non-invasive and ancient sources of DNA due to PCR requiring only tiny amounts of DNA. All these advantages led to microsatellites being regarded as the best molecular tools in population genetics, social structure, mating success and population movement (Schlötterer 2000; Sunnucks 2000), until the development of next generation sequencing.

1.4.2. Mitochondrial markers

In eukaryote cells the mitochondria have a short portion of DNA called the mitochondrial DNA (mtDNA). MtDNA is haploid in nature and therefore evolves four times faster than the average nuclear gene, as such mtDNA can be used to track divergence in very closely related taxa and even within species (DeSalle et al. 2017). Furthermore, due to its lack of recombination and simplicity to isolate mtDNA is often used in population and evolutionary genetics studies (Alves et al. 2003; Melo-Ferreira et al. 2012). MtDNA is often used as a marker when working with genetic non-invasive or ancient samples due to the ratio of mtDNA to nuclear copies being high which aids in its isolation and extraction from low quality samples (Merheb et al. 2019). Despite these advantages, mtDNA is solely maternally inherited, therefore may introduce bias as it only reflects the female portion of the species history, which might be different from the history of the species as a whole.

Despite its usefulness in the determination of phylogenetic relationships and species identification, mitochondrial introgression may occur (Melo-Ferreira et al. 2005; Alves et al. 2008b). This phenomenon is well described in hares. Examples of this phenomenon can be observed in Sweden with unidirectional hybridisation and introgression between native mountain hares and introduced European hares (Thulin et al. 1997) and in the Iberian peninsula with mountain hare introgressions into the European and Iberian hares (Alves et al. 2003). As such, the use of mitochondrial markers, in cases where introgression may be widespread, should be done in conjunction with nuclear markers, to obtain accurate results.

One of the mtDNA genes usually utilized as a mitochondrial marker is the cytochrome-b (cytb). Cytb is a protein found in the mitochondria of eukaryotic cells, it functions as part of the electron transport chain (Esposti et al. 1993) and it is commonly used to determine

phylogenetic relationships between organisms due to its sequence variability (Castresana 2001).

1.5. The broom hare

The broom hare (*Lepus castroviejo*; (Palacios 1976)) is an hare species endemic to the Iberian Peninsula (Figure 1). This species distribution is restricted to the Cantabrian Mountains, in the Northwest of Spain (Figure 2). Due to its limited distribution and endemic status it is listed as vulnerable in the IUCN Red list of endangered species (Ballesteros & Smith 2019).



Figure 1. The broom hare (*Lepus castroviejo*). Credit: uploaded by Marc in: <https://www.biodiversity4all.org/photos/157176339>

1.5.1 Broom hare description and habitat

The broom hare has an intermediate size between the Iberian and the European hare. It is larger than the Iberian hare but smaller than the European hare. Its weight can vary between 1.450 and 3.310 Kg (Ballesteros & Alves 2022) and females have a greater average weight than males (Ballesteros & Alves 2022). The broom hare has a greyish brown dorsal and lateral pelage, with a white ventral pelage and a white and black tail.

The facial design is characteristic with a lighter greyish band on the face which stands out against the brown colour of the area under the eye and snout (Ballesteros & Alves 2022). The ears and tail are shorter than both the Iberian and European hares most likely due to adaptations to the colder habitats it inhabits (Ballesteros & Alves 2022).

The habitats where the broom hare occurs have the typical vegetation from the high zones of the Cantabrian Mountains, between 1000 and 1900 m in altitude. The broom hare has an estimated distribution range around 5000 km² (230 km long, 25-40 km wide) (Ballesteros & Alves 2022) (Figure 2). This area is usually characterized by open pastures or small herbaceous clearings surrounded by broom formations, heathers, thorny bushes, and forests of beech and oak trees (Ballesteros & Alves 2022). Within its distribution, the broom hare occupies specific patches of habitat, such as the intercalation of brooms and heaths with wide mountain pastures in the hills at the top of the mountains, which might promote fragmentation (Ballesteros & Alves 2022). The fragmented habitat makes the broom hare a rare species within its own distribution range, and it is likely one of the reasons for its late description as a separate species from the Iberian hare.

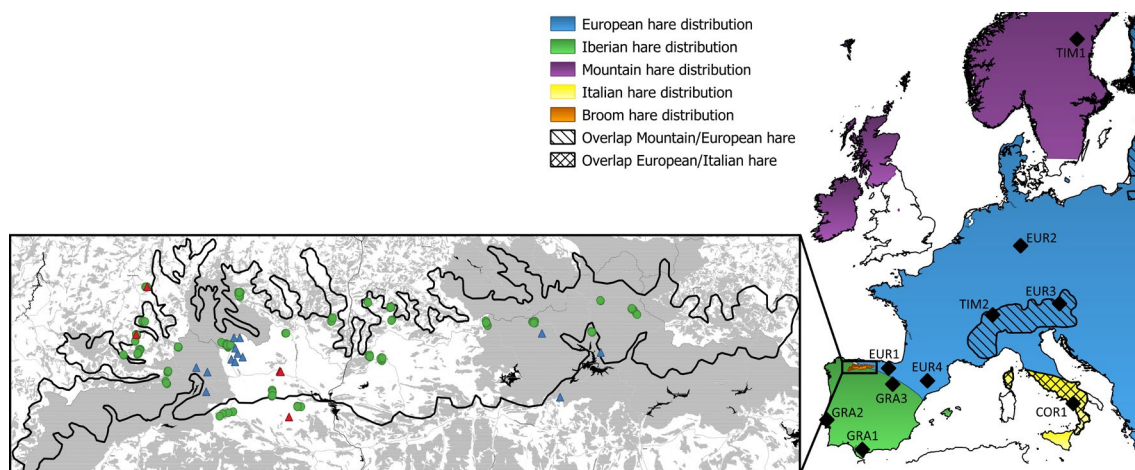


Figure 2. Distribution range of the European hare (Hackländer & Schai-Braun 2019), Iberian hare (Soriguer & Carro 2019), mountain hare (Smith & Johnston 2019), Italian hare (Randi & Riga 2019) and broom hare from (Ballesteros & Smith 2019) and respective sampled populations: EUR1, EUR2, EUR3 and EUR4 for the European hare; GRA1, GRA2 and GRA3 for the Iberian hare; TIM1 and TIM2 for the mountain hare; and COR1 for the Italian hare (right). Broom hare distribution range (Ballesteros & Smith 2019) and location of the collected broom hare samples (left). Sampling locations for genetic non-invasive samples (gNIS) are shown as green circles, while tissue samples are shown as triangles: red for samples collected in 2019/21 and blue for samples collected in 2003/07.

1.5.2 Evolutionary history

The broom hare was only firstly described in 1976 by Fernando Palacios (Palacios 1976), being previously considered a form of the Iberian hare (*Lepus granatensis*; (Rosenhauer 1856)) (Ballesteros & Smith 2019). Despite initial controversy about its specific status (Angermann 1983; Corbet 1986), comparative genetic and biochemical data confirmed the broom hare's identity as a separate species from either the Iberian (*Lepus granatensis*) and the European hare (*Lepus europaeus*; (Pallas 1778)) (Palacios 1979; Bonhomme et al. 1986; Pérez-Suárez et al. 1994). Later genetic studies, utilizing mtDNA, nuclear genes and microsatellite markers further confirmed this distinction (Alves & Niethammer 2003; Alves et al. 2003; Estonba et al. 2006; Alves et al. 2008a). These results were further confirmed in more recent studies based on large amounts of genomic data, such as whole exome and whole genome, though with more reduced population sampling (Giska et al. 2019; Ferreira et al. 2021). While the broom hare is evolutionarily distinct from the neighbouring hare species, it is closely related with the Italian hare (*Lepus corsicanus*; (de Winton 1898)) both in mitochondrial DNA (mtDNA) and nuclear DNA (Alves et al. 2003; Alves et al. 2008a; Mengoni et al. 2015; Ferreira et al. 2021). These species share not only genetic similarities but also phenotypic features (Palacios 1996) and ecological niche properties (Acevedo et al. 2014). As such these species are likely to have shared a common ancestor during the Pleistocene. This common ancestor has been suggested to have had a wider distribution in Europe, before the vicariance in the Iberian and Italian peninsulas during the last deglaciation, though this hypothesis lacks confirmation (Francesco Maria & Luca 2007; Alves et al. 2008a; Acevedo et al. 2014).

With the exception of the Italian hare, the broom hare shows quite a genetic differentiation from other hare species that inhabit Europe, this differentiation has been shown using nuclear genes, microsatellites, proteins and mtDNA markers (Bonhomme et al. 1986; Estonba et al. 2006; Alves et al. 2008a; Alves et al. 2008b).

Despite contacting with the Iberian hare (*L. granatensis*) in the southwest of Asturias, southeast of Galicia and a large part of the southern limit of its distribution range in León and with the European hare (*L. europaeus*) in the east of Cantabria and northeast of Palencia, no hybrids between these species and the broom hare have been identified so far. There are, however, evidences of ancestral hybridization between the broom hare and the mountain hare (*Lepus timidus*; (Linnaeus 1758)), a species that expanded to southern Europe during the glaciation events. This evidence is based on a mtDNA marker and using a reduced number of broom hare specimens (n=9) (Alves et al. 2008b).

Additionally, there are insights that the broom hare might have been affected by two separate events of mtDNA introgression from the mountain hare. This is due to the fact that the broom hare has two identified lineages of mtDNA, both of these lineages are related to the mountain hare despite nuclear genomic analysis evidencing its differentiation from this species (Melo-Ferreira et al. 2012). Coalescent simulations show that these lineages resulted from introgression (Melo-Ferreira et al. 2012). One of these lineages is shared with the Italian hare as such this introgression presumably affected both species before their split during the Pleistocene. The second lineage is closer to introgressed haplotypes present in the Iberian and European hares and may correspond to the same introgression events that affected these species at the end of the glacial period (Melo-Ferreira et al. 2012). However, it is not clear if this second mtDNA lineage came directly from the mountain hare or from introgressed Iberian or European hares (Melo-Ferreira et al. 2012), since analysis based on single nucleotide polymorphisms (SNPs) also suggested minor levels of admixture between the broom hare and Iberian hare, which might be either rare or ancient (Melo-Ferreira et al. 2009; Melo-Ferreira et al. 2012). These two mtDNA introgression events at different moments in the species history could be a unique case among mammals (Melo-Ferreira et al. 2009).

1.5.3. Genetic diversity

Despite the evidences of introgression and hybridization, preliminary studies show very low genetic diversity for this species. The broom hare genetic diversity was firstly addressed by Bonhomme et al. 1986, which found very low values of population heterozygosity (Mean $H_e = 0.284$) using ten individuals and six microsatellite markers (Bonhomme et al. 1986). This value is much lower than those found for the Iberian and European hares in the same study. A similar result was observed by Alzaga (2010), regarding the number of alleles ($N_a = 1.93$), observed heterozygosity ($H_o = 0.275$) and expected heterozygosity ($H_e = 0.339$). However, in this study only eight broom hare specimens and five microsatellites were used (Alzaga 2010). Despite both studies pointing out a low genetic diversity in this species, accurate genetic inference requires a robust sample size along the distribution range, as well as a high number of informative markers (Queirós et al. 2015).

1.5.4 Conservation efforts and Management

Due to its restricted distribution range and presumed low genetic diversity, the broom hare is listed as vulnerable in both the IUCN Red List (Ballesteros & Smith 2019) as well as in the Spanish National Red List (Ballesteros 2007). However, it is not included in the Spanish Catalogue of Endangered Species or the Spanish List of Wildlife Species of Special Protection Regime. Additionally, the broom hare is not included in the Habitats Directive or the Bern Convention, despite being both threatened and endemic. A large part of the broom hare's distribution range is included in natural protected areas, and many are included in the Natura 2000 network of the European Union, which protects the habitat against future and existing threats.

Despite the conservation concerns, the broom hare remains a hunting species in some areas, being legally hunted through part of its range. Due to the small distribution and possible low population size the effects of hunting in this species should be carefully evaluated. There is also the conservation issues associated with climate change and land use evolution in the mountainous rural environment which can influence availability and habitat use by hares (Ballesteros & Palacios 2009). The small distribution range, predictable small population size, the fragmented population and the occurrence of hunting constitute significant risk factors for the conservation of the broom hare.

Due to the overall lack of information, the population size and the detailed distribution range are not well-known. Moreover, peripheral populations of this species seem to be suffering a pronounced regression with even some local disappearances (Ballesteros & Palacios 2009).

1.6 Objectives

In this work we aimed to infer the genetic diversity and population structure of the broom hare to increase the knowledge of this endemic species and raise awareness for its conservation. Admixture with other hare species was also inferred by sampling several populations from other European hare species. We also aimed to explore some aspects of the evolutionary history of the broom hare, by utilizing a mtDNA fragment to study this species demographic history and introgression events. Additionally, genetic non-invasive sampling outside the species described distribution range was performed in an attempt to more accurately infer the species distribution and the putative hybridization with the neighboring hare species.

2. Materials and Methods

2.1. Study area

The Cantabrian Mountains stretch for over 300 km across northern Spain, from the western limit of the Pyrenees to the Galician Massif in Galicia, along the coast of the Cantabrian Sea. The broom hare inhabits the high zones characteristic of these mountains which are characterized by a biotope with scrub formations of broom and heath interspersed with grasslands and forests composed by beech, oak, and birch trees (Palacios 1976; Palacios & Meijide 1979).

2.2. Sample collection

A total of 185 genetic non-invasive samples (gNIS) were collected between 2019 and 2021 across the entire species distribution range in the Cantabrian mountains, encompassing 17 “Municipalities” (Figure 2, see details in Table S1, Supplementary Information). Sample collection started in the center of the species distribution and gradually moved outwards towards the edges, with final sampling efforts extending beyond the species supposed distribution range (Figure 2). Samples were systematically collected, with each sample being separated by approximately fifty meters. Feces with the same freshness and size within five meters or less from each other were considered as the same sample. Preference was given to fresh fecal pellets as older pellets have significantly lower amplification rates (Rehnus & Bollmann 2016). One to five pellets were collected for each sample and stored in tubes with ethanol 95%. The date, coordinates, location, number, and freshness of pellets were recorded at the time of collection. In addition to the gNIS, 22 specimens were opportunistically collected from hunting and road-kills. Five specimens were collected between 2019 and 2020 for this study, and the remaining seventeen were sampled between 2003 and 2007 in the scope of a previous studies (Alzaga 2010). Tissue samples from ear or liver were collected from these specimens.

In addition to the broom hare, populations from the European hare, the mountain hare, the Iberian hare and the Italian hare were analysed using samples from the CIBIO-InBIO tissue bank and from collaborators, to accurately compare the genetic diversity patterns

and infer admixture (samples originally obtained between 2002 and 2020). The analyzed populations were: Alava (EUR1, n=20), Germany (EUR2, n=15), Austria (EUR3, n=19) and Northeast Spain (EUR4, n=27) for the European hare (n=81 in total); Southern Spain (GRA1, n=15), Pancas (GRA2, n=29) and Soria (GRA3, n=24) for the Iberian hare (n=68 in total); Sweden (TIM1, n=20) and Switzerland (TIM2, n=57) for the mountain hare (n=77 in total); and Mainland Italy for the Italian hare (COR1, n=20) (Figure 2).

2.3. DNA extraction and species ID

DNA extraction for the gNIS (pellets) was performed using the E.Z.N.A tissue DNA kit (Omega Bio-Tek, GA, USA), following the manufacturer's protocol but employing an additional initial digestion step with a lysis wash buffer as described by (Maudet et al. 2004). Only one pellet was used per extraction. DNA extractions were performed in an exclusively dedicated laboratory for non-invasive samples to reduce the risks of DNA contamination. DNA extraction from the tissue samples was performed using the EasySpin Genomic DNA Tissue Kit (Citomed, Lisbon, Portugal), following the manufacturer's instructions. Negative controls were used for all DNA extractions. A 108bp mitochondrial (mtDNA) fragment of Cytochrome-b (cytB) and a 240bp fragment of the Transferrin (TF) gene were used to infer species ID, given their ability to distinguish the different European hare lineages and species (Alves et al. 2003; Melo-Ferreira et al. 2009). The primer pairs Lmtof1 and LmtNr1 were used for cytB and 08TFF_SS and 08TFR_SS for TF, the PCR amplification and sequencing conditions were followed as described in (Alves et al. 2003). Sequences were aligned with Geneious version 8.1.9 (<https://www.geneious.com>) and species identified using diagnostic cytB and TF SNPs (Palumbi et al. 1991; Alves et al. 2003).

2.4. Microsatellite genotyping and individual ID.

A panel of 15 microsatellites (Le15, Le17, Le19, Le2, Le51, Le23, Le28, Le30, Le4, Le46, Le3, Le43, Le47, Le48 and Le6) developed in CIBIO-InBIO for the European hare was used to infer individual ID, in three multiplexes (Table 1). To increase the specificity and sensitivity of the microsatellite amplification for gNIS, a nested polymerase chain reaction (PCR) protocol was employed (Piggott et al. 2004). Briefly, the first PCR was composed by 5 µl of Multiplex PCR kit (Qiagen), 1 µl of primer multiplex (forward and reverse primers), 2 µl of pure water and 2,5 µl of extracted DNA. The thermocycler PCR program included a first denaturation step of 95°C for 15min followed by 11 cycles of 95°C for

30s, 60°C for 90s, and 72°C for 30s, with the annealing temperature decreasing by 0.5°C for each cycle; finally, 9 cycles of 95°C for 30s, 55°C for 90s and 72°C for 30s were fixed, followed by a final extension step at 60°C for 15min. Afterwards, the second PCR was composed by 5 µl of Multiplex PCR kit (Qiagen), 1 µl of primer multiplex (reverse and tailed primers), 3 µl of pure water and 1,2 µl of the first PCR. Thermocycler PCR program included a first denaturation of 95°C for 15min, followed by 11 cycles of 95°C for 30s 60°C for 60s and 72°C for 30s, with the annealing temperature decreasing by 0.5°C for each cycle; finally, 34 cycles of 95°C for 30s, 55°C for 45s and 72°C for 30s, followed by 30 min at 60°C. A stepwise approach was used to amplify the three multiplexes. gNIS that were not amplified for the first multiplex-1 were discarded from the following two multiplex reactions, given the poor quality and quantity of DNA (Lau et al. 2003). Four PCR replicates were performed for each sample. In the case of high-quality DNA (tissue samples) samples, PCR reactions were performed using a single reaction instead of the nested approach and following the same conditions as described earlier. Negative controls were used in all PCR reactions. Fragment analysis of the microsatellites was performed using the 3130xl Genetic Analyzer from Applied Biosystems. The microsatellite alleles were visualized using the software GENEMAPPER, version 4.0 (Applied Biosystems), by two independent researchers.

Table 1. Microsatellite markers and multiplex PCR conditions used in this study.

MULTIPLEX-GROUP	LOCUS	PRIMER SEQUENCE 5'-3'	AMPLICON LENGTH (bp)	DYE	CONCENTRATION (μM)
1	Le23	F: CCAGTAACCTTCTTATGCTCCCC R: CCTTAAATGTAGGTGAATATTGAAAGG	105-133	FAM	0.4
	Le4	F: GGAACCTGCCCACATTCAACTG R: GCAGGTGAAATCGCTAAACC	102-144	VIC	0.3
	Le46	F: GGTCAACTGGGGATGGTAGA R: GGAAAATTATGAATGGACTTCTAAA	168-186	VIC	0.5
	Le28	F: GGTTTCATCCATGAGGCATCAAA R: ATCCCATCTCTGCCCATTCT	249-263	VIC	0.45
	Le30	F: TGAACCCGAAGAATTTCTGA R: GCTCTCCCCACTTTCTTCT	183-205	NED	0.4
2	Le51	F: ATAACATTGTGCTGGGCCTT R: GACGACAAAATTCCTGGAACA	108-132	FAM	0.4
	Le2	F: AAGGAGTAGTGAGGTCATTTCAA R: CTGGCATTGAGGAGTGGAT	171-205	FAM	0.5
	Le15	F: GGGGAGAGGGTGAGGGAGAGTT R: CCATTACAGCCCTTTGAGGA	113-137	VIC	0.5
	Le17	F: GGAGCTGCTGTTGCCTACCACT R: CTGGGCAGTCAGAAAGATCC	255-271	VIC	0.4
3	Le19	F: CTTGTCCCCATGTGCCTACT R: GGTTTCTGGGAAGTGAACCA	144-186	NED	0.4
	Le43	F: ACTGCCTTACAAATAAATGGAAA R: AGCAGTTTCTGCATTTCTGC	96-118	FAM	0.45
	Le3	F: GAATGTGAGATCACGCTCCA R: CATGCTTTCTGTGAACCTTTCTGA	235-257	FAM	1.2
	Le6	F: GGCTTCCTGCTACAGCCCTGAC R: CCCAGAACAGTCTGGCTCAT	254-272	VIC	0.7
	Le47	F: TTGTTCCATTTCCCTGTTCC R: GAATTGCAGAAAGGTCCAAA	146-158	NED	0.4
	Le48	F: TTGCTTTGGGAAAAGAGAGG R: GTGAAATGGTTGGAACCTGG	204-220	NED	0.5

2.5. Amplification of the longer mitochondrial Cytochrome-b fragment

For the broom hare individuals identified (n=76) a 541bp fragment of mtDNA cytB gene was amplified using the primer pair LGCYF and LGCYR and following the PCR amplification and sequencing conditions as described in (Alves et al. 2003). Sequencing was performed on the 3130xl Genetic Analyzer from Applied Biosystems using the respective forward primer. Base calling was performed using Sequencing Analysis v5.2

(Applied Biosystems) and haplotypes were visualized with DnaSP6 software v.6.12.03 (Rozas et al. 2017).

2.6. Data analysis

Consensus genotypes for the four microsatellite amplification replicates were created using the GIMLET software, version 1.3.3. (Valière 2002). The consensus genotypes for the four replicas were determined using the following rules: heterozygous genotypes were accepted if the same genotype was observed in two independent PCRs and homozygous genotypes were accepted if the genotype was observed in three independent PCRs. Broom hare genotypes obtained from both invasive and non-invasive samples were used to calculate the probability of identity (PI) and the Probability of Identity considering siblings (PI_{SIBS}) of the broom hare using GENEALLEX v.6.503 (Peakall & Smouse 2006; Peakall & Smouse 2012). Genotyping error rates (allelic dropout and false alleles) were evaluated for duplicate broom hare genotypes, obtained from gNIS, using PEDANT V.1 (Johnson & Haydon 2007). Additionally, MICRO-CHECKER v.2.2.3 was used to discard null alleles, scoring errors and allelic dropout (Van Oosterhout et al. 2004). Deviations from Hardy-Weinberg equilibrium and linkage equilibrium between loci were assessed for each hare population using Fisher's exact tests implemented in GENEPOP v.4.0.10 (Raymond 1995). Significance levels were estimated by Markov Chain Analysis (10^4 dememorization steps, 10^3 batches and 10^4 iterations per batch) and adjusted using Bonferroni's sequential method for multiple comparisons.

2.6.1. Nuclear and mitochondrial DNA genetic diversity

The nuclear genetic diversity parameters, number of alleles (N_a), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated for all populations of each species using GENEALLEX v.6.503 (Peakall & Smouse 2006; Peakall & Smouse 2012). The inbreeding coefficient (F_{is}) and allelic richness (Ar) were additionally calculated using the software FSTAT v.2.9.4 (Goudet 1995), assuming the Hardy-Weinberg equilibrium and using 5000 iterations. These genetic diversity parameters were calculated for the 15 microsatellite markers as well as for the 11 loci that showed polymorphism for the broom hare (see results). For the broom hare specimens, analyses were also conducted separately by sampling periods, 2003/2007 ($n=17$) and 2019/2021 ($n=59$). Statistically significant differences on nuclear genetic diversity parameters

among species and sampling periods were assessed using a non-parametric Mann-Whitney test (Mann & Whitney 1947) and considering the values for each locus obtained in the genotyped populations. Additionally, Mann-Whitney tests were also employed to contrast the broom hare's microsatellite diversity (N_a , A_r , H_e , H_o) with data available in literature for the European hare, Iberian hare, mountain hare and Italian hare (Estonba et al. 2006; Hamill et al. 2006; Ben Slimen et al. 2008; Alzaga 2010; Canu et al. 2013; Topchieva et al. 2013; Melo-Ferreira et al. 2014; Mengoni et al. 2015; Rehnus & Bollmann 2016) (Table S2, Supplementary Information).

Mitochondrial DNA genetic diversity was calculated for the broom hare population as nucleotide diversity (π) and haplotype diversity (H_d), and using DnaSp6 software v.6.12.03 (Rozas et al. 2017). As performed for the microsatellite data, Mann-Whitney tests were employed to contrast the nucleotide and haplotype diversity parameters of the broom hare with data available in literature for other European hare species (Pierpaoli et al. 1999; Kasapidis et al. 2005; Waltari & Cook 2005; Melo-Ferreira et al. 2007; Melo-Ferreira et al. 2011; Seixas et al. 2014; Mengoni et al. 2015) (Table S3, Supplementary Information).

2.7.2. Population differentiation and structure.

Utilizing the microsatellite data the degree of genetic differentiation between all the analyzed hare populations was assessed using F-statistics (Wright 1943, 1965) and Nei's standard genetic distance (Nei 1972) in Fstat v2.9.4 (Goudet 1995) and Populations software v.1.2.32 (Langella 1999), respectively. From the created pairwise F_{ST} distance matrixes neighbor-joining trees were constructed using MEGAX software (Kumar et al. 2018).

A factorial correspondence analysis (FCA) was also performed in the GENETIX software v.4.05.2 (Belkhir 2004).

Population structure and individual admixture analyses were performed on the STRUCTURE software v.2.3.4 (Pritchard et al. 2000). A hierarchical analysis was carried out using three different datasets: i) All specimens from the five hare species sampled in this study; ii) only the broom hare and the Italian hare individuals; and iii) only the broom hare individuals. The latter analysis was performed using only the 11 polymorphic markers for the broom hare, while the panels of 15 and 11 microsatellites were used on all the other analysis. The number of Markov chain Monte Carlo was set to 10^6 after a

burn-in period of 10^5 . Five to nine independent runs were tested for each cluster (K) and K was calculated from 1 to 10 for the first dataset and from 1 to 5 in the remaining datasets. The best K for the data in all datasets was estimated using the method of Evanno (Evanno et al. 2005), implemented in the Structure Harvester webserver (Earl & vonHoldt 2012), which enables the identification of the K value that captures the uppermost level of structure (Evanno et al. 2005). The CLUMPAK webserver (Kopelman et al. 2015) was also used to visualize and produce graphical representation of all runs obtained for each K value.

To assess the isolation-by-distance pattern within the broom hare distribution range, a Mantel test was performed using QG genetic distance (Queller & Goodnight 1989) and the logarithm of geographic distance among individuals in GENEALOX v.6.503 (Peakall & Smouse 2006; Peakall & Smouse 2012).

2.7.3. Phylogeographic and phylogenetic analyses

The mitochondrial cytb sequences obtained in this study for the broom hare were used to construct a Median-joining network using the PopART v.1.7 software (Bandelt et al. 1999; Leigh & Bryant 2015). In addition, the available cytb sequences for the *Lepus* species were downloaded from NCBI (N=2405), and aligned together using the MAFFT version 7 webserver, multiple sequence alignment tool (Kuraku et al. 2013; Katoh et al. 2019). The alignment was trimmed to the 541bp cytb fragment amplified in this study for the broom hare, using MegaX software (Kumar et al. 2018). DNACollapser was further used to collapse sequences onto single haplotypes (Villesen 2007). The final alignment had a total of 515 haplotypes. The most optimal substitution model for this alignment was inferred using PhyML 3.0 webserver (Guindon et al. 2010), and considering the AIC (Akaike Information Criterion) (Akaike 1998). A Bayesian phylogenetic tree was then performed using MrBayes v.3.2.7 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), and considering the European rabbit (*Oryctolagus cuniculus*; (Linnaeus 1758)) as an outgroup. The length of run was set 2 million iterations and at the end the standard deviation of split frequencies was 0.06. The consensus Bayesian tree was visualized using FigTree v.1.4.4.4 (Rambaut 2020). A maximum likelihood tree was also constructed using the IQTREE Webserver (Trifinopoulos et al. 2016), using the option of 1000 ultrafast bootstraps and considering the conditions used on the Bayesian analysis.

2.7.4. Demographic and evolutionary history

The current effective population size (N_e) for the broom hare was estimated utilizing the MLNe software package (Wang 2022). Both maximum likelihood (Wang 2001) and moment estimator (Nei & Tajima 1981) methods were employed under the isolation model. For this analysis temporal genotype data was utilized, considering a generation time of 2 years, and the sampling periods of 2003/2007 ($n=17$) and 2019/2021 ($n=59$) as temporal samples.

The signature of recent genetic bottleneck in the broom hare was assessed using the panel of 11 polymorphic markers for this species and the heterozygosity excess test implemented in the program BOTTLENECK, version 1.2.02 (Cornuet & Luikart 1996). This analysis compares the calculated H_e and H_o to establish if there is an heterozygosity excess or deficiency at each locus. A two-phase mutation model (Di Rienzo et al. 1994) with 30% of variation from the infinite allele model and of 70% from the stepwise-mutation model was set, and the significance of test was determined with a one-tailed Wilcoxon sign rank test (10 000 iterations) (Luikart & Cornuet 1998).

On the other hand, signature of population expansion or selection was inferred using the cytb sequences obtained in this study and the Fu/Li's D^* (Fu & Li 1993) and Tajima's D (Tajima 1989) test statistics. Tajima's D is calculated the difference between the mean number of pairwise differences and the number of segregating sites, each scaled so that they are expected to be the same in a neutrally evolving population of constant size. Fu's and Li's D is based on the differences between the number of singletons and the total number of mutations in a sequence. Furthermore, the divergence between the two broom hare clades was estimated using a reference database of 39 cytb haplotypes, representative of the original tree of 515 *Lepus* haplotypes, and the BEAST2 software (Bouckaert et al. 2019). The model was set as GTR, with estimated proportion of invariant sites and a gamma category count set to 4.0. To test the most fitting clock model a nested sampling approach was employed by utilizing the NS package (Russel et al. 2019) for BEAST2. This package calculates an estimate of the (log) Marginal Likelihood (ML) of each clock model as well as a variance of that estimate, which in turn allows for the calculation of the Bayes Factor (BF) and therefore ascertain which model possesses stronger support. The calibrated yule model was used as the tree prior and was calibrated using the most recent common ancestor (MRCA) prior with a value of 11.8 Myr (95% HPD, 10.8–12.8 Myr) for the *Lepus-Oryctolagus* divergence (Matthee et al. 2004; Melo-Ferreira et al. 2012; Pinheiro et al. 2013) and the same prior with a value of 47kya (95% HPD 27–67 Ka) for the broom hare – Italian hare divergence (Souto 2022).

The MCMC chain length was set to 10^9 and three independent runs were performed. The MCMC trace files were visualized and analyzed with the aid of Tracer v.1.7.1 (Rambaut et al. 2018), the independent runs were combined by using LogCombiner v.2.6.7 (Drummond & Rambaut 2007) and the trees produced by BEAST2 were summarized onto a single “target” tree with TreeAnnotator v.2.6.6 (Drummond & Rambaut 2007). The summarized divergence tree was then viewed using Figtree v.1.4.4 (Rambaut 2020).

A Bayesian skyline analysis employed on BEAST software v1.10.4 (Rambaut 2000) was performed with the mitochondrial data for the broom hare utilizing the “Coalescent: Bayesian Skyline” prior and a mutation rate of 1% per Myr calculated by BEAST2 software (Bouckaert et al. 2019). Three independent runs of 10^7 states and with 10^6 burn-in were performed. The independent runs log and tree files were combined with LogCombiner v.2.6.7 (Drummond & Rambaut 2007). The independent runs were analyzed by Tracer v.1.7.1 (Rambaut et al. 2018) and the Bayesian skyline reconstruction option of Tracer was used with the combined log and tree files of the three independent runs in order to produce the Bayesian Skyline plot.

3. Results

3.1. Species and individual ID of the broom hare gNIS

From the 185 gNIS collected, 150 (81%) successfully amplified the 108bp cytB fragment and 88 (47%) amplified the TF gene fragment. In total, 87(47%) successfully amplified both genes, and upon SNP analysis, all the samples were identified as broom hare specimens, even though 19 (21%) of gNIS showed cytB haplotypes matching the mountain hare, that were previously reported to have introgressed in the Iberian hare and European hare. From these 87 samples 80 (91%) were successfully amplified for the microsatellite markers. In total, 54 individuals were identified from gNIS, with 26 recaptures, with a range of 1 to 3 recaptures per individual and an average number of samples per individual of 1.48. From these 54 individuals, 42 successfully amplified for the long cytB fragment (541bp). After adding the 22 tissue samples, 76 and 64 broom hare individuals were retained for further nuclear and mitochondrial DNA genetic analyses, respectively. Considering the 76 available individuals, the microsatellite markers utilized showed a Probability of Identity of $PI=4.8E-04$ and Probability of Identity

considering siblings of $PI_{SIBS} = 4.2E-02$ (See details in tables S5, S6 and S7, Supplementary Information).

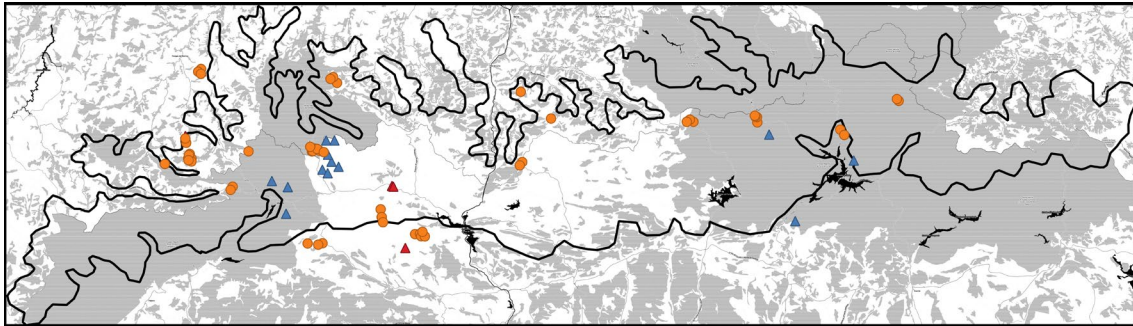


Figure 3. Distribution map of broom hare individuals identified through microsatellite analysis utilizing the 15-microsatellite panel. Broom hare distribution outlined in black. Individuals detected through gNIS are shown as orange circles, while individuals collected by hunting or roadkill are shown as triangles: red for individuals collected in 2019/21 and blue for individuals collected 2003/2007

A total of 14 out of 76 individuals were found outside the previously established distribution range of the species, although all were collected above the 1000m altitude mark (Figure 3).

3.2. Microsatellite markers and nuclear genetic diversity

All microsatellites were found to follow Hardy-Weinberg equilibrium (HWE) expectations within each population, except at locus Le23 in CAS1 and COR1, Le15 in EUR3 and EUR4, Le30 and Le51 in TIM1 and TIM2, and Le17 in TIM1. All markers were in linkage equilibrium (LE), except the loci pair Le4 and Le46 in EUR4, and Le28 and Le43 in GRA2. As deviations from HWE and LE were not consistent among populations and across loci, all markers were considered for further genetic analysis. The same reasoning was applied to the putative presence of null alleles and scoring errors suggested by Micro-checker in some locus and populations (Table 2). For the broom hare gNIS samples Maximum likelihood estimates from PEDANT gave a mean allelic dropout rate per allele of 4% and a mean false allele rate per allele of 0.005% (Table S4, Supplementary Information).

Table 2. Micro-checker outputs for the putative presence of null alleles and scoring errors by microsatellite marker and population analysed. Scoring errors marked by an x and null alleles marked with an asterisk.

	Le15	Le17	Le19	Le2	Le51	Le23	Le28	Le30	Le4	Le46	Le3	Le43	Le47	Le48	Le6
CAS1									x*						
EUR1															
EUR2		*													
EUR3	*												*		
EUR4	x*	*									*				
GRA1															
GRA2															
GRA3															
TIM1		*			x*			*							
TIM2					x*			*							
COR1	*					*						*	x*	x	
Total Scoring errors	1	0	0	0	2	0	0	0	1	0	0	0	1	1	0
Total Null Alleles	3	3	0	0	2	1	0	2	1	0	1	1	2	0	0

Regarding the nuclear genetic diversity, the broom hare population showed the lowest levels among the analyzed *Lepus* species, with lower number of alleles ($N_a=2.667$; $SE=0.540$), allelic richness ($Ar=2.265$), and observed and expected heterozygosity ($H_o=0.168$; $SE=0.062$ and $H_e=0.186$; $SE=0.65$) when compared to all the populations/species analyzed, both for the panel of 15 (Table 3) and 11 loci (Table 4).

Table 3. Nuclear genetic diversity parameters inferred from 15 microsatellite markers for all populations sampled in this study.

Species	Population	N	Na		Allelic Richness	Ho		He		Fis	Loci in HWD
			Mean	SE		Mean	SE	Mean	SE		
Broom hare (<i>L. castroviejo</i>)	2019/2021 [#]	59	2.533	0.515	2.264	0.160	0.061	0.180	0.063	0.117	Le23
	2003/2007 [#]	17	2.267	0.473	2.267	0.196	0.072	0.198	0.069	0.042	0
	CAS1	76	2.667	0.540	2.265	0.168	0.062	0.186	0.065	0.101	Le23
Italian hare (<i>L. corsicanus</i>)	COR1	20	3.867	0.844	3.689	0.301	0.076	0.378	0.085	0.229	Le23
European hare (<i>L. europaeus</i>)	EUR1	20	4.600	0.349	4.374	0.635	0.035	0.645	0.024	0.042	0
	EUR2	15	5.200	0.619	5.200	0.613	0.044	0.617	0.039	0.04	0
	EUR3	19	5.733	0.547	5.567	0.647	0.043	0.712	0.027	0.119	Le15
	EUR4	27	6.933	0.539	5.867	0.631	0.039	0.687	0.038	0.101	Le15
	All	81	9.067	0.859	5.252	0.633	0.026	0.728	0.024	0.137	
Iberian hare (<i>L. granatensis</i>)	GRA1	15	5.867	0.515	5.867	0.618	0.059	0.609	0.052	0.021	0
	GRA2	29	5.267	0.547	4.636	0.556	0.054	0.583	0.053	0.063	0
	GRA3	24	5.733	0.605	5.157	0.569	0.053	0.597	0.054	0.067	0
	All	68	7.600	0.659	5.220	0.575	0.050	0.617	0.052	0.076	
Mountain hare (<i>L. timidus</i>)	TIM1	20	6.667	0.637	5.610	0.63	0.073	0.706	0.040	0.113	Le17,Le51,Le30
	TIM2	57	6.667	0.659	6.328	0.700	0.034	0.724	0.026	0.042	Le51,Le30
	ALL	77	8.400	0.850	5.969	0.682	0.041	0.747	0.026	0.094	

N, number of individuals; Na, Mean number of alleles, Ho, observed heterozygosity, He, expected heterozygosity, Fis, inbreeding coefficient, SE, standard deviation; # no statistical differences on genetic diversity parameters were observed in Mann-Whitney test: P-values for each comparison; Na 0.5697; AR 0.8981; Ho 0.7657; He 0.9661.

Table 4. Nuclear genetic diversity parameters inferred for all analysed populations using the 11 polymorphic microsatellite markers in the broom hare.

Species	Species/Pop	N	Na		Allelic Richness	Ho		He		Fis
			Mean	SE		Mean	SE	Mean	SE	
Broom hare (<i>L. castroviejoii</i>)	2019/2021	59	3.091	2.723	2.723	0.219	0.076	0.246	0.077	0.117
	2003/2007	17	2.727	2.727	2.727	0.267	0.090	0.271	0.085	0.042
	CAS1	76	3.273	2.725	2.725	0.23	0.077	0.254	0.079	0.101
Italian hare (<i>L. corsicanus</i>)	COR1	20	4.636	4.418	4.418	0.383	0.091	0.470	0.101	0.209
European hare (<i>L. europaeus</i>)	EUR1	20	4.636	4.408	4.408	0.629	0.046	0.638	0.032	0.04
	EUR2	15	5.727	5.727	5.727	0.655	0.051	0.661	0.041	0.044
	EUR3	19	5.636	5.471	5.471	0.631	0.056	0.697	0.036	0.123
	EUR4	27	7	5.910	5.910	0.634	0.048	0.677	0.05	0.082
	All	81	9.545	5.378	5.378	0.637	0.035	0.725	0.031	0.127
Iberian hare (<i>L. granatensis</i>)	GRA1	15	6.182	6.182	6.182	0.673	0.069	0.653	0.06	0.004
	GRA2	29	5.727	4.995	4.995	0.058	0.638	0.054	0.053	0.06
	GRA3	24	5.909	5.296	5.296	0.583	0.07	0.61	0.069	0.065
	All	68	8.091	5.491	5.491	0.615	0.056	0.656	0.059	0.070
Mountain hare (<i>L. timidus</i>)	TIM1	20	7.545	5.930	5.930	0.073	0.758	0.042	0.04	0.096
	TIM2	57	7.182	7.142	7.142	0.732	0.026	0.74	0.029	0.02
	All	77	9.364	6.536	6.536	0.725	0.036	0.778	0.026	0.069

N, number of individuals; Na, Mean number of alleles, Ho, observed heterozygosity, He, expected heterozygosity, Fis, inbreeding coefficient, SE, standard deviation;

Significant differences on Na, AR, H_e and H_o were estimated between the broom hare (considering all samples) and the other species, with exception of the Italian hare (Figure 4 and Table S8, Supplementary Information).

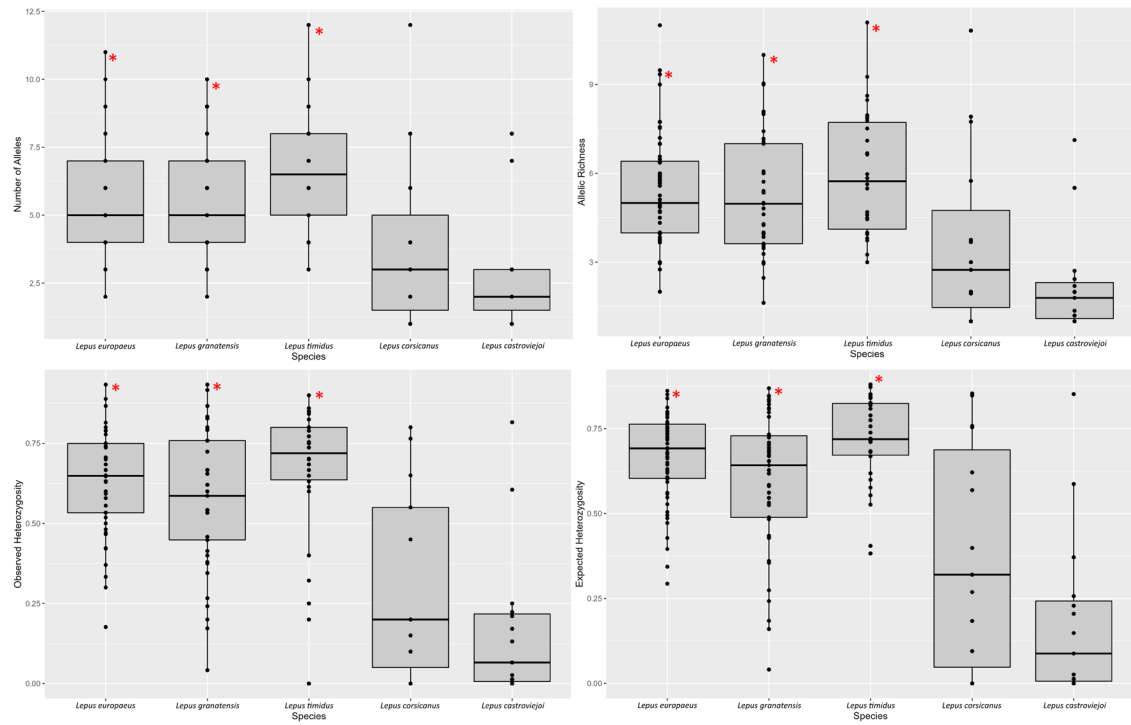


Figure 4. Values of number of alleles, allelic richness, observed and expected heterozygosity obtained for each one of the 15 microsatellite markers and populations analyzed in this study. Statistically significant differences between the broom hare (*L. castroviejo*) and the other hare species: European hare (*L. europaeus*), Iberian hare (*L. granatensis*), mountain hare (*L. timidus*) and Italian hare (*L. corsicanus*) - are represented by a red asterisk.

Similar results were obtained when comparing the estimates obtained from microsatellite data available in literature (Figure 5 and Table S9, Supplementary Information).

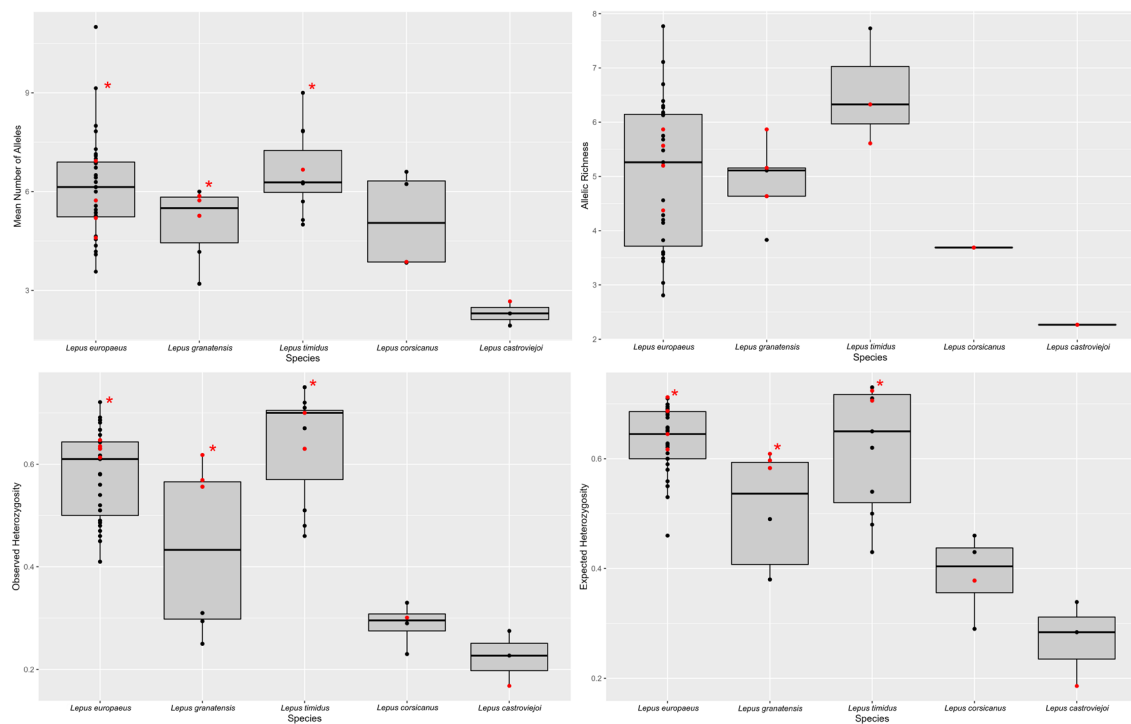


Figure 5. Values of mean number of alleles, allelic richness, observed and expected heterozygosity obtained from the literature (black dots) and values obtained for each one the populations analyzed in this work using the 15 microsatellite markers (red dots). Values of statistically significant differences between the broom hare (*L. castroviejo*) and the other hare species: European hare (*L. europaeus*), Iberian hare (*L. granatensis*), mountain hare (*L. timidus*) and Italian hare (*L. corsicanus*) are represented by a red asterisk.

Within the broom hare, no significant differences on nuclear genetic diversity parameters were observed when compared by sampling periods, even though the inbreeding coefficient (F_{IS}) increased from $F_{IS}=0.042$ in 2003/07 up to $F_{IS}=0.117$ in 2019/2020 (Table 3).

3.3. Population differentiation and structure

The degree of genetic differentiation among populations was consistent with species origin, except for the broom hare and Italian hare that clustered together in the Neighbor joining trees (NJT) inferred from the pairwise Nei genetic distance (Figure 6A) and pairwise F_{ST} (Figure S1, Supplementary information). Notably, the mountain hare populations showed the lowest distance towards the broom and Italian hare in the NJT (Figure 6A see details in Table S10 Supplementary information), with F_{ST} values ranging from 0.282 to 0.606 (Table S11, Supplementary information).

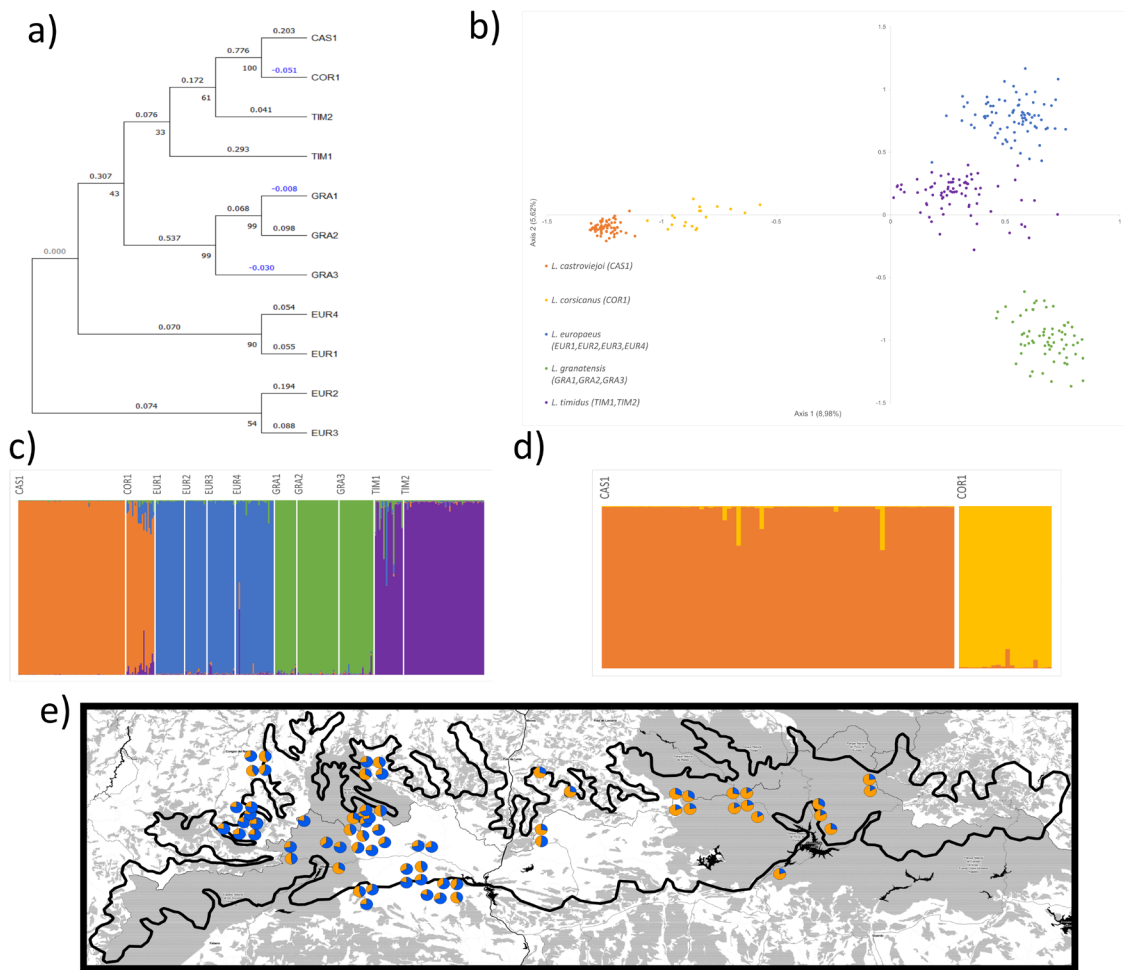


Figure 6. Population differentiation and structure: **a)** Neighbor-Joining tree inferred from the pairwise Nei genetic distance among populations of each species (European hare - *L. europaeus*, EUR1, EUR2, EUR3, EUR4); Iberian hare - *L. granatensis*, GRA1, GRA2, GRA3; mountain hare - *L. timidus*, TIM1, TIM2; Italian hare - *L. corsicanus*, COR1; and broom hare - *L. castroviejoi*, CAS1). Node labels represent bootstrap support for each node, while branch labels represent branch length according to Nei's standard genetic distance; **b)** Factorial correspondence analysis (FCA) plot obtained for the hare populations and using the 15 microsatellite markers; **c)** Bayesian clustering results obtained in STRUCTURE for K=4 (the best DeltaK value) using all hare populations and 15 microsatellite markers. Bar color represents the membership proportion (qi) obtained for each cluster; **d)** Bayesian clustering results obtained in STRUCTURE for k=2 (the best DeltaK value) using only broom and Italian hare populations and 15 microsatellite markers; **e)** Bayesian clustering results obtained in STRUCTURE for K=2 (the best DeltaK value) using only the broom hare population and the 11 polymorphic markers for this species. Pie charts represent the individual membership proportion obtained for each cluster.

The FCA (Figure 6B) results were concordant with the NJT, with the broom hare and Italian hare clustering together and the mountain hare showing the least differentiation towards these species.

The same pattern occurred in the STRUCTURE analysis (Figure 6C) where, the highest DeltaK value was reached for K = 4, segregating the broom/Italian hares from the other three species (Figure S2, Supplementary information). The hierarchical analysis carried out with only the broom/Italian cluster showed the highest DeltaK value for k = 2,

segregating the broom hare from the Italian hare (Figure 6D and see details in Figure S3, Supplementary Information). In this analysis, however, some small levels of admixture between these two parapatry species was evidenced. Regarding the other European cluster, the highest DeltaK value was obtained for $k = 3$, segregating the Iberian hare, European hare and mountain hare populations by species (Figure S4, Supplementary Information).

Considering only the broom hare individuals and 11 polymorphic loci, the highest DeltaK value was obtained for $k = 2$, segregating the species into two clusters that showed a shallow geographic distribution across east and west of the Cantabrian mountains (Figure 6E, see details in Figure S5, Supplementary information). The mean $\text{LnP}(K)$ value for $k=1$ (-885.06) was higher than for $k=2$ (-910.82). However, the difference between the values of mean $\text{LnP}(K)$ for $k=1$ and $k=2$ was inferior to the standard deviation for $\text{LnP}(K)$ of $k=2$ (Table S12, Supplementary Information). This could be an indication that the population differentiation evidenced for $k=2$ is shallow or that the species may be in the early stages of differentiation, since both values of k have very similar $\text{LnP}(K)$ values. The pattern of differentiation observed in STRUCTURE, however, is not evidenced in the FCA (Figure 7).

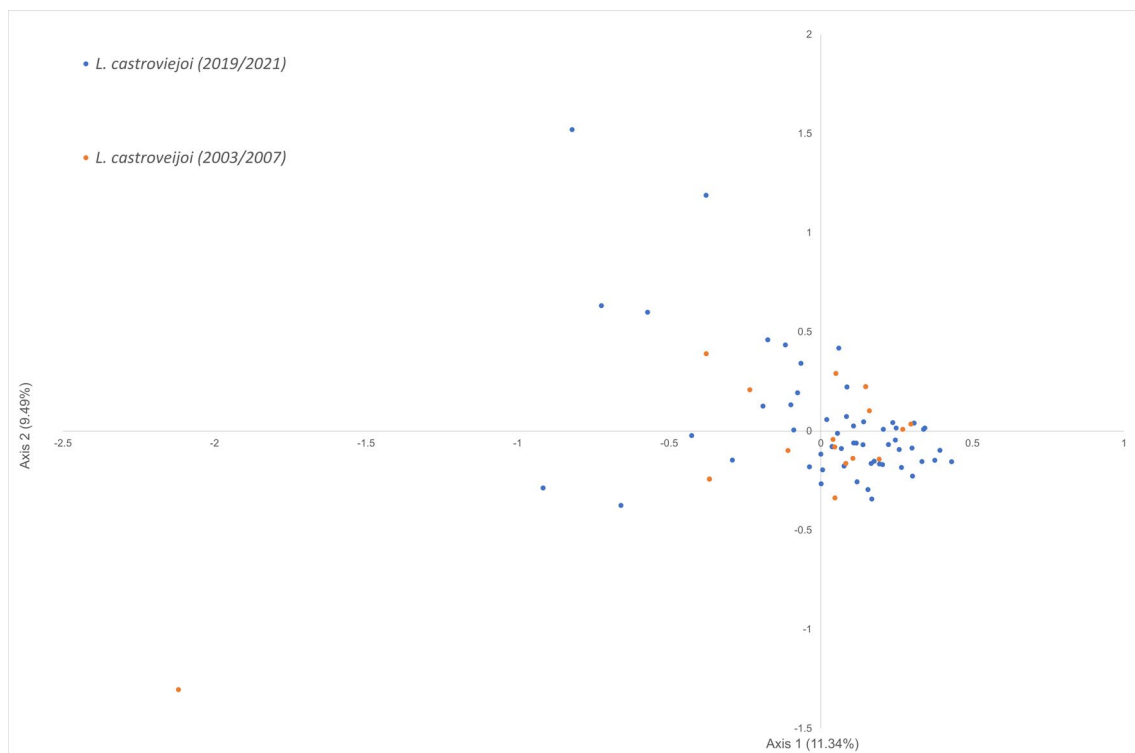


Figure 7. Factorial Correspondence Analysis (FCA) plot obtained for the 11 microsatellite loci using only the broom hare population (CAS1), divided by sampling periods (2003/2007 and 2019/2021).

A pattern of isolation-by-distance was inferred from Mantel test ($R_{xy} = -0.316$, $p\text{-value} < 0.05$), even though with small correlation coefficient ($r^2 = 0.10$) (Figure 8).

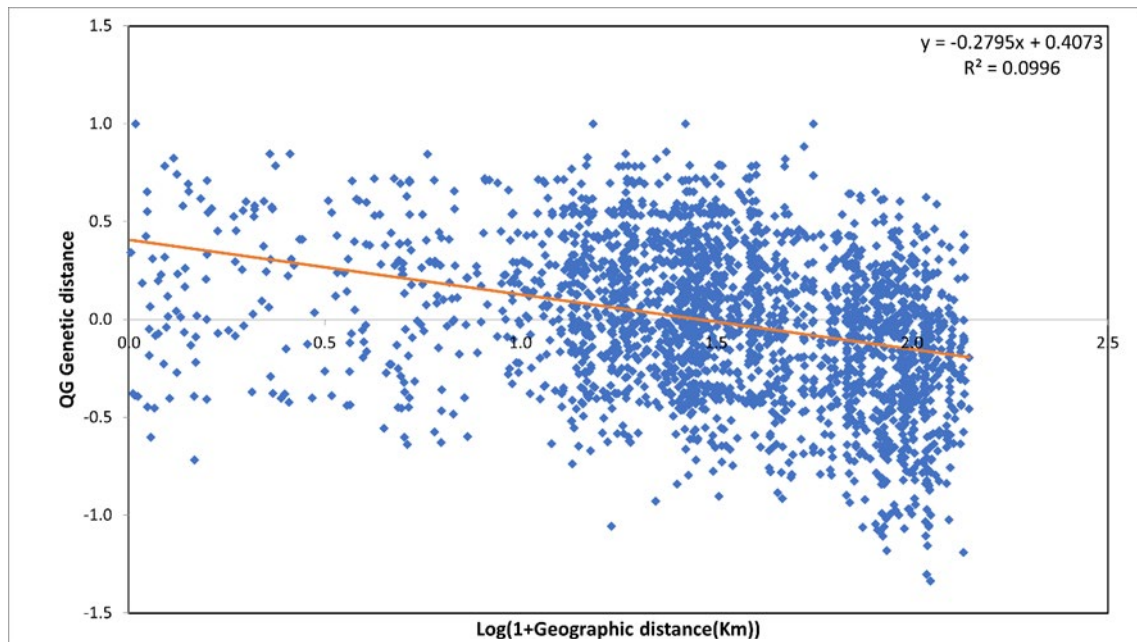


Figure 8. Isolation-by-Distance. Mantel test performed utilizing GenAlEx to test the occurrence of a correlation between the genetic relatedness of individuals (QG Genetic Distance) and the logarithm of the geographic distance in Km. Each blue dot represents a pairwise comparison between two samples, trendline is displayed in orange and correlation coefficient (R^2) displayed on the top right of the graph. The genetic and geographic distances had a negative significant correlation ($R_{xy} = -0.316$ $p = 0.001$).

3.4. Mitochondrial diversity and phylogeography

Eight mitochondrial cytB haplotypes were identified from the 64 analyzed broom hare specimens, representing a nucleotide and haplotype diversity of $\pi = 0.01543$ and $H_p = 0.743$, respectively (Table 5). When compared with the values for these parameters calculated using the sequences extracted from NCBI the broom hare showed the lowest values of haplotype diversity than all analyzed species with exception of the Italian hare and lower nucleotide diversity values than both the European and Iberian hare (Table 5).

Table 5. Mitochondrial genetic diversity parameters inferred for the broom hare (*L. castroviejo*), the European hare (*L. europaeus*), the Iberian hare (*L. granatensis*), the mountain hare (*L. timidus*) and the Italian hare (*L. corsicanus*) utilizing the 541bp cytb haplotype sequences.

Species	N	h	H _p	π
<i>Lepus castroviejo</i>	64	8	0.743	0.01543
<i>Lepus europaeus</i>	81	31	0.944	0.04219
<i>Lepus granatensis</i>	253	55	0.915	0.04773
<i>Lepus timidus</i>	206	67	0.931	0.01053
<i>Lepus corsicanus</i>	7	3	0.762	0.00194

N, number of individuals; h, number of haplotypes, H_p, haplotype diversity; π, nucleotide diversity.

No statistical differences were observed for these parameters when compared with the population data available in literature for the other European species (Figure 9 and Table S13 Supplementary Information).

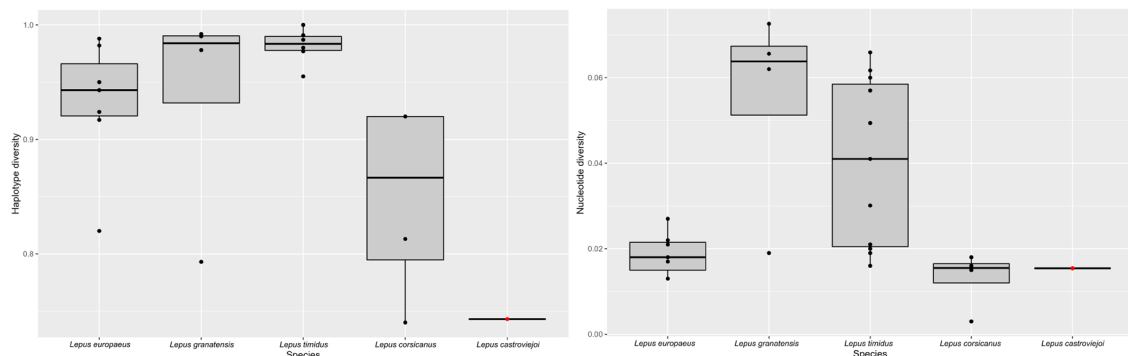


Figure 9. Values of haplotype and nucleotide diversity obtained from the literature for the European hare (*L. europaeus*), the Iberian hare (*L. granatensis*), the mountain hare (*L. timidus*) and the Italian hare (*L. corsicanus*) (black dots) and same values calculated for the broom hare (*L. castroviejo*) for the 541 cythochrome-b fragment analyzed (red dots).

Within the broom hare, no differences between mitochondrial DNA genetic diversity parameters were estimated when the two different sampling periods were compared. Two divergent lineages of haplotypes (Haplotypes 1, 2, 3, 5, 6, 7 and Haplotypes 4 and 8) were estimated from the network analysis (Figure 10). The “ancestral” lineage haplotypes (1, 2, 3, 5, 6, and 7) were found fairly distributed across the species range, while the “introgressed” lineage haplotypes (4 and 8) were found in opposing areas of the species distribution (Figure 11).

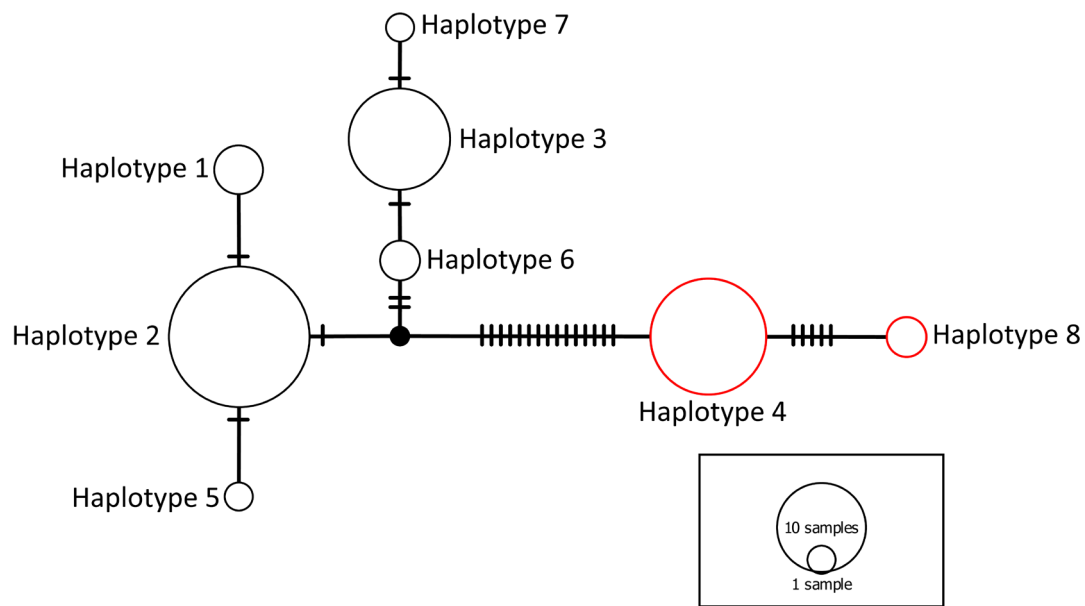


Figure 10. Mitochondrial cytochrome-b haplotypes (541bp) found in this study for the broom hare. Median joining network estimated using PopART (Bandelt et al. 1999; Leigh & Bryant 2015). Size of haplotypes is proportional to the number of samples; dashes represent number of mutations between each haplotype. The haplotypes belonging to the mountain hare (*L. timidus*) lineage introgressed in the Iberian (*L. granatensis*) and European (*L. euroapeus*) are outlined in red.

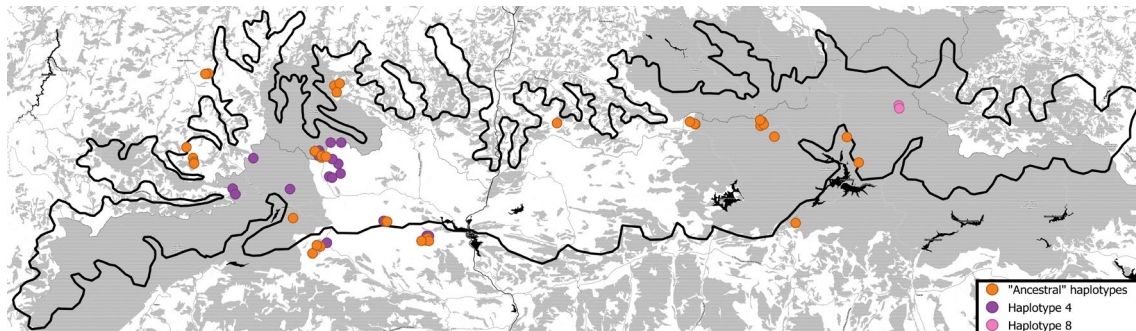


Figure 11. Distribution map of the broom hare haplotype lineages. Broom hare distribution outlined in black. Individuals carrying the identified haplotype lineages are represented by coloured dots.

A similar pattern of divergence was found in the Bayesian and Maximum likelihood evolutionary trees (Figures S6 and S7, Supplementary Information). These two lineages were clustered within the wide mountain hare clade (Figure 12). Haplotypes 1, 2, 3, 5, 6 and 7 were found to be closely related to haplotypes from the Italian hare, while haplotypes 4 and 8 clustered closely with mountain hare haplotypes, previously inferred to have introgressed into the European and Iberian hares.

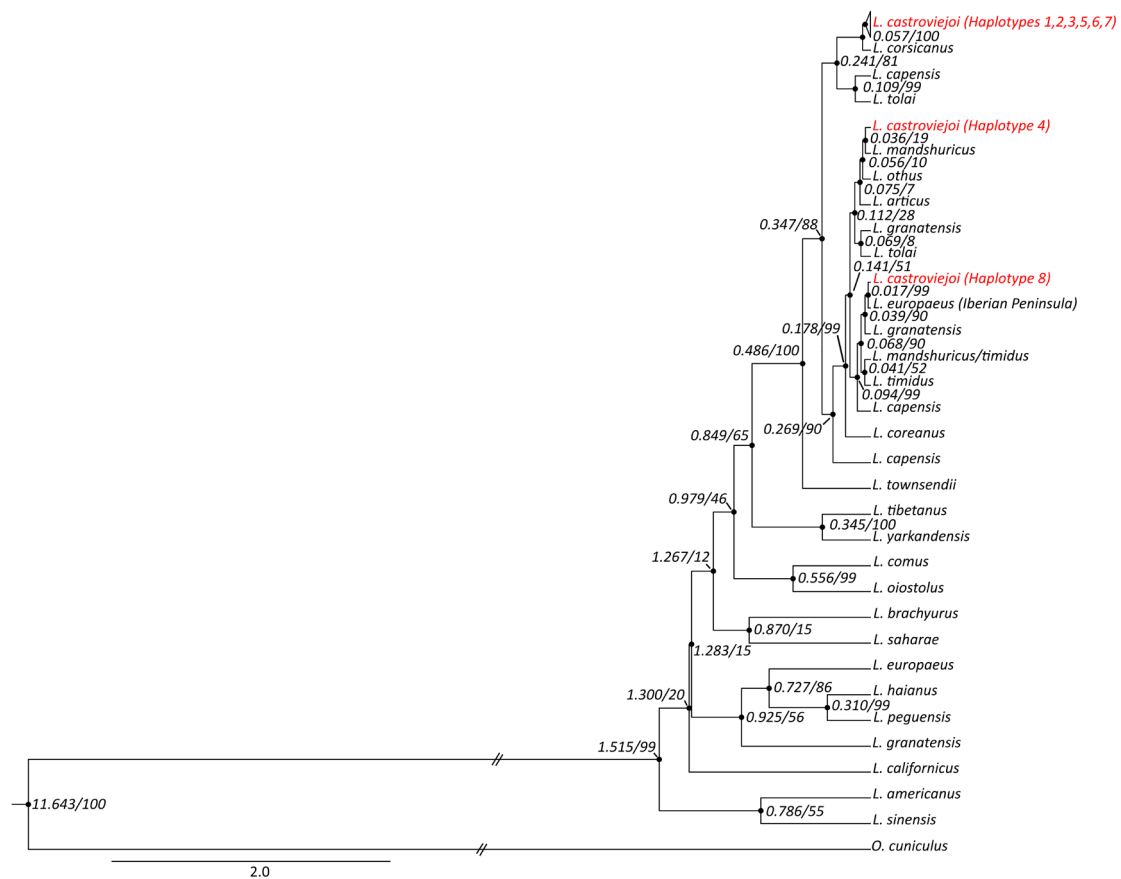


Figure 12. Divergence time tree estimated with BEAST2 and calibrated with the *Lepus-Oryctolagus* and *L. corsicanus* – *L. castroviejoi* divergence times. Nodes labels represent divergence time in million years before the present and posterior probability, respectively.

3.5. Demographic and evolutionary history

Effective population size for the broom hare was low, with a point estimate of 404 (95% CI 111 - >10000) for the maximum likelihood method and a point estimate of 634 for the moment estimator method.

No signals of recent population bottlenecks were evidenced from the heterozygosity excess test performed in Bottleneck (Wilcoxon one tail with p-value > 0.05). Additionally, no evidence of recent population expansion was inferred from Tajima's D, Fu's and Li's D test statistics (p-value > 0.05)(Table 6). However, the Bayesian skyline plot showed smooth population decline of population effective size (N_e) around 50.000 YBP, (Figure 13), even though with broad confidence limits.

Table 6. Fu's and Tajima's D test statistics inferred for the broom hare using the 541bp fragment of cytochrome-b gene.

Species	Broom hare (N=64)
Fu's and Li's D* test statistic	1.13198 Not significant
Tajima's D	1.82555 Not significant

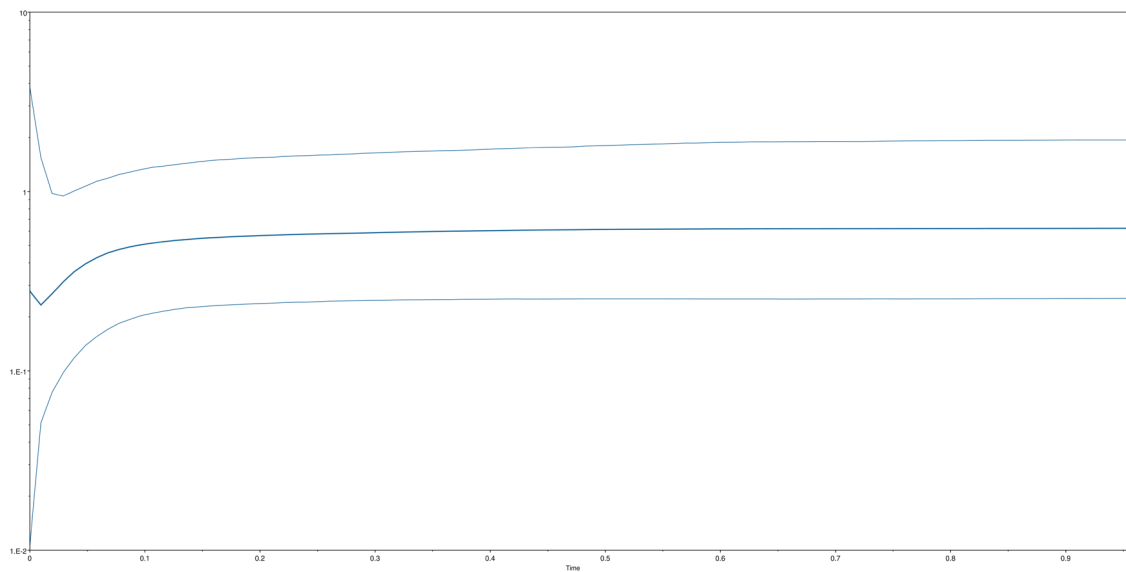


Figure 13. Bayesian skyline plot (BSP) calculated using the 541 bp mitochondrial cytochrome-b fragment of the broom hare and calibrated with a mutation rate of 1% per Mya.

To date the time of divergence between the broom hare and the other European hare species we used the relaxed clock model, since it showed overwhelming support (log marginal likelihood of -3144.6) as compared with the strict clock model (log ML of -3387.3). Thus, the relaxed clock divergence tree (Figure 12), placed the divergence of the introgressed mountain hare haplotypes in the broom hare (Haplotype 4 and 8) and the “ancestral” broom hare haplotypes (Haplotype 1,2,3,5,6 and 7) at around 0.347 Myr ago (95% HPD 0.109–0.647 Myr).

4. Discussion

Using genetic non-invasive sampling (feces) coupled with tissue collection along the distribution range of the broom hare in the Cantabrian Mountains we gain novel insights on the genetic diversity of this endemic and threatened species (Ballesteros & Smith 2019). The broom hare showed the lowest levels of nuclear genetic diversity ($N_a = 2.667$, $A_r = 2.265$, $H_o = 0.168$ and $H_e = 0.186$) and very low values of mitochondrial DNA diversity (541bp fragment of *cytB*), when compared either with the studied populations of the Iberian hare, European hare, mountain hare and Italian hare (Figure 4), or the data published so far in literature for these species (Figures 5 and 9).

Nuclear genetic diversity of the broom hare varied slightly between sampling periods, with N_a , H_o and H_e showing lower values in 2019/21 in comparison with 2003/2007. Although no statistically significant differences were found between sampling periods, this might be indicative of a tendency for genetic diversity loss over time. The higher number of individuals sampled in 2019/2021 ($n=59$) when compared with 2003/2007 ($n=17$), coupled with their widespread distribution range provides additional support for this hypothesis. The recent (2019/2021) estimates of genetic diversity parameters (H_e , H_o and N_a) were even lower than those observed in previous studies using small number of individuals and markers (Bonhomme et al. 1986; Alzaga 2010). Although the consequences of low genetic diversity for this species are unknown, it may represent a serious risk to population fitness and adaptive potential (Reed & Frankham 2003; Spielman et al. 2004), which together with the restricted distribution (Palacios & Meijide 1979) might represent an increased risk of extinction (Spielman et al. 2004; Sgrò et al. 2011).

Notably, the Italian hare is the only species that showed similarly low levels of nuclear DNA diversity (Figure 4). This might be explained by their common evolutionary history (Alves & Melo-Ferreira 2007; Alves et al. 2008a; Acevedo et al. 2014; Mengoni et al. 2015) as well as the restricted distribution range of the Italian hare when compared with the other European hare species, which can result in lower effective population sizes and lower genetic diversity.

Our findings as well as recent evidence, suggest that both species derived from a very recent common ancestor (Alves et al. 2003; Alves & Melo-Ferreira 2007; Alves et al. 2008a; Acevedo et al. 2014) (Figure 12), and that the divergence and further isolation in the Iberian and Italian Peninsulas occurred around 50kya (Souto 2022), overlapping with the last glacial period (Head 2021). Similar patterns of retraction and isolation in glacial

refugia are well documented in the literature for a wide range of species such as, the red deer (*Cervus elaphus*; (Linnaeus 1758)), beetles (*Carabus irregularis*; (Fabricius 1792)), conifers (*Cryptomeria japonica*; (D. Don.)) and frogs (*Rana sylvatica*; (LeConte, 1825)) (Lee-Yaw et al. 2008; Schmitt 2009; Homburg et al. 2013; Kimura et al. 2014; Queirós et al. 2019). The recent divergence between species is also supported by nuclear DNA data (Figure 6). High levels of similarity between these two species are still currently inferred from microsatellite loci analysis (Figure 6), reinforcing previous findings using molecular SNP markers (Alves et al. 2008a; Melo-Ferreira et al. 2012).

The reduced nuclear DNA diversity of the broom and the Italian hare populations also suggests that genetic diversity estimates were not greatly biased for the broom hare by the amplification of cross-specific markers previously developed for the European hare. This could happen due to ascertainment bias introduced during the optimization procedures for the targeted species, as well as the criteria used for marker selection (Queirós et al. 2015). The high genetic diversity values observed in the Iberian and mountain hare populations are an additional support for the reduced impact of ascertainment bias introduced by our panel of microsatellites (Table 3). Despite a slight increase of nuclear DNA diversity estimates when the broom hare's monomorphic loci (n=4) were removed from the analysis (Table 4), the significant differences among species remained consistent (Table S14, Supplementary Information), highlighting the accuracy of our panel of markers.

The mitochondrial DNA diversity of broom hare was also low. For the comparisons utilizing the 541bp cytb fragment only the Italian hare showcased lower values for haplotype diversity. Although this could be due to the low number of analyzed sequences for this species (n=7) when compared with the broom hare (n=64). Regarding nucleotide diversity both the mountain and the Italian hare showcased lower values than the broom hare. The higher nucleotide diversity in the broom hare when compared with the mountain hare might be an effect of the diverging haplotype lineages encountered in the broom hare which might slightly increase nucleotide diversity.

For the population comparisons the broom hare also showed low values, but equivalent to values estimated in some populations from other hare species (Figure 9). These comparative analyses were restricted to the data available in the literature, which, due to the wide variability of genes and fragments amplified, together with the distinct number of individuals used in each study, hampered a robust comparison among populations/species, as done previously for nuclear diversity parameters (Pierpaoli et al. 1999; Kasapidis et al. 2005; Waltari & Cook 2005; Estonba et al. 2006; Hamill et al. 2006;

Melo-Ferreira et al. 2007; Ben Slimen et al. 2008; Alzaga 2010; Melo-Ferreira et al. 2011; Canu et al. 2013; Topchieva et al. 2013; Melo-Ferreira et al. 2014; Seixas et al. 2014; Mengoni et al. 2015; Rehnus & Bollmann 2016).

Nevertheless, the reduced mtDNA diversity of the broom hare might be a result of ancient population bottlenecks as a consequence of species retraction and isolation during the glacial cycles (Widmer & Lexer 2001). Moreover, it might be shaped by the mtDNA introgression from the mountain hare with consequent replacement of the original broom hare haplotypes, or a combination of factors such as population demography and introgression (Muñoz & De la Rúa 2021). The low mitochondrial DNA diversity described for some populations of European hare, mountain hare and Italian hare might be explained by similar introgression events and/or biogeographic phenomena such as population isolation as well as other factors such as bottlenecks.

The phylogenetic relationships of the two described broom hare mtDNA lineages positioned them within the wide mountain hare mitochondrial DNA clade, together with the Italian hare haplotypes and the other mountain hare haplotypes introgressed in the Iberian hare and the European hare (Figure 12; see details in Figures S6 and S7, Supplementary Information). While the “ancestral” lineage comprising haplotypes 1, 2, 3, 5, 6 and 7 includes only broom hare sequences, despite being closely related to the Italian hare sub-clade, the “introgressed” lineage comprising the haplotypes 4 and 8 is closely related to the mountain hare haplotypes, which are known to have been introgressed into the Iberian and European hares (Melo-Ferreira et al. 2005; Alves et al. 2008b) (Figure 12; see details in Figures S6 and S7, Supplementary Information). Given that phylogenies based on an extensive nuclear genomic dataset showed that the broom hare is not closely related to the mountain hare (Melo-Ferreira et al. 2012; Ferreira et al. 2021), it has been previously suggested that both mtDNA lineages present in the broom hare resulted from introgression at two different times: an older event replacing the broom and the Italian hare native mtDNA before the split of these species, and a more recent one resulting from the introgression events that also affected the other species in the Iberian Peninsula (Melo-Ferreira et al. 2012). Our results are consistent with this hypothesis. Moreover, they suggest that the upper bound for the first introgression event may be around 241.000 (95% HPD 0.071–0.474 Myr) years before the present, represented by the most recent common ancestor between the broom hare and the Italian hare mtDNA clade and the closest *timidus*-like clade. The second introgression event might have occurred more recently with the upper bound for this introgression being ~57k (95% HPD 39–76 Ka) years before the present since it most likely occurred

in the Iberian Peninsula after the split between the broom and Italian hare (Figure 12). Previous genomic analyses from the Iberian hare, suggested that these more recent introgression events occurred after the last glacial maximum (Seixas et al. 2018). An alternative hypothesis for the latter mitochondrial lineage is a secondary introgression event of the broom hare through hybridization with introgressed Iberian and European hare species (Alves et al. 2008b).

The haplotype phylogeography showcases the “ancestral” lineage of haplotypes distributed across the species range. However, haplotype 4 and 8 despite belonging to the “introgressed” lineage are found in opposing areas of the distribution. Additionally, these two haplotypes despite clustering within mountain hare introgressions do not cluster together as do the haplotypes from the “ancestral” lineage. As such it is possible that these two haplotypes might have also been introgressed in the broom hare in two separate occasions by hybridization with neighboring introgressed Iberian hares, given previous evidence of admixture between the broom and Iberian hares (Melo-Ferreira et al. 2009). However, the data collected in this work is not sufficient to fully support this hypothesis.

The identification of broom hare individuals using gNIS collected outside the presumed geographic distribution of the species (Palacios & Meijide 1979) highlights not only the usefulness of this methodological approach, but also the possible wider distribution range of the species. Moreover, it also indicates an absence of hybridization between the broom hare and the Iberian hare, hinting at their putative parapatry behavior and reproductive isolation (Barton & Bengtsson 1986). No signals of hybridization with European hare were also observed in the northern-east limits, although sampling in this region was only conducted in the core distribution of broom hare (Figure 2). These findings reinforce previous evidence of the lack of widespread recent hybridization between the broom hare and their putative sympatric species (Alves et al. 2008b; Ballesteros & Smith 2019). However, future studies are needed to accurately infer the distribution limits of these species in and around the Cantabrian Mountains. A large gNIS sampling scheme, particularly in the uppermost limits of its known distribution range, using the efficient approach developed here, may provide a better understanding of the interaction dynamics between species, and assist in the implementation of management and conservation strategies to preserve the broom hare. Moreover, it is also important to clarify the existence or not of genetic barriers to gene flow.

Our findings evidenced a shallow population differentiation and structure between individuals sampled in the western and in the eastern regions of the Cantabrian

Mountains, raising additional concerns regarding the preservation of the overall gene pool of the species (Kool et al. 2013). Similar patterns of population differentiation along the Cantabrian mountains have also been reported for other mountain species. With population splits being reported in the Brown Bear (*Ursus arctos*; (Linnaeus 1758)) (Gonzalez et al. 2016), the Cantabrian Capercaillie (*Tetrao urogallus cantabricus*; (Castroviejo 1967))(Vázquez et al. 2012) and the Cantabrian chamois (*Rupicapra pyrenaica parva*; (Cabrera 1914))(Fernández-Morán et al. 1997). Regarding the brown bear (García et al. 2007) suggests that the area between the two subpopulations sports lithological constraints indicating poorer habitat quality. These lithological constraints could also have affected the population splits in the Cantabrian Capercaillie as well as the Cantabrian chamois (García et al. 2007). These gaps in population distribution might also be accounted for by human disturbances since there is an accumulation of human made structures and roads in this area (Gonzalez et al. 2016). The combination of these two factors, the low-quality habitat and the accumulation of structures and roads, might have contributed for the pattern of population differentiation evidenced not only in the broom hare but also in various other species across the Cantabrian mountains.

The signs of isolation-by-distance in the broom hare (Figure 13) were minor and there were no indications of population genetic bottleneck. However, this shallow differentiation might be rapidly magnified by the progressive population declines associated with anthropogenic impacts, habitat modifications due to climate change and ongoing pastoral changes (Sgrò et al. 2011; Ballesteros & Smith 2019). Thus, this work shows the relevance of the implementation of a gNIS program to accurately assess the broom hare distribution range, monitor genetic diversity and gene flow, and ascertain putative hybridization with the other Iberian hare species.

5. Conclusion

In this work we evaluated for the first time the genetic diversity, population structure and evolutionary history of the broom hare population over its entire distribution range. This species showed very low levels of nuclear diversity, with values of $N_a = 2.667$, $A_r = 2.265$, $H_o = 0.168$ and $H_e = 0.186$. These parameters were significantly lower than those observed for other European hare species analyzed in this study, except for the sister species, the Italian hare. In contrast, no significant differences on mitochondrial genetic diversity parameters ($\pi = 0.01543$ and $H_p = 0.743$) was evidenced when compared with data available in literature for the European hare species. The broom hare shows a clear

differentiation from the Iberian hare, European hare and mountain hare in all performed genetic analyses, with no evidence of recent hybridization. Shallow population differentiation and structure was inferred within the broom hare distribution, with a significant isolation-by-distance pattern. In contrast, no signals of recent genetic bottleneck and population expansion were evidenced. However, an ancient decrease of effective population size was estimated around 50.000 YBP in the BSP, overlapping with divergence time inferred between the broom hare and Italian hare. Two broom hare *cytB* lineages were detected from the network and phylogenetic trees. Both lineages clustered within the wide mountain hare clade, with an estimated divergence time of around 0.347 Myr between the two. These two lineages most likely resulted from two events of mountain hare introgression in the broom hare.

Overall, the reduced genetic diversity of the broom hare coupled with the apparent population differentiation and the restricted distribution range of the species might represent a serious risk of losing population adaptability and increasing the likelihood of population extinction. Efforts should be employed to continue studying this endemic and threatened species, particularly considering the recent evidence of population declines as a result of anthropogenic impacts and climate change.

6. Future Prospects

This thesis provided novel insights on species distribution, population structure and genetic diversity of the broom hare, however aspects such as the accurate detailed distribution range of the species, its population size and recent population trends as well as its interactions with other hare species remain mostly unknown.

To tackle this, an even wider sampling effort should be employed, with sampling locations extending beyond the species distribution range mainly in the west and east regions of the distribution. A genetic non-invasive sampling program should be implemented, including areas below 1000m in altitude to assess the possibility of altitudinal structuring of the species, with the broom hare replacing the Iberian hare at higher altitudes. The extensive sampling efforts, using gNIS, could offer a deeper understanding of the distribution range of the broom hare, its population structure as well as possible hybridization with the neighbouring European and Iberian hares. The existence or lack of hybrids could provide insights on the origin of the “introgressed”

mountain hare mtDNA lineage found in the broom hare, whether this occurred through direct or secondary introgression.

Population monitoring studies, using gNIS, should also be performed to understand the population size and trends of the broom hare. Due to its low genetic diversity, supposed restricted range and endemic status, the broom hare might be facing a high risk of extinction and as such its population should be monitored and adequate conservation measures should be employed such as monitoring hunting as to not cause irredeemable population decreases or further decreases to its genetic diversity.

Additionally, genomic analysis could be performed to further clarify the evolutionary history of the broom hare, such as possible past bottlenecks and introgression events.

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8. Supplementary Information

Table S1. Number of the broom hare samples collected at each Spanish Municipality.

Municipality	N
Cangas de Narcea	39
Villablino	5
Villamanín	10
Palacios del Sil	3
Aller	7
Lena	5
Somiedo	19
Teverga	3
Puebla de Lillo	10
Maraña	10
Teverga	10
Burón	4
Posada de Valdeón	9
Boca de Huérgano	3
Murias de Paredes	11
Sena de Luna	11
San Emiliano	26
Total	185

Table S2. Summary of the nuclear genetic diversity parameters compiled in the literature for the European hare species.

Species	N	Microsatellite number	Na	He	Ho	Allelic Richness	Fis	Reference
<i>L. castroviejo</i>	11	6	2.3	0.284	0.227	-	-	Estonba et al.2006
<i>L. granatensis</i>	19		3.2	0.49	0.294	-	-	
<i>L. europaeus (Bulgaria)</i>	31		8	0.695	0.486	-	-	
<i>L. europaeus (Iberia)</i>	39		6.5	0.559	0.644	-	-	
<i>L. euroapeus (Italy-HD1)</i>	40	7	9.14	0.652	0.617	6.3	-	Canu et al.2013
<i>L. euroapeus (Italy-HD2)</i>	15		7.29	0.688	0.667	7.11	-	
<i>L. euroapeus (Italy-HD3)</i>	19		7.14	0.687	0.61	6.7	-	
<i>L. euroapeus (Italy-ANG)</i>	20		6.86	0.685	0.691	6.18	-	
<i>L. euroapeus (Italy-BRO)</i>	20		6.86	0.628	0.657	6.13	-	
<i>L. euroapeus (Italy-CAV)</i>	15		6.43	0.656	0.681	6.27	-	
<i>L. euroapeus (Italy-CHI)</i>	19		6.43	0.699	0.631	6.14	-	
<i>L. euroapeus (Italy-CIV)</i>	20		6.14	0.684	0.686	5.75	-	
<i>L. euroapeus (Italy-CRE)</i>	21		6.14	0.692	0.721	5.68	-	
<i>L. euroapeus (Italy-CRO)</i>	20		5.57	0.657	0.643	5.26	-	
<i>L. euroapeus (Italy-ESS)</i>	15		6.29	0.621	0.581	6.15	-	
<i>L. euroapeus (Italy-MON)</i>	5		3.57	0.625	0.686	-	-	
<i>L. euroapeus (Italy-PIE)</i>	20		6	0.675	0.643	5.48	-	
<i>L. euroapeus (Italy-Total)</i>	249		12.57	0.68	0.651	-	-	
<i>L. corsicanus</i>	149	13	6.6	0.43	0.29	-	0.31	Mengoni et al.2014
<i>L. corsicanus (Peninsular Italy)</i>	77		6.23	0.46	0.33	-	0.25	
<i>L. corsicanus (Sicily)</i>	66		3.84	0.29	0.23	-	0.25	
<i>L. euroapeus</i>	146		11	0.58	0.58	-	0.14	
<i>L. Timidus</i>	72		9	0.48	0.48	-	0.28	
<i>L. europaeus (HUL)</i>	10	11	4.64	0.58	0.48	3.599	0.2297	Slimen et al.2008
<i>L. europaeus (URF)</i>	7		4.36	0.6	0.49	3.599	0.2535	
<i>L. europaeus (CEV)</i>	17		6.73	0.71	0.56	4.56	0.2433	
<i>L. europaeus (MAN)</i>	11		5.36	0.65	0.58	4.145	0.1427	
<i>L. europaeus (TRK)</i>	13		5.27	0.6	0.46	3.8242	0.2739	
<i>L. europaeus (LOT)</i>	12		5.73	0.69	0.63	4.286	0.1271	
<i>L. europaeus (VRT)</i>	23		7.09	0.68	0.54	4.199	0.2231	
<i>L. europaeus (YAM)</i>	10		4.64	0.6	0.56	3.605	0.1148	
<i>L. europaeus (PLV)</i>	23		5.36	0.59	0.48	3.568	0.2138	
<i>L. europaeus (MOL)</i>	9		4.18	0.6	0.45	3.491	0.3041	
<i>L. europaeus (LAS)</i>	42		5.45	0.61	0.52	3.435	0.163	
<i>L. europaeus (NIE)</i>	24		4.55	0.53	0.51	3.037	0.0546	
<i>L. europaeus (MEM)</i>	22		4.09	0.46	0.41	2.808	0.0274	
<i>L. granatensis (Parental)</i>	79	6	4.17	0.38	0.25	3.83	-	Melo-
<i>L. granatensis (Contact zone)</i>	115		6	0.38	0.31	5.11	-	Ferreira et
<i>L. euroapeus (contact zone)</i>	40		7	0.55	0.47	6.39	-	al.2014
<i>L. euroapeus (parental)</i>	69		7.83	0.55	0.49	7.77	-	
<i>L. timidus (Italian alps/Sweden)</i>	39		7.83	0.54	0.46	7.73	-	
<i>L. castroviejo</i>	8	5	1.932	0.339	0.275	-	-	Alzaga 2010
<i>L. timidus (Swiss alps)</i>	26	10	5	0.5	0.51	-	-	Rehnus and Bollmann 2016
<i>L. timidus (Northern Finland)</i>	9	7	5.7	0.73	0.7	-	-	Hamill et al. 2006
<i>L. timidus (Southern Finland)</i>	27		7.85	0.62	0.67	-	-	
<i>L. timidus (Northern Sweden)</i>	11		6.28	0.65	0.7	-	-	
<i>L. timidus (Central Sweden)</i>	9		6.28	0.73	0.75	-	-	
<i>L. timidus (Norway)</i>	7		5.14	0.71	0.72	-	-	
<i>L. timidus (Republic of Karelia)</i>	31	4	6.25	0.43	0.71	-	-	Topchieva et al. 2013

N, number of individuals; Na, average number of Alleles, He, expected Heterozygosity; Ho, observed Heterozygosity; Fis, Inbreeding Coefficient; Ar, Allelic Richness

Table S3. Summary of the mitochondrial genetic diversity parameters compiled in the literature for the European hare species.

Species	n	MtDNA Region	Size of fragment	h	π	(Hd)	Reference
<i>L. corsicanus</i>	136			37	0.016	0.92	
<i>L. corsicanus (Italy)</i>	76			24	0.018	0.92	
<i>L. corsicanus (Sicily)</i>	56	CR	439bp	13	0.003	0.74	Mengoni et al.2015
<i>L. euroapeus</i>	143			70	0.018	0.95	
<i>L. timidus</i>	63			40	0.041	0.98	
<i>L. granatensis</i>	183			67	0.019	0.978	
<i>L. europaeus (Iberian Peninsula)</i>	70			11	0.017	0.82	
<i>L. timidus (Northern Europe)</i>	47	CytB /	617bp / 471-	39	0.02	0.987	Melo-Ferreira et al.2007
<i>L. timidus (Alps)</i>	47	CR	473bp	24	0.019	0.955	
<i>L. timidus (Eastern Europe)</i>	4			4	0.016	1	
<i>L. timidus (Eastern Russia)</i>	26			23	0.021	0.991	
<i>L. corsicanus</i>	36			13	0.015	0.813	
<i>L. euroapeus</i>	41	CR-I	293-295bp	22	0.013	0.917	Pierpaoli et al.1999
<i>L. timidus</i>	19			16	0.057	0.977	
<i>L. timidus (Fennoscandia)</i>	5			5	0.0617	-	
<i>L. timidus (E Europe)</i>	7			5	0.0301	-	
<i>L. timidus (Central Russia)</i>	26	CR	473bp	22	0.0494	-	Waltari et al.2005
<i>L. timidus (S Central Siberia)</i>	7			6	0.06	-	
<i>L. timidus (Far East Russia)</i>	11			9	0.0659	-	
<i>L. timidus (Total/Average)</i>	69			59	0.0617	-	
<i>L. euroapeus (Clade A)</i>	25			16	0.027	0.943	
<i>L. euroapeus (Clade B)</i>	117			71	0.022	0.982	
<i>L. euroapeus (Greece and Bulgarian; Clade B)</i>	73	CR	293bp	50	0.021	0.988	Kasapidis et al.2005
<i>L. euroapeus (Italian and Central Europe)</i>	44			24	0.013	0.924	
<i>L. granatensis (Iberian peninsula)</i>	429	CR	450bp	166	0.0726	0.99	Seixas et al.2014
<i>L. granatensis (Mallorca)</i>	25			8	0.0656	0.793	
<i>L. granatensis</i>	429	CytB / CR	617bp / 472-474bp	186	0.062	0.992	Melo-Ferreira et al.2011

h, number of haplotypes; Hd haplotype diversity; π , nucleotide diversity.

Table S4. Maximum likelihood genotyping error rates for the polymorphic microsatellites of the broom hare individuals detected through gNIS.

Locus	E1 (allelic dropout)	E2 (false alleles)
Le15	0.000	0.000
Le17	0.064	0.000
Le19	0.000	0.000
Le2	0.000	0.000
Le23	0.009	0.000
Le28	0.209	4.65E-04
Le4	0.000	0.000
Le46	0.000	0.000
Le47	0.000	0.000
Le6	0.112	0.000
All loci	0.039	4.65E-05

Table S5. Probability of Identity (PI) and Probability of Identity considering siblings (PIsibs) of all analysed species utilising the 15 microsatellite panel.

<i>Species</i>	<i>N</i>	<i>PI</i>	<i>Pisibs</i>
<i>L. castroviejo</i>	76	4.8E-04	4.2E-02
<i>L. europaeus</i>	81	1.5E-15	1.7E-06
<i>L. granatensis</i>	68	7.6E-13	1.5E-05
<i>L. timidus</i>	77	1.6E-16	1.0E-06
<i>L. corsicanus</i>	20	4.5E-08	1.2E-03

Table S6. Probability of Identity(PI) per locus for all analysed species.

<i>Species</i>	<i>Le15</i>	<i>Le17</i>	<i>Le19</i>	<i>Le2</i>	<i>Le51</i>	<i>Le23</i>	<i>Le28</i>	<i>Le30</i>	<i>Le4</i>	<i>Le46</i>	<i>Le3</i>	<i>Le43</i>	<i>Le47</i>	<i>Le48</i>	<i>Le6</i>
<i>L. castroviejo</i>	0.04	0.64	0.97	0.59	1.00	0.20	0.73	0.97	0.46	0.84	1.00	1.00	0.95	1.00	0.62
<i>L. europaeus</i>	0.05	0.12	0.08	0.10	0.18	0.04	0.19	0.04	0.09	0.21	0.11	0.09	0.30	0.08	0.15
<i>L. granatensis</i>	0.03	0.14	0.22	0.10	0.57	0.04	0.17	0.04	0.61	0.38	0.26	0.10	0.16	0.27	0.18
<i>L. timidus</i>	0.04	0.22	0.02	0.07	0.29	0.05	0.12	0.04	0.09	0.13	0.14	0.07	0.17	0.15	0.06
<i>L. corsicanus</i>	0.10	0.55	1.00	1.00	1.00	0.04	0.82	0.09	0.04	0.24	1.00	0.51	0.44	0.68	0.22

Table S7. Probability of Identity considering siblings (PIsibs) per locus for all analysed species.

<i>Species</i>	<i>Le15</i>	<i>Le17</i>	<i>Le19</i>	<i>Le2</i>	<i>Le51</i>	<i>Le23</i>	<i>Le28</i>	<i>Le30</i>	<i>Le4</i>	<i>Le46</i>	<i>Le3</i>	<i>Le43</i>	<i>Le47</i>	<i>Le48</i>	<i>Le6</i>
<i>L. castroviejo</i>	0.33	0.81	0.99	0.77	1.00	0.51	0.86	0.99	0.68	0.92	1.00	1.00	0.97	1.00	0.79
<i>L. europaeus</i>	0.34	0.41	0.39	0.41	0.47	0.34	0.49	0.34	0.39	0.49	0.40	0.38	0.56	0.38	0.44
<i>L. granatensis</i>	0.33	0.43	0.50	0.41	0.76	0.34	0.46	0.33	0.79	0.64	0.55	0.40	0.45	0.57	0.48
<i>L. timidus</i>	0.33	0.50	0.31	0.37	0.57	0.35	0.42	0.33	0.39	0.42	0.43	0.37	0.46	0.44	0.37
<i>L. corsicanus</i>	0.40	0.75	1.00	1.00	1.00	0.34	0.91	0.39	0.33	0.53	1.00	0.72	0.66	0.83	0.49

Table S8. Statistical results of species pairwise comparisons of the nuclear genetic diversity parameters inferred for the studied populations using the 15 microsatellite markers.

Comparison	Na		Ar		Ho		He	
	W	p-value	W	p-value	W	p-value	W	p-value
<i>Lepus castroviejo</i> - <i>Lepus corsicanus</i>	90	0.349	78	0.155	91	0.379	79	0.167
<i>Lepus castroviejo</i> - <i>Lepus europaeus</i>	120.5	1.13E-05	91	2.02E-06	86	1.45E-06	74	6.56E-07
<i>Lepus corsicanus</i> - <i>Lepus europaeus</i>	244.5	0.006	242	0.006	176	2.89E-04	239	0.005
<i>Lepus castroviejo</i> - <i>Lepus granatensis</i>	81.5	1.08E-05	72	6.02E-06	75	7.51E-06	82	1.34E-05
<i>Lepus corsicanus</i> - <i>Lepus granatensis</i>	177	0.006	184.5	0.009	162.5	0.003	215	0.037
<i>Lepus europaeus</i> - <i>Lepus granatensis</i>	1342	0.961	1315	0.826	1189	0.300	116	0.131
<i>Lepus castroviejo</i> - <i>Lepus timidus</i>	39.5	7.48E-06	35	5.01E-06	43.5	1.29E-05	33	3.98E-06
<i>Lepus corsicanus</i> - <i>Lepus timidus</i>	94	0.002	96	0.002	72.5	2.48E-04	102	0.003
<i>Lepus europaeus</i> - <i>Lepus timidus</i>	686	0.065	731.5	0.150	674.5	0.054	685	0.066
<i>Lepus granatensis</i> - <i>Lepus timidus</i>	511.5	0.074	541.5	0.150	484	0.039	419	0.006

Na, number of Alleles; Ar, allelic Richness; Ho, observed Heterozygosity; He, expected Heterozygosity, W Wilcoxon test statistic

Table S9. Statistical results of species pairwise comparisons of the nuclear genetic diversity parameters inferred for the studied populations using the 15 microsatellite markers and all data available in the literature for the European hare species.

Comparision	Na		Ar		Ho		He	
	W	P-value	W	P-value	W	P-value	W	P-value
<i>L. castroviejo</i> - <i>L. corsicanus</i>	0	0.057	0	1.000	1	0.114	1	0.114
<i>L. castroviejo</i> - <i>L. europaeus</i>	0	0.005	0	0.104	0	0.005	0	0.005
<i>L. corsicanus</i> - <i>L. europaeus</i>	44	0.238	8	0.448	0	0.001	0.5	0.001
<i>L. castroviejo</i> - <i>L. granatensis</i>	0	0.024	0	0.333	1	0.048	0	0.028
<i>L. corsicanus</i> - <i>L. granatensis</i>	10	0.762	0	0.333	6	0.257	4	0.109
<i>L. europaeus</i> - <i>L. granatensis</i>	59	0.093	71.5	0.801	46	0.031	28	0.005
<i>L. castroviejo</i> - <i>L. timidus</i>	0	0.013	0	0.500	0	0.012	0	0.013
<i>L. corsicanus</i> - <i>L. timidus</i>	9	0.102	0	0.500	0	0.005	1.5	0.009
<i>L. europaeus</i> - <i>L. timidus</i>	155.5	0.347	18	0.089	112.5	0.041	177	0.699
<i>L. timidus</i> - <i>L. granatensis</i>	11	0.031	1	0.071	9	0.018	14	0.063

Na, number of Alleles; Ar, allelic Richness; Ho, observed Heterozygosity; He, expected Heterozygosity, W Wilcoxon test statistic

Table S10. Pairwise Nei's standard genetic distance matrix obtained for the studied populations using the 15 microsatellite markers.

Population	CAS1	EUR1	EUR2	EUR3	EUR4	GRA1	GRA2	GRA3	TIM1	TIM2	COR1
CAS1	0.0000										
EUR1	1.8424	0.0000									
EUR2	1.3540	0.3931	0.0000								
EUR3	1.5446	0.2761	0.2819	0.0000							
EUR4	1.7634	0.1088	0.3930	0.2954	0.0000						
GRA1	2.1919	0.9213	1.1988	1.1544	0.9205	0.0000					
GRA2	2.0030	1.0948	1.3173	1.2982	1.0760	0.0900	0.0000				
GRA3	1.7238	0.8858	1.1400	1.0356	0.8929	0.0503	0.1170	0.0000			
TIM1	1.6877	0.8028	1.0525	0.7361	0.8587	0.8606	0.9887	0.8359	0.0000		
TIM2	0.9749	0.8176	0.8718	0.7879	0.7814	0.8412	0.9817	0.8604	0.2889	0.0000	
COR1	0.1518	1.6077	1.1534	1.2942	1.3853	1.8018	1.8281	1.5437	1.3775	0.8094	0.0000

Table S11. Pairwise Fst matrix obtained for the studied populations using the 15 microsatellite markers.

Population	CAS1	EUR1	EUR2	EUR3	EUR4	GRA1	GRA2	GRA3	TIM1	TIM2	COR1
CAS1	0.0000										
EUR1	0.6332	0.0000									
EUR2	0.6332	0.1310	0.0000								
EUR3	0.6021	0.0809	0.0859	0.0000							
EUR4	0.5937	0.0228	0.1246	0.0706	0.0000						
GRA1	0.6735	0.2377	0.2812	0.2281	0.2219	0.0000					
GRA2	0.6410	0.2824	0.3125	0.2693	0.2616	0.0302	0.0000				
GRA3	0.6358	0.2448	0.2866	0.2328	0.2314	0.0031	0.0522	0.0000			
TIM1	0.6061	0.1894	0.2242	0.1459	0.1801	0.2051	0.2450	0.2158	0.0000		
TIM2	0.4640	0.1878	0.1999	0.1557	0.1713	0.1991	0.2352	0.2131	0.0748	0.0000	
COR1	0.3108	0.4181	0.4030	0.3641	0.3748	0.4538	0.4547	0.4346	0.3728	0.2824	0.0000

Table S12. Evanno table for the STRUCTURE analysis considering only broom hare individuals and the 11 polymorphic loci.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	Delta K
1	9	-885.0556	0.2351	NA	NA	NA
2	9	-910.8222	36.719	-25.7667	132.0222	3.595472
3	9	-1068.6111	34.2565	-157.789	67.02222	1.956479
4	9	-1159.3778	79.9327	-90.7667	191.2444	2.39257
5	9	-1058.9	121.1439	100.4778	NA	NA

Table S13. Statistical results of species pairwise comparisons of the mitochondrial genetic diversity parameters inferred for the studied populations using the 541bp fragment of cytochrome-b gene and all data available in the literature for the European hare species.

Comparision	Nucleotide Diversity		Haplotype diversity	
	W	p-value	W	p-value
<i>L. castroviejo</i> - <i>L. corsicanus</i>	2	1.000	1	0.717
<i>L. castroviejo</i> - <i>L. europaeus</i>	2	0.661	0	0.250
<i>L. corsicanus</i> - <i>L. europaeus</i>	7.5	0.255	4	0.072
<i>L. castroviejo</i> - <i>L. granatensis</i>	4	0.400	0	0.400
<i>L. corsicanus</i> - <i>L. granatensis</i>	0	0.029	3	0.191
<i>L. europaeus</i> - <i>L. granatensis</i>	3	0.047	9	0.412
<i>L. castroviejo</i> - <i>L. timidus</i>	0	0.167	0	0.286
<i>L. corsicanus</i> - <i>L. timidus</i>	1.5	0.009	0	0.014
<i>L. europaeus</i> - <i>L. timidus</i>	13.5	0.026	7	0.051
<i>L. timidus</i> - <i>L. granatensis</i>	11.5	0.191	11	0.914

W Wilcoxon test statistic.

Table S14. Statistical results of species pairwise comparisons of the nuclear genetic diversity parameters inferred for the studied populations using the 11 polymorphic microsatellite markers in the broom hare.

Comparision	Na		Ar		Ho		He	
	W	P-value	W	P-value	W	P-value	W	P-value
<i>L. castroviejo</i> - <i>L. corsicanus</i>	48.5	0.437	41	0.212	49	0.470	40	0.189
<i>L. castroviejo</i> - <i>L. europaeus</i>	88.5	0.001	66	2.21E-04	62	1.58E-04	54	7.96E-05
<i>L. corsicanus</i> - <i>L. europaeus</i>	172	0.141	175	0.162	126.5	0.015	169	0.127
<i>L. castroviejo</i> - <i>L. granatensis</i>	55	0.001	50	3.84E-04	51	4.20E-04	55	0.001
<i>L. corsicanus</i> - <i>L. granatensis</i>	119.5	0.091	125.5	0.132	101	0.030	138	0.244
<i>L. europaeus</i> - <i>L. granatensis</i>	689	0.704	699	0.785	721	0.963	704	0.825
<i>L. castroviejo</i> - <i>L. timidus</i>	22.5	1.63E-04	20	1.24E-04	20.5	1.32E-04	22	1.68E-04
<i>L. corsicanus</i> - <i>L. timidus</i>	58	0.016	60	0.021	36	0.001	65	0.034
<i>L. europaeus</i> - <i>L. timidus</i>	305	0.014	325.5	0.032	296.5	0.011	299.5	0.012
<i>L. timidus</i> - <i>L. granatensis</i>	249	0.048	263	0.087	254.5	0.063	214	0.011

Na, number of Alleles; Ar, allelic Richness; Ho, observed Heterozygosity; He, expected Heterozygosity, W Wilcoxon test statistic

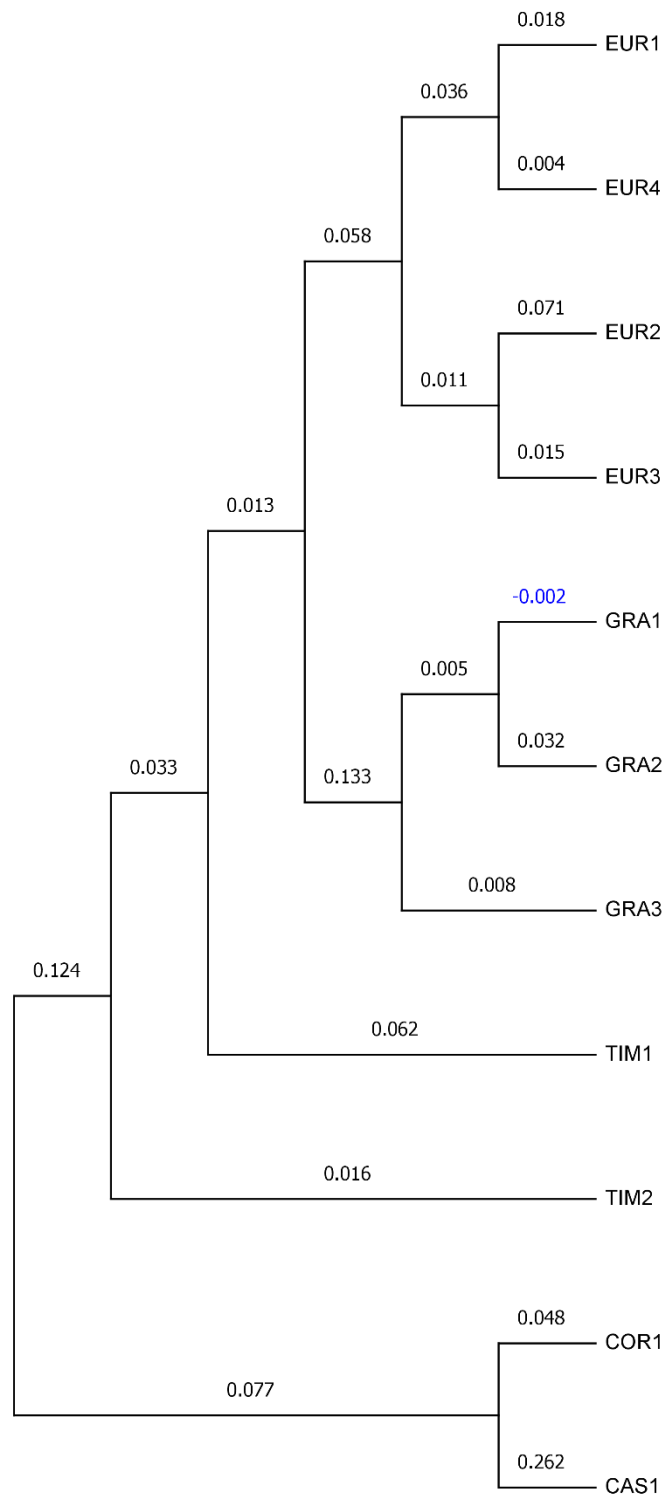


Figure S1. Neighbor-joining tree inferred from the pairwise F_{st} distances among populations of each species: European hare - *L. europaeus*, EUR1, EUR2, EUR3, EUR4; Iberian hare - *L. granatensis*, GRA1, GRA2, GRA3; mountain hare - *L. timidus*, TIM1, TIM2; Italian hare - *L. corsicanus*, COR1; and broom hare - *L. castroviejo*, CAS1. Branch labels represent branch length according to the pairwise F_{st} distances

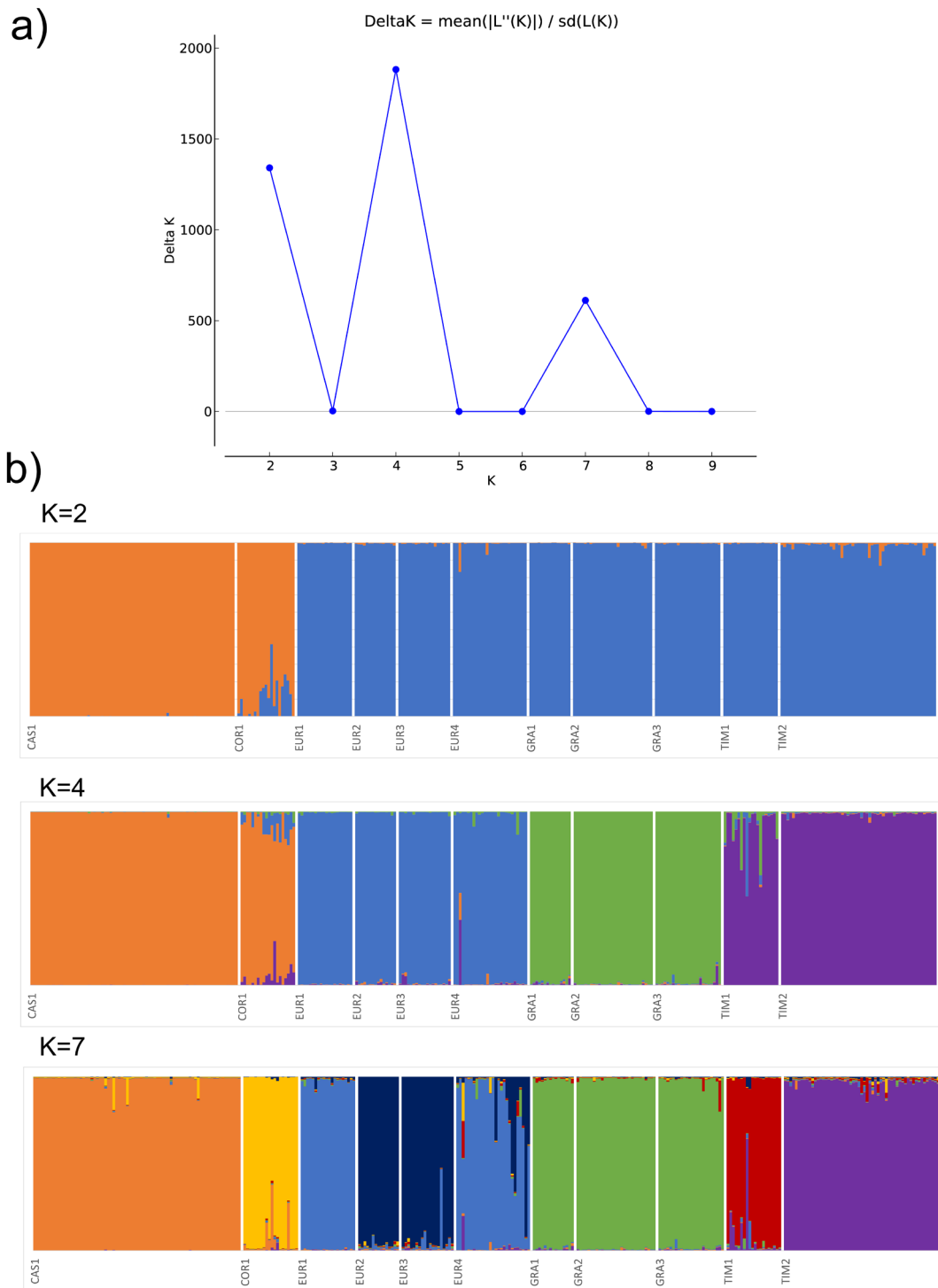


Figure S2. Bayesian clustering results obtained in STRUCTURE using all hare populations analyzed (European hare: EUR1, EUR2, EUR3 and EUR4; Iberian hare: GRA1, GRA2 and GRA3; mountain hare: TIM1 and TIM2; Italian hare: COR1 and broom hare: CAS1) and the 15 microsatellite markers: a) DeltaK vales for each value of K calculated trough the method of Evanno; b) Structure results for K equal 2, 4 and 7. Bar color represents the membership proportion (q_i) obtained for each cluster.

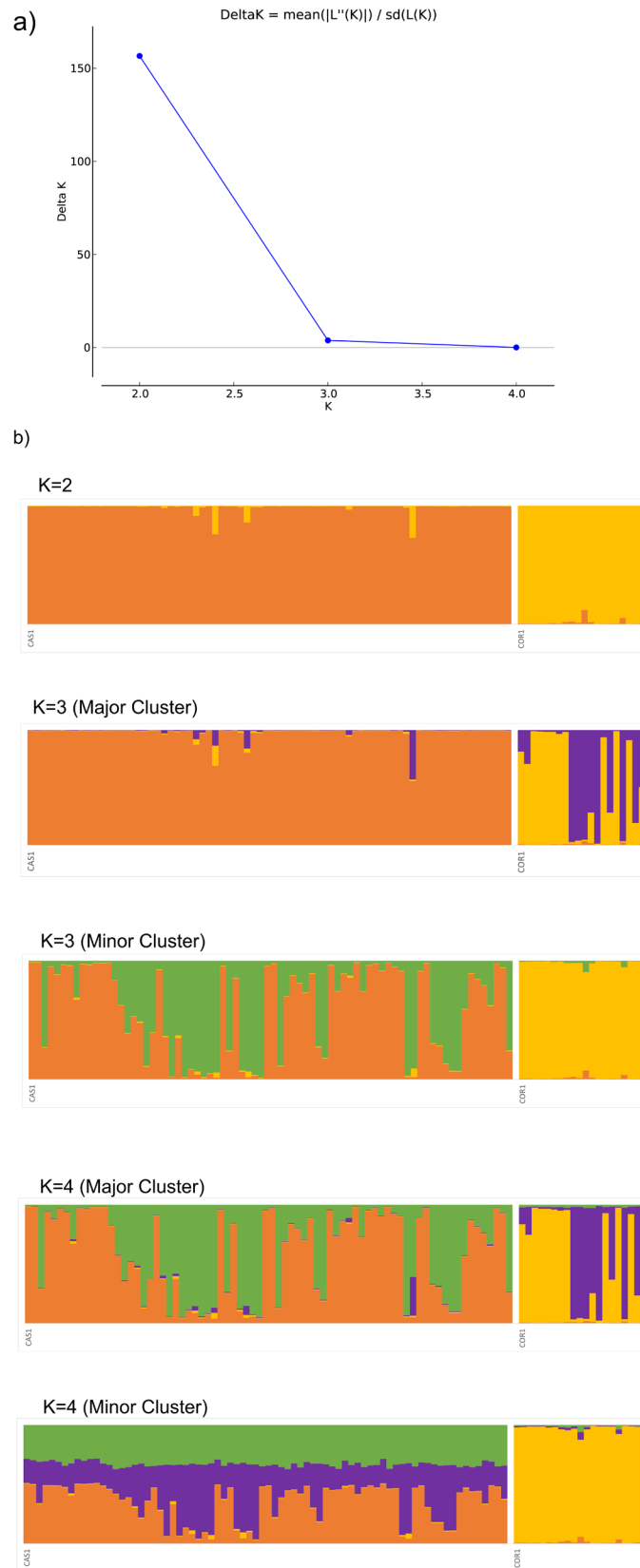


Figure S3. Bayesian clustering results obtained in STRUCTURE using the Italian (COR1) and broom hare (CAS1) populations and the 15 microsatellite markers: a) DeltaK vales for each value of K calculated trough the method of Evanno; b) Structure results for k equal 2, 3 and 4. Bar color represents the membership proportion (q_i) obtained for each cluster. Minor clusters represent the clusters that were not supported by the majority of replicates.

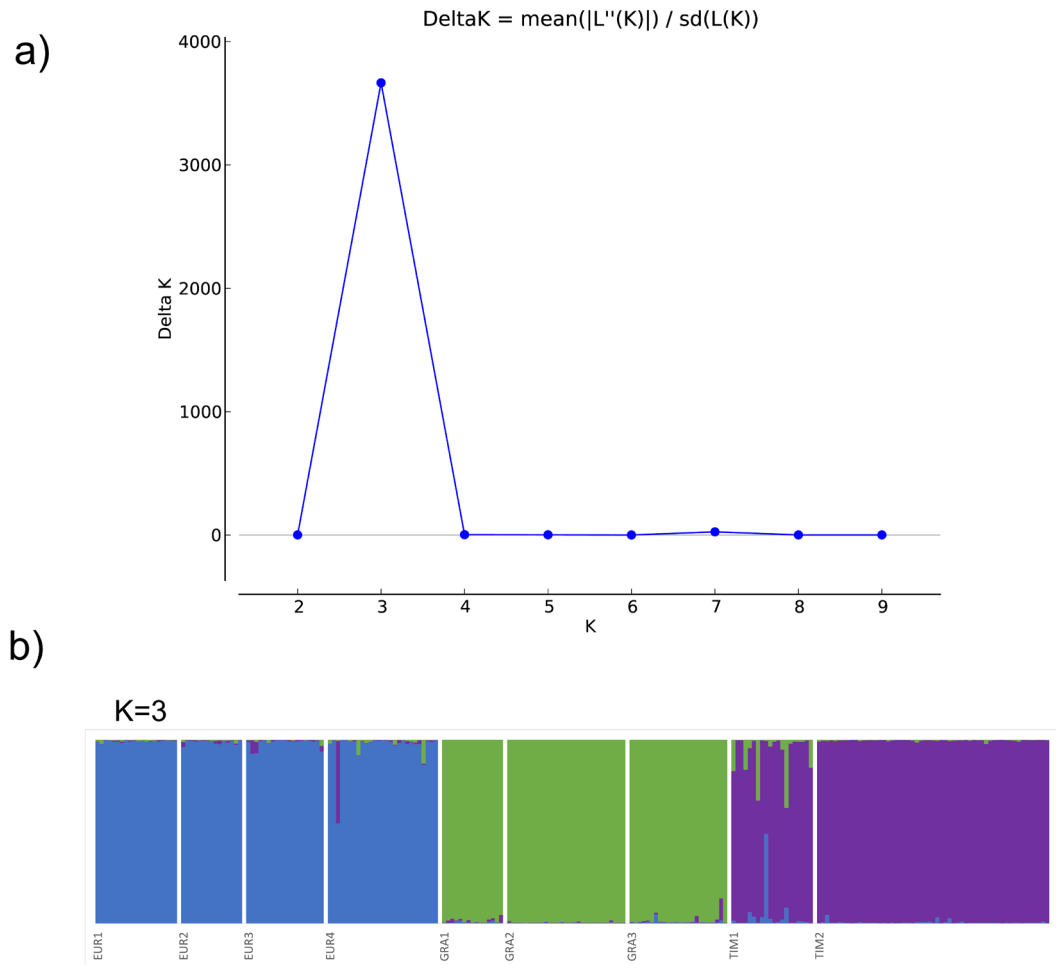


Figure S4. Bayesian clustering results obtained in STRUCTURE using the European hare (EUR1, EUR2, EUR3 and EUR4), the Iberian hare (GRA1, GRA2 and GRA3) and the mountain hare (TIM1 and TIM2) and the 15 microsatellite markers: a) DeltaK vales for each value of K calculated trough the method of Evanno; b) Structure results for the best Deltak value (K=3). Bar color represents the membership proportion (q_i) obtained for each cluster.

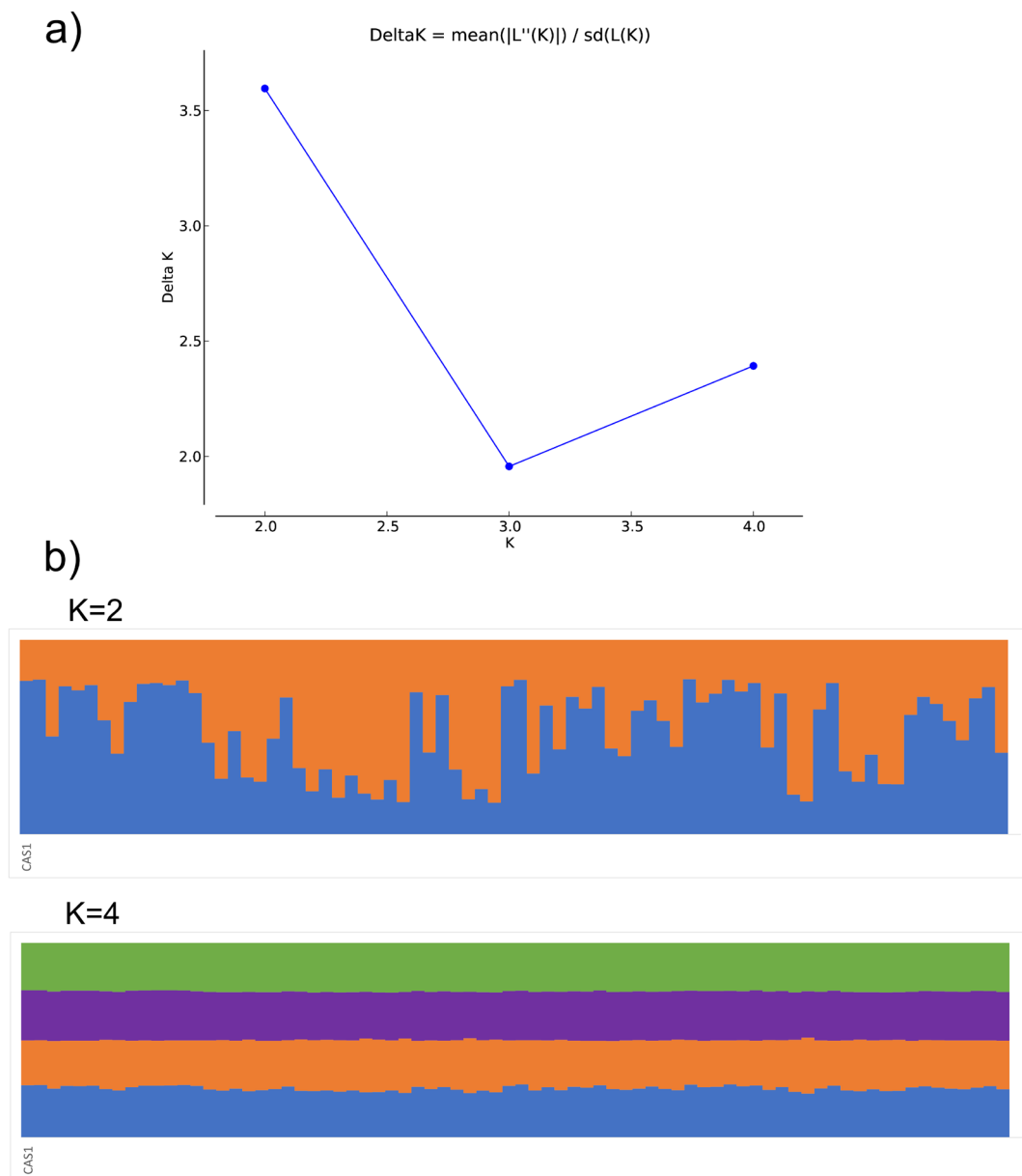


Figure S5. Bayesian clustering results obtained in STRUCTURE using the Broom hare population (CAS1) and the 11 microsatellites polymorphic for this species: a) DeltaK values for each value of K calculated through the method of Evanno; b) Structure results for k equal 2 and 4. Bar color represents the membership proportion (q_i) obtained for each cluster.

