



# The evolutionary pathways for local adaptation in mountain hares

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## Abstract

Understanding the evolution of local adaptations is a central aim of evolutionary biology and key for the identification of unique populations and lineages of conservation relevance. By combining RAD sequencing and whole-genome sequencing, we identify genetic signatures of local adaptation in mountain hares (*Lepus timidus*) from isolated and distinctive habitats of its wide distribution: Ireland, the Alps and Fennoscandia. Demographic modelling suggested that the split of these mountain hares occurred around 20 thousand years ago, providing the opportunity to study adaptive evolution over a short timescale. Using genome-wide scans, we identified signatures of extreme differentiation among hares from distinct geographic areas that overlap with area-specific selective sweeps, suggesting targets for local adaptation. Several identified candidate genes are associated with traits related to the uniqueness of the different environments inhabited by the three groups of mountain hares, including coat colour, ability to live at high altitudes and variation in body size. In Irish mountain hares, a variant of *ASIP*, a gene previously implicated in introgression-driven winter coat colour variation in mountain and snowshoe hares (*L. americanus*), may underlie brown winter coats, reinforcing the repeated nature of evolution at *ASIP* moulding adaptive seasonal colouration. Comparative genomic analyses across several hare species suggested that mountain hares' adaptive variants appear predominantly species-specific. However, using coalescent simulations, we also show instances where the candidate adaptive variants have been introduced via introgressive hybridization. Our study shows that standing adaptive variation, including that introgressed from other species, was a crucial component of the post-glacial dynamics of species.

## KEYWORDS

adaptive evolution, genome scans, introgression, *Lepus timidus*

## 1 | INTRODUCTION

During their evolution, species face environmental changes that challenge their distributions, potentially leading to local extinctions, range shifts or new adaptations (Nogués-Bravo et al., 2018). These changes in local environmental conditions and the colonization or recolonization of habitats with distinct selective pressures are potential triggers of divergent local adaptation. Identifying instances of adaptive evolution is a central aim in evolutionary biology, and also provides important insights to delineate conservation strategies aiming at preserving adaptive uniqueness and evolutionary potential (Funk et al., 2012; Mills et al., 2018; Teixeira & Huber, 2021). For example, the existence of locally adapted populations across the range of widely distributed species may justify delineating guided conservation actions to preserve adaptive genetic variation. The advent of high throughput sequencing provides exceptional tools to detect signatures of adaptation along the genome, which can be linked to the morphological, physiological, behavioural, and ecological uniqueness of populations, and ultimately to fitness (Hendricks et al., 2019; Savolainen et al., 2013). However, establishing a relationship between genetic variation and its adaptive underpinnings is challenging because it implies differential fitness in alternative habitats, which is not trivial to test. Scanning the genome for extraordinary differentiation between recently diverged populations, that is incompatible with neutral demographic expectations, can be used as an important first step towards identifying candidate genomic regions, genes and evolutionary pathways that underlie trait differences between locally adapted populations (Hoban et al., 2016).

The mountain hare (*Lepus timidus*) is an arctic-alpine species currently widely distributed in the northern Palearctic, from Fennoscandia to East Russia, with some isolated populations in Ireland, the Alps, Scotland, Faroe Islands and Poland, for example (Angerbjörn, 2018). Pleistocene fossil records show that the species was distributed further south at the Last Glacial Maximum (LGM), as in the Iberian Peninsula or Southern France (Altuna, 1970; Lopez-Martinez, 1980). Phylogeographic analyses of mitochondrial DNA and microsatellite variation (Hamill et al., 2006; Melo-Ferreira et al., 2007) as well as ancient DNA (Smith et al., 2017) have shown that the fragmented populations of the mountain hare share substantial variation, suggesting that they have diverged recently, potentially from a large range south of the ice rim. Thus, the current distribution of mountain hares is likely to have resulted from population fragmentation and colonization of deglaciated areas. The evolutionary history of the species, however, is far from established. Contemporary isolated mountain hare populations in Europe inhabit diverse biogeographical regions and habitats, such as the insular Atlantic habitats of Ireland, the alpine environment of the Alps, or the boreal forests and tundra of Scandinavia. These three regions are categorised in distinct climate zones, according to the Köppen-Geiger classification (Peel et al., 2007). Ireland is in a temperate oceanic climate zone (Cfb), with mild winter temperatures usually above 0°C. Winter snow cover in Ireland is ephemeral, with an annual mean

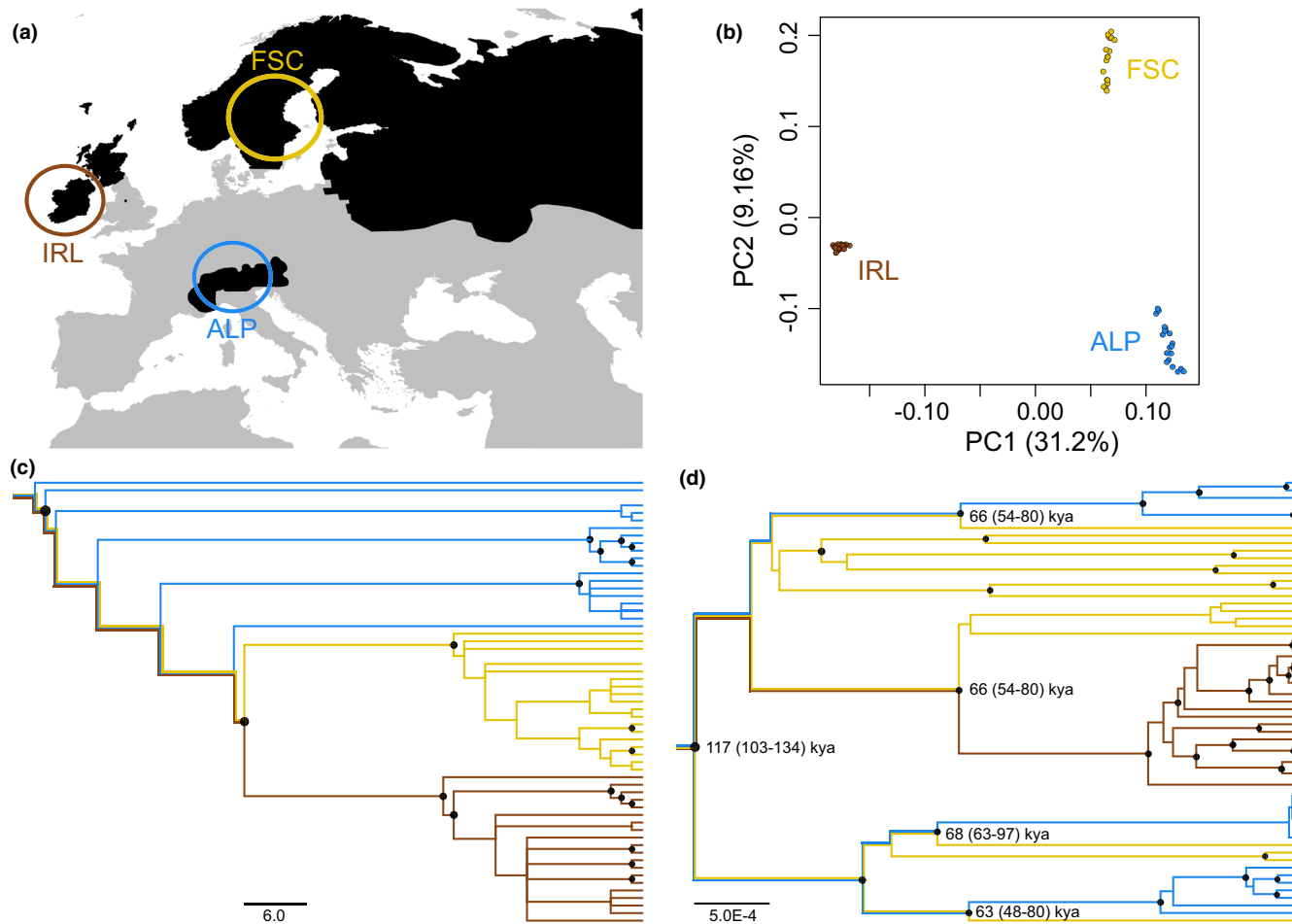
of 3.7 days of snow or sleet (recorded between 1981 and 2010), most regions being snow-free year round (The Irish Meteorological Service, <https://www.met.ie>). The Alps are in the alpine (ET) and boreal (Dfc) climate zones, depending on elevation, with temperatures permanently below 0°C at high elevations, and long snow cover duration (annual average consistently over 150 days above 1500 m of elevation in the period between 1971 and 2019) (Matiu et al., 2021). The Alps are characterized by a complex physiography and high elevation, where mountain hares are present above 1300 m above sea level and up to ~4000 m (Angerbjörn, 2018; Angerbjörn & Schai-Braun, in press). Scandinavia is in the oceanic climate zone in the south (Cfb) and boreal climate zones in the north (Dfc and Dfb), with annual mean snow cover duration over 125 days even in low altitude regions (Dietz et al., 2012). Mountain hares from these isolated areas are characterised by distinct traits that may have arisen from local adaptation during or after the colonization of these regions. These include the distinctive brown winter coat colour of the Irish mountain hare (instead of the typical summer-brown to winter-white pelage colour change), the smaller body size of the Alpine mountain hare and its ability to inhabit high altitudes, or the larger body size of Scandinavian mountain hare. These differences have supported their taxonomic classification into distinct subspecies, *L. t. hibernicus* in Ireland, *L. t. varronis* in the Alps, or *L. t. timidus* and *L. t. sylvaticus* in Fennoscandia (Angerbjörn & Flux, 1995). Furthermore, the range shifts of mountain hares since the LGM have led to transient or persistent contacts with other species of hares. These contacts have resulted in hybridization with non-negligible levels of introgression between mountain hares and other hare species (Giska et al., 2019a; Melo-Ferreira et al., 2009; Seixas et al., 2018). Often, these introgression events resulted from ancient species interactions, such as between the currently allopatric mountain and the Iberian hare (*L. granatensis*), which may have been driven by natural selection in many instances (Giska et al., 2019a; Melo-Ferreira et al., 2012; Seixas et al., 2018). The mountain hare is therefore a privileged model to assess genomic signatures of selection resulting in local adaptation and to understand the evolutionary origin of adaptive variants.

Here, we analyse restriction site-associated DNA and whole-genome sequencing data from three isolated populations of mountain hares. We perform genome scans of differentiation and selection to identify candidates for local adaptation and genetic uniqueness. Using genomic data across several species of hares, we dissect the evolutionary trajectories underlying the candidate adaptive variation. We then discuss the relevance of our findings in the context of conservation strategies for the species.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling and data collected

Mountain hares from Ireland, the Alps, and Fennoscandia are included in this study (Figure 1). These three regions represent disjoint ranges of species' distribution, have very distinct



**FIGURE 1** Mountain hare sampling and genetic relationships. (a) Distribution of the mountain hare in western Europe (black), according to Mitchell-Jones et al. (1999), and sampled regions: Ireland, IRL; Alps, ALP; Fennoscandia, FSC. (b) Principal component analysis (PCA) based on whole-genome data, inferred using 125,196 polymorphic sites, sampled at least 20 kb apart along the genome. (c) Neighbour-joining tree based on individual pairwise genetic distances estimated from 128,019 SNPs derived from whole-genome datasets. Nodes with bootstrap support values  $\geq 0.90$  are shown as black circles. (d) mtDNA phylogeny. Ages of key nodes are displayed (mean and 95% HDI) and nodes with posterior probability  $\geq 0.90$  are shown as black circles

environmental and climatic envelopes and harbour mountain hares with distinctive morphological features that could have resulted from local adaptation. Irish mountain hares were sampled in five counties (Mayo, Cavan, Limerick, Laois, Waterford), Alpine hares originated from France, Italy, Switzerland as well as Austria, and Fennoscandian hare samples derived from Sweden, Norway and Finland (see sampling details in Table S1). Hereafter we will use “population” to designate each sampled geographic region: Ireland, Alps and Fennoscandia.

Genetic data from these populations and for other *Lepus* species was collated: genotype data sets from RAD-sequencing and whole-genome low individual coverage data sets from mountain hare populations (the latter was further used to reconstruct mitochondrial genome sequences), and individual medium to high coverage whole genome sequencing data from representatives of each mountain hare populations and five other *Lepus* species (details are given below; see Tables S1 and S2).

## 2.2 | RAD sequencing

Double-digest restriction site-associated DNA sequencing (RAD-seq) libraries were prepared for a total of 62 mountain hares, from Ireland ( $N = 20$ ), Fennoscandia ( $N = 26$ ), and the Alps ( $N = 16$ ) (Figure 1a, Table S1), following a protocol adapted from Peterson et al. (2012). Genomic DNA was extracted using the EasySpin Genomic DNA tissue kit (Citomed) and quantified with Qubit 2.0 fluorometer (Invitrogen). For each individual, up to 400 ng of genomic DNA was digested with the SbfI-HF and MspI restriction enzymes (New England BioLabs) for 3 h at 37°C, purified, quantified and ligated to adapters with compatible overhangs (P1-SbfI with a 5–7 bp inline barcode, and P2-MspI). Individual DNA samples were then combined in pools based on (i) DNA intactness, assessed with gel electrophoresis before digestion and (ii) DNA concentration measured with Qubit after digestion but before ligation (the same amount of total DNA per sample was

pooled). After electrophoresis on a 2.5% agarose gel, fragments between 450 and 700 bp were excised and purified with the minElute gel extraction kit (Qiagen). Indexed Illumina sequencing adapters were added by PCR enrichment of ligated fragments with phusion high-fidelity DNA polymerase using 10–12 cycles (Thermo Fisher Scientific). Amplified libraries were analysed on a 2200 TapeStation (Agilent Technologies) to confirm the size range of the recovered fragments. After quantification by qPCR, libraries were pooled in equimolar ratios and sequenced as part of two lanes of an Illumina HiSeq 2500 platform (paired-end, 150 bp reads) at Novogene (Novogene Corporation Ltd.).

The raw RAD-seq reads were demultiplexed based on the unique barcode-index combination and filtered using the program `process_radtags` included in the Stacks 2.52 pipeline (Catchen et al., 2013) with the following filters: `-c -q -r --retain_header --adapter_mm 1 -w 0.1 -s 20 -t 143 --len_limit 143`. Reads were mapped onto a hare pseudoreference, originally generated through iterative mapping of reads from three hare species onto the rabbit genome (Seixas et al., 2018, 2019), which allowed retrieving the ancestral state and polarizing variants for downstream analyses. We used BWA-MEM with default parameters (Li & Durbin, 2009) to map the reads, which were locally realigned around indels using the `RealignerTargetCreator` and `IndelRealigner` functions from GATK v.3.8.1 (McKenna et al., 2010). Then, we ran the program `ref_map.pl` from Stacks for loci assembly with the following filters: `"gstacks: --min-mapq 20 --max-clipped 0.2"` and `"populations: -p 3 -r 0.5"`. To avoid the inclusion of paralog loci and poorly mapped regions we excluded RADtags with excessive polymorphism detected by `ref_map.pl` (more than 10 SNPs). The filtered data set was used for final SNP calling, which was performed in the next steps based on genotype likelihoods. The set of used samples is indicated in Table S3.

### 2.3 | Whole genome sequencing

Genomic DNA of 20 Irish mountain hares (details in Table S1) was used to prepare double-indexed individually barcoded libraries, following Meyer and Kircher (2010) with slight modifications (see Giska et al., 2019a). These libraries were sequenced in ~33% of four lanes of an Illumina HiSeq 1500 at CIBIO-InBIO's New-Gen sequencing platform, to obtain 125 bp paired-end reads. The whole genome sequencing (WGS) data was complemented using similar data from Alpine (*L. t. varronis*,  $N = 20$ ) and Fennoscandian (*L. t. timidus*,  $N = 19$ ) specimens analysed previously by Giska et al. (2019a, 2019b). In addition, a data set of whole genome sequence data at higher individual coverage was put together, including four mountain hare specimens, from Ireland, the Alps, Fennoscandia and the Faroe Islands (6.9–32.8x; from Giska et al., 2019a, 2019b; Marques, Seixas, et al., 2019; Marques et al., 2020a; Seixas, Boursot, et al., 2019; Seixas et al., 2017, 2018), and five other *Lepus* species: *L. americanus*, *L. californicus*, *L. europaeus*, *L. granatensis*, and *L. castroviejo* (6.1–21.8x; from Giska et al., 2019a; Jones et al., 2017, 2018; Marques et al., 2019; Seixas, 2017; Seixas Boursot et al., 2017, 2018, 2019; Seixas et al., 2019, 2019) (details in Table S2).

The quality of raw Illumina reads was examined using FastQC (available at <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and the reads were trimmed for adapters and quality using Trimmomatic, TRAILING:15, SLIDINGWINDOW:4:15, MINLEN:30 (Bolger et al., 2014). Reads were mapped onto the hare pseudo-reference genome, and analyses were repeated using also the Irish mountain hare de novo draft reference genome (Marques et al., 2020a, 2020b). PCR duplicates were removed with PICARD (<http://broadinstitute.github.io/picard/>) and local realignment around indels was done with GATK IndelRealigner.

Complete mitochondrial DNA (mtDNA) genomes were recovered for each mountain hare individual for which the whole genome was sequenced at lower coverage, using the ABySS assembler (Jackman et al., 2017; Simpson et al., 2009). The standard ABySS run was done with  $k$ -mer size of 64 bp ( $k = 64$ ), but modified to  $k = 96$  and/or randomly subsampling input reads, when it allowed increasing the contig length. For each individual, the nearly full mtDNA sequence was retrieved by inspecting the longest contigs and then aligning with a mountain hare mtDNA reference (GenBank Acc. Nr KJ397605; Melo-Ferreira et al., 2014). Final alignments were produced using ClustalW (Thompson et al., 1994), followed by manual corrections in BioEdit (Hall, 1999).

### 2.4 | Evolutionary relationships, population structure and demography

A principal component analysis (PCA) based on the mountain hare RAD-seq and WGS data sets was carried out using PLINK 1.9 (Chang et al., 2015) and the single-read sampling approach implemented in ANGSD (Korneliussen et al., 2014), respectively. In all ANGSD analyses we used the following set of quality filters: `-minMapQ 20 -minQ 20, -trim 0, -SNP_pval 1e-6`, and genotype likelihoods were estimated using SAMtools model (-GL 1), adjusting additional options to the needs of a particular analysis and data set. In the RAD-seq data set, we performed SNP calling for each population using ANGSD with the following additional options: `-doMajorMinor 5, -doMaf 1, -doCounts 1, -setMinDepthInd 4, -doGeno 4, -doPost 1`, and assuming the reference variant as ancestral. We removed sites with over 20% of missing data per population, sequencing depth higher than three times the mean depth times the number of samples in each region, and the minor allele frequency was set to remove singletons. Then, using PLINK 1.9, we removed SNPs with a missing rate higher than 10%, and excluded SNPs failing the Hardy-Weinberg test ( $p = .05$ ). In the WGS data set, SNP calling was performed using sites represented by at least six individuals in each population and maximum coverage three times the average coverage (-setMaxDepth). The following additional filters were applied: `-uniqueOnly 1, -remove_bads 1, -baq 2`. To minimize linkage, SNPs in both data sets were sampled at least 20 kb apart. Thus, PCA was based on 1,196 and 125,196 SNPs, for RAD-seq and WGS data, respectively. To further examine the genetic relationships of mountain hares from the different areas, we used individual pairwise distances

to construct a neighbour-joining (NJ) tree based on the WGS data. ANGSD genotype posterior probabilities at 128,019 SNPs filtered for a minimum distance of 20 kb were used to calculate pairwise genetic distances with ngsDist (Vieira et al., 2016). Branch support values were generated through bootstrapping (100 replicates, 100 SNPs blocks, with replacement), and the trees were inferred using FastME (Lefort et al., 2015) and plotted with FigTree v1.4.4 (available at <http://tree.bio.ed.ac.uk/software/figtree/>). We then used allele counts to construct a TreeMix tree using both the WGS and RAD-seq data and the European rabbit variant as the ancestral allele (Carneiro et al., 2014). TreeMix (Pickrell & Pritchard, 2012) analyses (1000 bootstrap replicates of 100 and 20 SNPs blocks for WGS and RAD-seq data, respectively) were performed using allele frequency estimates from ANGSD converted into allele counts at SNPs at least 20 kb apart (1268 and 136,874 SNPs, for RAD-seq and WGS data, respectively). A high coverage genome of the snowshoe hare was used as outgroup for both the NJ and TreeMix trees. In addition, using ANGSD and the RAD-seq data, we computed the *D* statistics (Durand et al., 2011; Green et al., 2010), for all possible topological conformations of the three mountain hare populations and using the snowshoe hare as outgroup (Soraggi et al., 2018). A 20 SNP jackknife block size was used, to test whether the minimum *D* values across the possible population trees deviated from incomplete lineage sorting expectations (Malinsky et al., 2018).

A mitochondrial DNA phylogeny was inferred using the MultiTypeTree method (Vaughan et al., 2014) implemented in the BEAST v2.6.1 package (Bouckaert et al., 2019). This method is based on a structured coalescent model in which the phylogenetic inference incorporates known structure across samples (here set to Ireland, Alps and Fennoscandia). Three independent replicate runs of 100 million MCMC generations were carried out, using the HKY + G + I site model (determined using jmodeltest2; Durraba et al., 2012) and a Log Normal relaxed clock (Drummond et al., 2006). The runs were assessed for convergence with Tracer v1.7.1 (Rambaut et al., 2018), and the first 10% of each run was discarded as burnin. The resulting trees were merged using LogCombiner and summarized using TreeAnnotator, which is part of the BEAST package, and plotted with FigTree v1.4.4. Node ages were converted to years using a mutation rate estimated from the *L. timidus* - *L. granatensis* average corrected mtDNA distance, considering a split time of 1.86 million years (Giska et al., 2019a).

Finally, the RAD-seq data was used to model the demographic history of European mountain hares, first considering each sampled area - Ireland, Alps, Fennoscandia - as a separate population and modelled independently, and second using the single-population estimates to guide multipopulation models. Using ANGSD, we estimated the site allele frequency likelihoods based on individual genotype likelihoods from bam alignments: `-GL 1 -doSaf 1`. We filtered sites following the same criteria as those applied for PCA. The site frequency spectrum (SFS) for single populations and the 2D SFS were inferred applying the expectation-maximization (EM) algorithm optimization with realSFS program of ANGSD. 2D SFSs were estimated using sites commonly covered across the three populations.

SFS for single populations and 2D SFSs were inferred using all sites available after filtering. The coalescent-based method implemented in FASTSIMCOAL v.2.6.0.3 (Excoffier et al., 2013) was used to infer the demographic parameters. The separate demographic models for each population were first used to get accurate estimations of current effective population sizes. Then, the demographic history of the divergence of the three considered mountain hare populations was inferred based on two different models (following the neighbour-joining and TreeMix inferences - see Results): (1) considering a simultaneous split (same time model - STM), and (2) considering two splits forward in time, first of the Alpine mountain hares and then between Fennoscandian and Irish mountain hares (different time model - DTM) (Table S4). We were particularly interested in understanding the timings of the establishment of the current allopatric ranges of these populations, and therefore considered models without post-split gene flow, which assume that shared variation results from incomplete lineage sorting during the differentiation process (supported by the *D* statistics - see Results). For the STM and DTM models, the current effective populations sizes were fixed, based on the estimations from the single population models, to reduce the large parameter space of the multi-population models. The three individual population scenarios and the two models of population divergence were run for 250,000 simulations and for 100 replicates with random parameter values as starting point. To avoid overfitting of the model, we used a threshold of 10 (-C) for observed SFS entry count, pooling all entries with less than 10 SNPs. We optimized the fit between estimated and observed likelihoods by performing a total of 100 expectation-conditional maximization (ECM) cycles. For the first 50 cycles, the likelihood was computed with monomorphic and polymorphic sites, while for the remaining 50 cycles, we used only polymorphic sites. Parameters were estimated using a maximum composite likelihood from the unfolded SFS, and the run with the best likelihood was retained. The method calculates a maximum observed likelihood by replacing the estimated SFS by the empirical SFS, thus obtaining the maximum possible likelihood. Small differences between the estimated and maximum observed likelihood indicate good fit. For the three-population models, density intervals for parameters values were estimated by parametric bootstrapping using fastsimcoal. The parameters generated from the run with the best likelihood were used to generate 100 pseudodata sets using the following options: `"-n 100 -j -d -s0 -x -l -q"`. For each of these pseudodata sets, fastsimcoal was run under the same conditions as the empirical data. In each case, the parameters values were retrieved, and 95% high-density intervals (HDI) were estimated with R using the function HPDinterval included in the package coda (Plummer et al., 2006).

## 2.5 | Genome-wide scans for adaptive differentiation and selective sweeps

Genomic regions underlying local adaptation in a given population are expected to show extraordinary levels of differentiation

relative to the other two populations. Using the low individual coverage whole-genome sequencing data, we performed genome scans of the population branch statistics (PBS), which has been shown to have power to detect recent positive selection (Yi et al., 2010). PBS scans were run with PBSscan (Hamala & Savolainen, 2019) on genotype likelihoods estimated with ANGSD, using only sites represented by at least six individuals per area. The sites were filtered for minimum coverage of 10 (-setMinDepth), maximum coverage three times the average coverage (-setMaxDepth), and the quality filters mentioned earlier. Additionally, we applied a minimum of 4 read counts per base and minor allele frequency  $\text{maf} = 0.05$ . The final data set consisted of 8,978,539 SNPs, including 8,770,557 autosomal SNPs, and the PBSscan was run in nonoverlapping windows of 50 SNPs. We used Hudson's  $F_{ST}$  for differentiation estimates (-div 0). Outlier detection was based on  $p$ -values derived from neutral data simulated with msms (Ewing & Hermisson, 2010) using demographic parameters inferred according to the STM and DTM models. We simulated 10,000 fragments of 10 kb as the median length of 50 SNPs window in PBS scan was 10,104 bp. We ran PBSscan separately with data simulated based on mean demographic parameters of each model, supplied with -ms option. For each underlying demographic model, outliers were defined as genomic regions with at least two consecutive windows with  $p \leq .01$ . The union of the identified outliers for the two models was retained as candidates for local adaptation. Because the three sampled areas are isolated and thus populations are strongly structured, which could lead to spurious outliers of differentiation, we also used BAYPASS v.2.2 (Gautier, 2015) to reassess PBS outliers taking this structure into account. We ran the core model with a contrast statistics (C2; Olazuaga et al., 2020) based on allele frequencies corrected for structure, using the scaled covariance matrix of population allele frequencies ( $\Omega$ ). The model was run for three possible settings of two groups, the first including the area of interest, and the second containing the two remaining areas. The input file was generated with POOLSTAT R package (Hivert et al., 2018) from a sync file, and included the same set of autosomal sites as used for PBS. The analysis was run per SNP with default parameters, except -npilot 30 and -dOyij 8. Then, we calculated mean C2 statistics in nonoverlapping windows of 50 SNPs, in keeping with window sizes used in the PBS scans. We considered regions with at least two consecutive windows within 0.1% top C2 values as outliers. This procedure was used for three independent runs with different seed numbers, but given highly concordant results across runs we kept the results of a single run.

To validate if the low coverage individual data allowed properly recovering allele frequency differences, we selected 28 SNPs for genotyping in four genomic regions showing increased differentiation in Irish mountain hares (chr03 - NR3C1, chr04 - HMGA2, chr13 - DNM3, chr18 - EXOC6). Target SNPs were selected in regions of high differentiation determined using Fisher exact tests implemented in Popoolation2 (Kofler et al., 2011). Fisher's exact tests were based on sync input files and run with minimum count of four, minimum coverage of ten, maximum coverage 3x the average coverage,

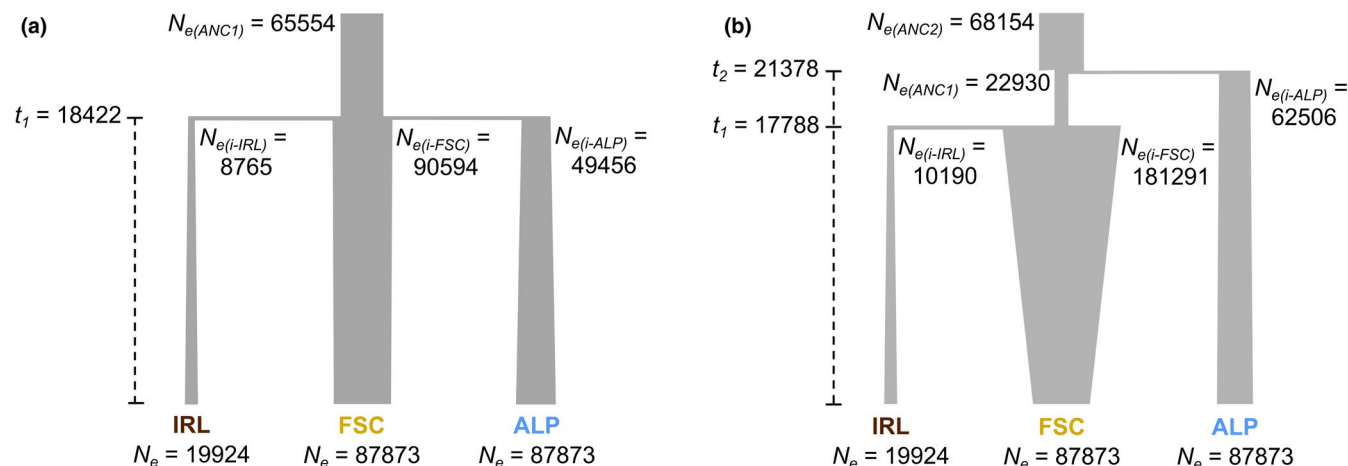
nonoverlapping windows of 20 kb covered in at least 25%, and the resulting  $p$ -values were corrected using the Bonferroni criterion. Six to eight SNPs were genotyped per genomic region in 103 individuals, using the AgenaBio iPLEX technology at Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal.

We next scanned the genomes of mountain hares for within-population signatures of selective sweeps using three statistics: nucleotide diversity ( $\pi$ ), Tajima's D and the composite likelihood ratio (CLR) of SweeD (Pavlidis et al., 2013). The decorrelated composite of multiple signals (DCMS) approach (Ma et al., 2015) was used to summarize the statistics. In particular, we tested whether high differentiation coincided with population-specific signatures of selective sweeps, thus allowing differentiating local adaptation from global adaptation (Booker et al., 2021). Tajima's D was calculated in 100 kb windows with 20 kb steps using PoPoolation1 (Kofler, Orozco-Wengel, et al., 2011), based on the mpileup file subsampled to a uniform coverage of 10, the classic option selected (--min-count 1), and the following options: --min-qual 20, --min-covered-fraction 0.25, --dissable-corrections. Nucleotide diversity was calculated using PoPoolation1, with the mpileup file used as input employing the following parameters: --min-count 4, --min-coverage 10, --min-qual 20, --min-covered-fraction 0.25, and maximum coverage 3 times the average coverage. The inference of composite likelihood ratio implemented in SweeD was performed based on ANGSD-derived allele frequency estimates, assuming the European rabbit variant as the ancestral state. Grid size was set to define non-overlapping 20 kb windows along the whole-genome, and CLR values were averaged in 100 kb windows with 20 kb steps. Only windows overlapping across the three inferences were retained for DCMS, which was run using R package MINOTAUR (Verity et al., 2017). Raw statistics were first converted to  $p$ -values based on a left-tailed (nucleotide diversity and Tajima's D) or right-tailed test (SweeD CLR). Then DCMS score was calculated for each window along the genome and visualized as a Manhattan plot with the top 1% genome-wide distribution values indicated. Outliers of DCMS were considered coincidental with the PBS ones if the distance between them was not greater than 80 kb (four windows in the DCMS scan).

## 2.6 | Comparative genomic analyses

To understand the origin of candidate locally adapted variants in the context of the evolution of the genus, we analysed the high coverage whole genome data of six *Lepus* species (1 to 4 individuals per species), including representatives of Irish, Alpine, Fennoscandian and Faroese mountain hares (Table S2). Maximum-likelihood phylogenies were built using RAxML with the GTR + G model (Stamatakis, 2015) for alignments containing all autosomes, and then each genomic region identified as candidate to underlie local adaptation. The European rabbit (*Oryctolagus cuniculus*) was used as outgroup in these phylogenetic reconstructions.

We then focused on cases where the mountain hare candidate adaptive variant shared the most recent common ancestor with



**FIGURE 2** Demographic history of the mountain hare populations. Models were inferred from RAD-seq data (see detailed inferred and 95% high density intervals in Table S4). (a) The simultaneous split (STM) model. (b) The sequential split (DTM) model. Parameter estimates are shown:  $N_e$ , effective population size in number of diploid individuals ( $l$ , initial; ANC, ancestral);  $t$ , time of splits in years, considering 2 years per generation (Marboutin & Peroux, 1995). The two models showed similar fit to the data with  $\Delta$ Lhood of 3870 and 3802 for the STM and DTM model, respectively. Ireland, IRL; Alps, ALP; Fennoscandia, FSC

another species and not with their conspecifics. Such phylogenetic pattern could either result from the retention of ancestral variation or from secondary introgression. Given that introgression would lead to low local sequence divergence to the donor species, we estimated genetic divergence, both raw ( $d_{XY}$ ) and mutation-scaled divergence (relative node depth [RND], using the European rabbit as outgroup) (Feder et al., 2005) in 20 kb overlapping windows with 2 kb step along two chromosomes of interest, 1 and 8, using `genomics_general` scripts ([https://github.com/simonhmartin/genomics\\_general](https://github.com/simonhmartin/genomics_general)). Only windows with a minimum of 25% of the sites covered were included for these estimates. To further test whether low empirical local divergence could be simply driven by the variance of the coalescence during species divergence, we simulated the expected local sequence distances under a strict isolation model, following Giska et al. (2019a). We first used G-PhoCS (Gronau et al., 2011) to estimate demographic parameters of the species divergence (effective population sizes, divergence times, and migration rates) using the higher coverage whole-genome data (Table S2). For *L. timidus*-*L. castroviejo* inferences, G-PhoCS runs were based on 9,256 fragments of 1 kb length sampled along the genome and fulfilling the following criteria: within intergenic regions, at least 50 kb apart, maximum 40% missing sequence after masking repeats with Ns. Estimates of *L. timidus*-*L. europaeus* divergence were from Giska et al. (2019a). We then used the estimated demographic parameters in *msms* (Ewing & Hermisson, 2010) to simulate the expected sequence divergence using a mutation rate of  $2.8 \times 10^{-9}$  substitutions/site/generation (Giska et al., 2019a), and a model with no post-divergence migration. We also simulated an extreme case of a strong selective sweep (selection coefficient  $s = 0.1$ ) starting 25 kya prior to the species split, forcing the time of allelic divergence between species to coincide with the species split, thus setting a conservative lower bound for the expected divergence under no gene flow.

### 3 | RESULTS

#### 3.1 | Sequencing and demographic history

The RAD-seq data set consisted of 62 samples, including 20 from Ireland, 16 samples from the Alps and 26 from Fennoscandia (Giska et al., 2021b). The whole genomes of 20 Irish mountain hares were sequenced for a total population coverage of 20.5 $\times$  (mean 1.0 $\times$  per individual) (Giska et al., 2021b), and combined with similar published mountain hare data from the Alps ( $N = 20$ ; total coverage 17.1 $\times$ ; mean individual coverage 0.85 $\times$ ) and Fennoscandia ( $N = 19$ ; total coverage 18.0 $\times$ ; mean individual coverage 0.95 $\times$ ) (Giska et al., 2019a). Mitochondrial DNA sequences were reconstructed from the low individual coverage whole genome sequencing data, resulting in an alignment of 16,223 bp ( $N = 59$ ) (Giska et al., 2021a).

The PCA confirmed the expected strong population structure of the analysed fragmented species (Figure 1b, Figure S1). The Irish mountain hare showed larger differentiation (average pairwise  $F_{ST}$  of 0.22 and 0.25, with Fennoscandian and Alpine populations, respectively;  $F_{ST} = 0.12$  between the Alpine and Fennoscandian population; based on WGS data), which agrees with its inferred smaller effective population size (Figure 2), and the distinct mtDNA clade (Figure 1). The Alpine and Fennoscandian populations showed similar nucleotide diversity (genome-wide mean 0.00367 and 0.00350, respectively), which was slightly higher than that of the Irish population (genome-wide mean 0.00292; Figure S2). The genomic neighbour-joining tree suggested a closer relationship between the Irish and Fennoscandian populations (Figure 1c), an evolutionary pattern that was also supported by TreeMix and the mitochondrial DNA haplotype phylogeny (Figure 1d; Figure S3). However, the NJ whole-genome phylogeny showed paraphyly of the Alpine genetic variation (Figure 1c), while the mtDNA phylogeny showed monophyly only in the Irish mountain hare (Figure 1d). The  $D$  statistics

tests, based on patterns of shared derived variants among the mountain hare populations, did not suggest deviation from incomplete lineage sorting expectations ( $D$  ranged between 0.00755 and 0.01106;  $p$ -value  $>.05$  for all trio combinations). Single population demographic models simulated with fastsimcoal showed good fit to the empirical SFSs (difference between the maximum observed and the estimated likelihoods,  $\Delta L_{\text{hood}}$  of 146, 620, and 462 for Irish, Alpine, and Fennoscandian mountain hares, respectively). The estimated effective population sizes were used to infer the history of divergence among the three mountain hare populations using two alternative models (simultaneous split (STM) or a sequential split (DTM)), which showed similar fit to the data ( $\Delta L_{\text{hood}}$ , 3870 and 3802 for the STM and DTM model, respectively, Table S4C). Similar time-frames for the population split were inferred: 18.4 kya (thousand years ago; assuming 2 years per generation; 95% HDI 17.5–22.1 kya) for the STM model, and 21.2 kya (95% HDI 19.1–25.3 kya) and 17.8 kya (95% HDI 15.9–20.1 kya) for the DTM model (Figure 2; see also Table S4C for detailed parameter estimates). Slightly different demographic trends were suggested from the STM or DTM models but with overlapping 95% HDI (Figure 2; Table S4C). Despite the natural uncertainties of molecular calibrations and large confidence intervals of the estimates (Table S4C), our results are consistent with simultaneous or near-simultaneous fragmentation of the three mountain hare populations occurring at late Pleistocene, possibly around the Last Glacial Maximum.

### 3.2 | Differentiation outliers and signatures of selective sweeps

Population branch statistics (PBS) scans between Irish, Alpine and Fennoscandian mountain hares revealed several genomic regions with extreme levels of differentiation. We considered as autosomal outlier genomic regions for interpopulation differentiation those with at least two consecutive windows of 50 SNPs with PBS of  $p \leq .01$  (established from simulations based on the demographic models), confirmed by BayPass contrast scans (Figures S4 and S5; Tables S5 and S6). We identified 12 differentiation outliers in Irish, 18 in Alpine, and nine in Fennoscandian mountain hares. These genomic regions overlapped 14, nine, and 12 protein coding genes, respectively (Figure 3; Table S5). Consistent results were obtained when analyses were based on the de novo Irish mountain hare draft genome reference (Figure S6, Tables S7 and S8). Our independent validation of allele frequency inferences, based on SNP genotyping in four candidate regions in the Irish mountain hare, confirmed the patterns inferred from the whole genome data of low individual coverage (Figure S7, Table S9).

The combined evidence of PBS and BayPass, identifying differentiation outliers, and population-specific DCMS, identifying intra-population selective sweeps (Figure S8, Table S10) pinpointed two candidate genomic regions in Irish mountain hares, one overlapping gene *ASIP*, three candidates in Alpine mountain hares, one

overlapping gene *PCCA*, and two candidates in Fennoscandian mountain hares, one overlapping genes *EPRS1* and *SLC30A10*. Additional selective sweep signals were detected in other PBS peaks, but were found to be shared among two or all three populations, suggesting possible global and not local selection (Table S10). These regions under global selection included genes *IPO7*, *GCKR*, *FNDCA4*, *IFT172*, *HMGA2*, *RFX6*, *DNM3*, and *EXOC6*.

### 3.3 | Comparative genomics

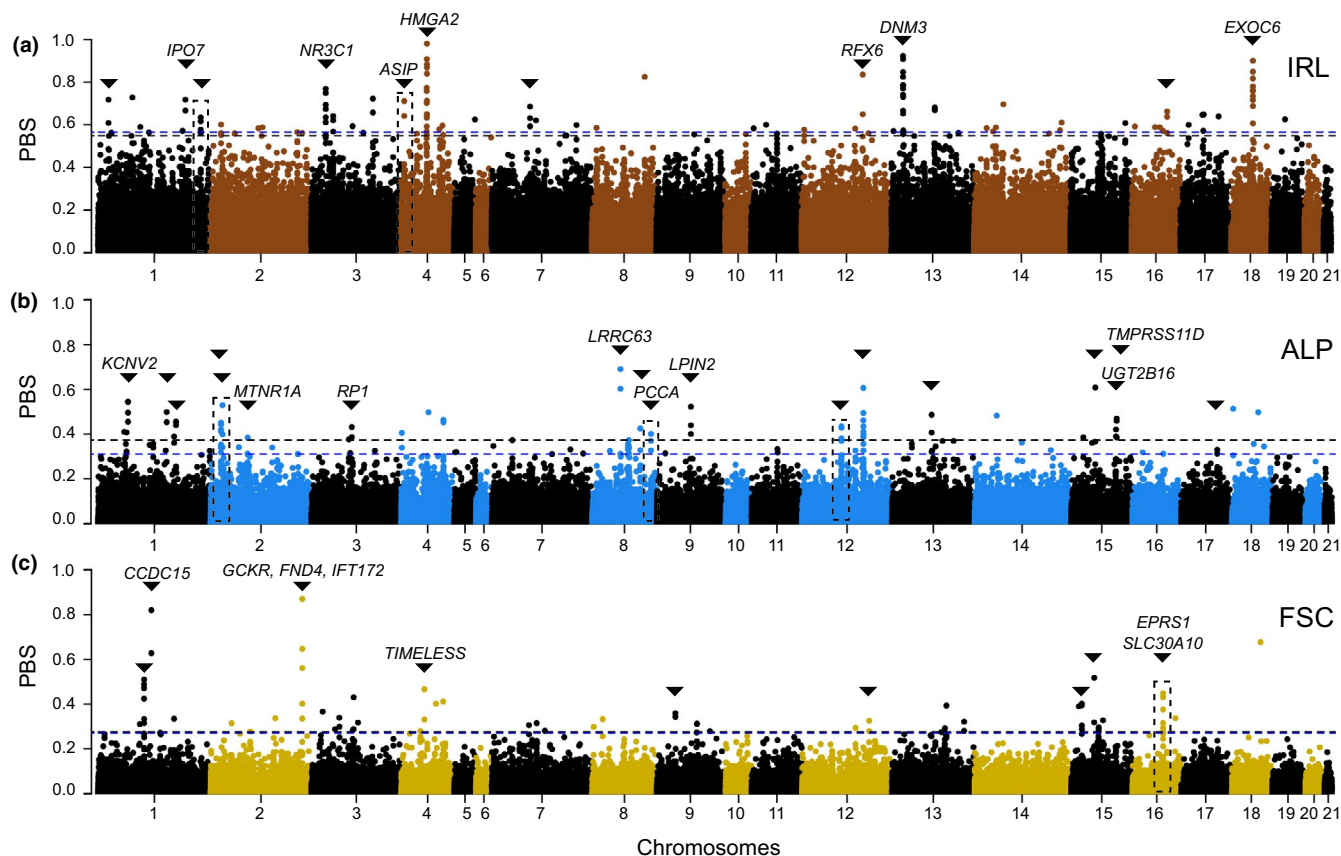
From the analyses of the higher coverage whole genome data across species, we found that local gene trees of candidate regions for local adaptation predominantly recovered the mountain hare as monophyletic lineage (Figure 4). However, in two cases, the local phylogenetic tree was discordant from the presumable species tree, with the candidate variant grouping with variants from another species with strong bootstrap support (Figure 4c,e). These patterns of shared variation between species could either result from interspecies incomplete lineage sorting or secondary introgression. We found that within both these regions, chromosome 1 (177.8 Mb, unknown gene content) and chromosome 8 (101.7 Mb, *PCCA*), the divergence to the potential donor species is lower than intraspecific divergence between populations, and the pattern is not influenced by local mutation rate when controlled using the *RND* (Figure 5a,c). Simulating the expected distribution of divergence between species (based on the inferred parameters of species divergence; Table S11) showed that, in both cases, the empirical divergence between the mountain hare and the presumable donor species along the candidate region is smaller than 1% lowest values simulated under a species divergence model with strong selection and no post-split gene flow (Figure 5). In the case of the candidate region on chromosome 8, this pattern was found along a predominant portion of the region, while for the chromosome 1 it affected a shorter fragment of the candidate genomic stretch. These results suggest that in both cases of phylogenetic discordance, introgressive hybridization introduced the adaptive variants in the mountain hare, from *L. europaeus* into Irish *L. timidus* (unknown gene content) and from *L. castroviejoi* into Alpine *L. timidus* (gene *PCCA*) (Figure 4).

## 4 | DISCUSSION

In the present work we studied genomic variation of a typically cold-adapted species, currently fragmented into isolated populations in Western Europe, characterized by vast environmental differences. We identified candidate targets of positive selection, the biologically relevant genetic variation underlying local adaptation, and the evolutionary origin of some of these variants through introgression from related species.

Our demographic modelling suggested that the current Irish, Alpine and Fennoscandian mountain hare populations resulted



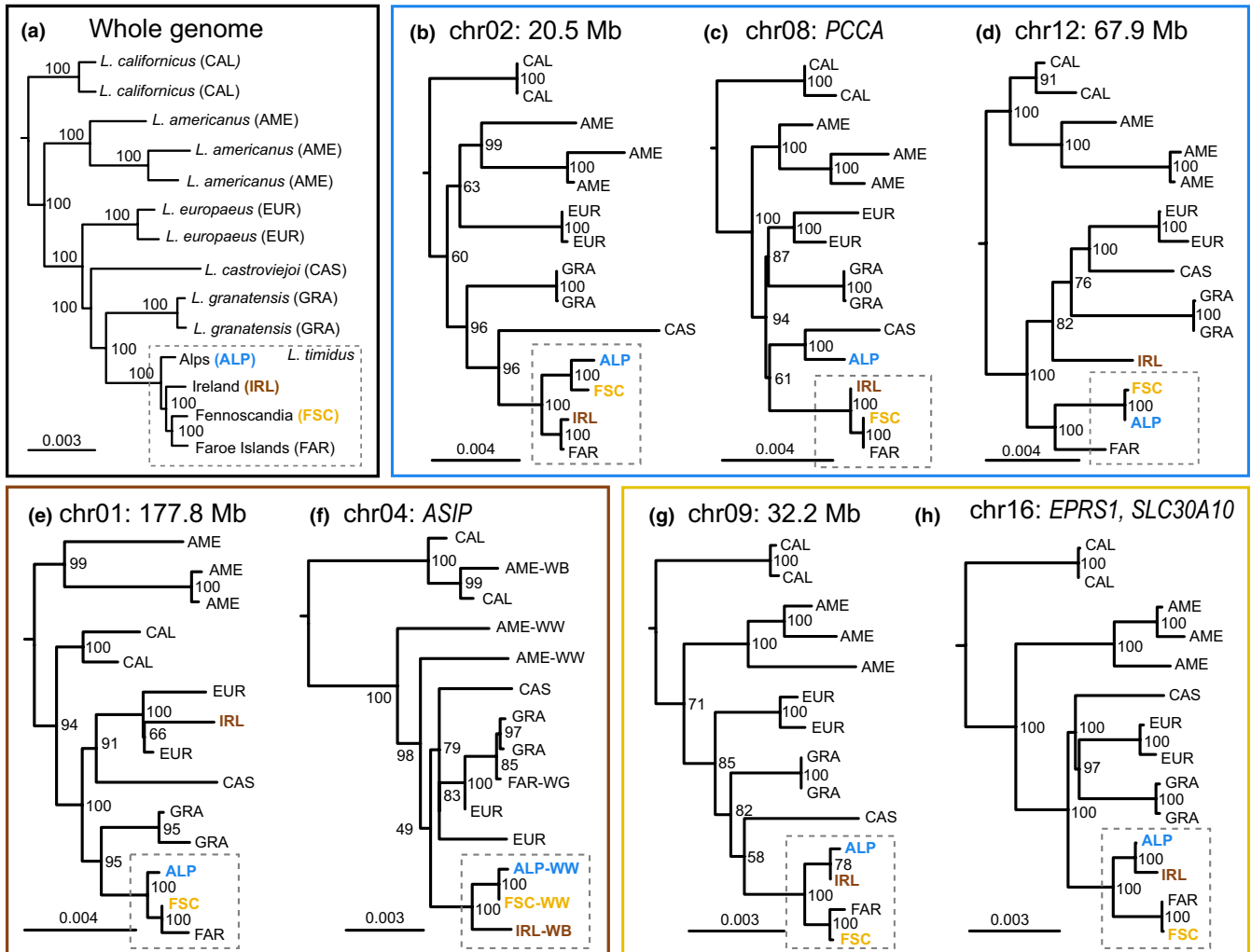


**FIGURE 3** Population branch statistic and selective sweep (DCMS) scans along the autosomes for each mountain hare population (A, Ireland; B, Alps; C, Fennoscandia). The peaks marked with black triangles represent regions with at least two consecutive windows with PBS  $p \leq .01$  (blue dashed horizontal lines indicate thresholds based on the STM model simulations, and black dashed horizontal lines indicate thresholds based on the DTM model simulation), confirmed by BayPass contrast analysis (see Figure S3). Codes indicate genes within the outlier regions (see Table S5). Dashed rectangles indicate PBS peaks that overlap with signatures of population-specific selective sweeps, evidenced by DCMS analysis (see Figure S6 and Table S10). See Figure S5 for PBS scans along chromosome X

from a nearly simultaneous fragmentation from a common gene pool in Europe during the late Pleistocene, with cessation of gene flow around the Last Glacial Maximum ~20 kya (Figure 2; Table S4). Previous analyses of mountain hares, based on few genetic markers, have shown sharing of genetic variation across the range of the species, which supports a scenario of late Pleistocene/post-glacial fragmentation (Hamill et al., 2006; Melo-Ferreira et al., 2007; Smith et al., 2017). Notwithstanding uncertainties linked to molecular calibrations and modelling, our demographic estimates are in line with expectations for the species and support fragmentation patterns inferred for other organisms. Our time estimate for the fragmentation of Irish mountain hares (~17–21 kya) is compatible with the earliest paleontological record of the species in Ireland, 28 kya, and the last land bridge to the region, 16 kya (Montgomery et al., 2014). These time estimates are in agreement with phylogeographic patterns described for other mammals, such as stoats, *Mustela erminea*, for which the presumed origin of the Irish populations predates the LGM (Martinkova et al., 2007; Montgomery et al., 2014). In continental Europe, the progressive deglaciation of the ice caps in the Alps and Northern Europe after the LGM allowed colonization, which implies

that putative fragmentation of the ranges of species colonizing both regions should not predate the LGM (Schmitt, 2017). As a consequence, the populations of some species present in the Alps and in Scandinavia show marked genetic similarities, from animals, such as wolf spiders, *Pardosa saltuaria* (Muster & Berendonkschm, 2006), to plants, such as the alpine rock-cress, *Arabis alpina* (Ehrich et al., 2007). Overall, our inferred demographic history appears compatible with a phylogeographic pattern characterised by periodical shifts in geographical range distribution accompanied by further fragmentation caused by retreats and advances of the ice streams during deglaciation after the LGM, typical of cold-adapted species (Stewart et al., 2010). Our inference sets the upper limit for the time-frame of local adaptation in the species in the newly colonized territories at late Pleistocene.

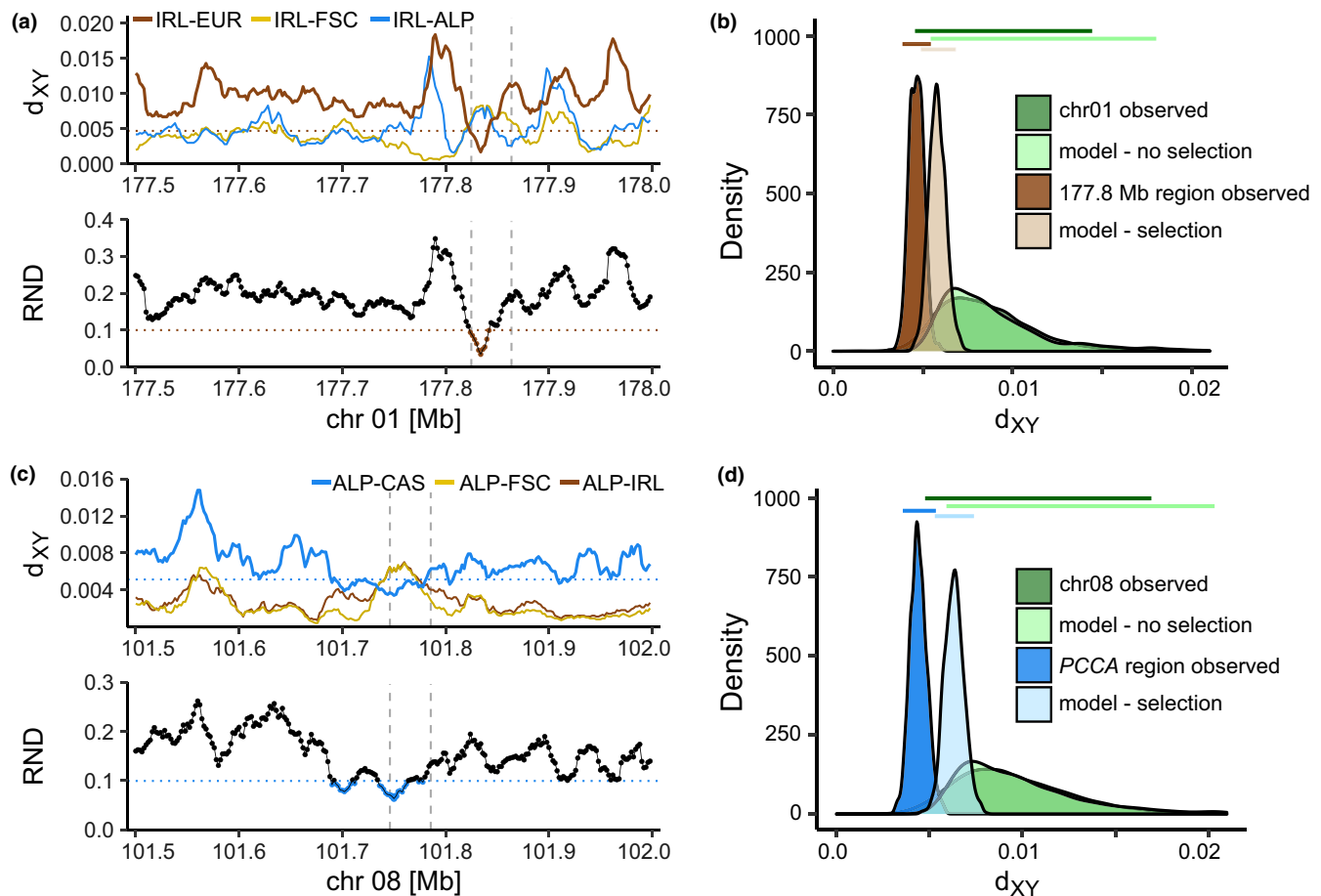
Our scans of population branch statistics identified highly differentiated regions of the genome in all three analysed isolated populations (Figure 3, Table S5). These extreme differentiation regions could be spurious signals of extreme differentiation, given the structured nature of the contrasted populations (Hoban et al., 2016), but our analyses showed that the patterns remain when taking the



**FIGURE 4** Maximum-likelihood trees for the whole genome and local trees of candidate regions for local adaptation in the mountain hare (genes in the regions are noted, unless no annotation is known, in which case the coordinate is noted). (a) Global tree based on the concatenated whole genome. Local phylogenies of candidate regions in (b–d) Alpine mountain hares, (e–f) Irish mountain hares, and (g–h) Fennoscandian mountain hares. In the *ASIP* phylogeny, notations WW (winter-white), WB (winter-brown) and WG (winter-grey), indicate winter colouration morphs of the sequenced individual

covariance of allele frequencies into account, thus controlling for population structure (Figure S4, Table S6) (Gautier, 2015). However, spurious patterns of variation resembling local adaptation could result from spatially uniform positive or negative selection, decreasing variation at linked sites, and increasing the variance of pairwise differentiation estimated locally in the genome (Cruickshank & Hahn, 2014; Hoban et al., 2016). While our candidate PBS outliers were population-specific, we did detect several instances of candidate regions where a signal of selective sweep based on intra-population statistics was shared across two or three populations analysed, which could result from simultaneous sweeps or uniform background selection (Figure S8, Table S10). Nevertheless, we identified seven genomic regions with population-specific signatures of extreme local differentiation and population-specific selective sweeps, which are supportive of signatures of local adaptation during or after the colonization of these territories.

We inspected the gene content of these candidates for local adaptation and found that some gene functions match phenotypic uniqueness of the populations. For instance, in the Irish mountain hare, the pigmentation gene *ASIP* was identified as a candidate. This protein (agouti-signalling protein) is an antagonist of the melanocortin 1 receptor (MC1R), present in the membranes of melanocytes, shifting the production of pigment from dark eumelanin to light pheomelanin or inhibiting the production of pigment (Hoekstra, 2006). It is a well-known pigmentation gene that underlies adaptive coat colour variation in many mammals (Barrett et al., 2019; Fontanesi et al., 2010; Kerns et al., 2004; Miranda et al., 2021). Mountain hares typically undergo brown-white seasonal moults to remain camouflaged year-round on seasonal snow (Zimova et al., 2018). Yet, winter coats in Irish mountain hares present typically a unique brown winter coat pattern. This suggests a local adaptation, as individuals with white coat would have higher fitness costs by



**FIGURE 5** Signatures of introgression. Left panels: scan of  $d_{XY}$  (top; horizontal dotted line indicates 1% lowest values in interspecific  $d_{XY}$  simulated under a model with selection; IRL, Ireland; ALP, Alps; FSC, Fennoscandia; EUR, *L. europaeus*; CAS, *L. castroviejoi*) and RND between focal mountain hare and potential donor species (bottom; horizontal dotted line indicates 1% lowest values in the observed chromosome-wide distribution), with vertical dashed lines representing the candidate regions. Right panels: distribution of  $d_{XY}$  between the focal mountain hare population and the putative donor species, including the observed distribution along the whole chromosome and the candidate region, and simulated distribution under the model with and without selection. Top horizontal bars represent 95% range for each distribution. (a–b) Irish *L. timidus* - candidate region chr01:177.8 Mb and possible introgression from *L. europaeus*, EUR. (c–d) Alpine *L. timidus* - candidate region chr08:101.7 Mb, overlapping PCCA gene, and possible introgression from *L. castroviejoi*, CAS

coat colour mismatch due to absence of significant or prolonged snowfall in Ireland (Zimova et al., 2016). *ASIP* has been previously linked with coat colour variation in hares, namely the winter brown morphs of snowshoe hares (*L. americanus*) in the Pacific Northwest of the USA (Jones et al., 2018) and the winter grey morph of Faroese mountain hares (Giska et al., 2019a) via *cis*-regulatory changes. Our results thus suggest that *ASIP* is also a strong candidate to be involved in the determination of the winter brown colour morph of the Irish mountain hare. In Alpine mountain hares, which inhabit high altitude environments up to ~4000 m above sea level (Angerbjörn & Schai-Braun, in press; Hackländer & Jenny, 2011), the *PCCA* gene showed pattern of population specific positive selection. This gene encodes the propionyl-CoA carboxylase enzyme responsible for catabolism of odd chain fatty acids (OCFAs) by carboxylation of their metabolite, propionyl-CoA. It was shown that due to cold exposure the level of OCFAs in the white adipose tissue of mice got changed (Xu et al., 2019). Propionyl-CoA can be transported from

mitochondria to the cytoplasm and there used as an intermediate in the Krebs cycle to fight hypothermia when mitochondrial energy production is weakened. It has been hypothesized that OCFAs may increase energy production when temperature is low. Thus, *PCCA* may be involved in adaptations to cold Alpine habitat by intensifying the use of OCFAs as energy source. In Fennoscandian mountain hares, we found evidence of selection overlapping the *SLC30A10* and *EPRS1* genes, which in human GWAS studies have been linked to body mass, bone density and adipose tissue physiology (Arif et al., 2017), and may thus underlie larger body sizes of Fennoscandian hares. Among the genes with signals of global selection (i.e., overlapping signs of selective sweeps in several populations) (Figure S8, Table S10), *HMGA2* has been vastly linked to body size differences in mammals, including dwarfism in rabbits (Carneiro et al., 2017; Jones et al., 2008), and may underlie body size differences across mountain hare populations. The functional relevance of the inferred adaptive variation remains naturally tentative (see complete list of candidate

genes in Table S5), but our results can guide future functional testing to validate these hypotheses.

We then analysed the candidate adaptive genetic variation of the mountain hare in the context of the evolutionary history across species. When comparing local and whole-genome phylogenies, we found that the local phylogenies of most of the candidate genomic regions, recovered the mountain hare as monophyletic (Figure 4). This suggests that most of the adaptive variation inferred here is species-specific. With the current data set, we cannot determine whether adaptation resulted from standing variation or from de novo mutation, but standing variation appears more plausible, given the recent post-glacial time-frame of the divergence (Barrett & Schluter, 2008) (Figure 2). However, in some cases we found that the local phylogenetic signal was discordant from the species-tree, with the candidate variant grouping with another species. Our simulations show that for *PCCA*, the empirical divergence between the broom hare found in the Cantabrian Mountains in northern Spain and the Alpine mountain hare variant is lower than expected under a no-gene flow scenario, and hence originated from introgression (Figures 4 and 5). The detection of the introgressed variant only in the Alps could either result from interspecific interactions specific to Alpine mountain hares after the fragmentation of the mountain hare populations, or from earlier hybridization and persistence of introgressed standing variation in the Alps but not elsewhere (or missed in our sampling). Also, given the close genetic similarity of the broom hare and the Italian hare (*L. corsicanus*) (Alves et al., 2008), present in the Italian Apennines, we cannot discard that the latter species, not included in our phylogeny, is the actual donor of the introgressed variation. Even though mountain, broom and Italian hares are not currently in contact, the post-glacial dynamics of hare species in southern Europe implied frequent cases of historical hybridization and the persistence of introgressed variants in the gene pool of the species (Melo-Ferreira et al., 2012). Therefore, the introgression detected here may result from those historical events. We also found evidence of introgression from the European hare into the Irish mountain hare (Figures 4 and 5), but in this case no known gene was annotated to this genomic region, and the putative source of adaptive variation and timing of the introgression events remain elusive. Even though our approach was rather conservative, these results suggest that adaptive variants may originate from introgression. This can be particularly common in hares, given both historical and contemporary contact between species resulting in interspecific exchanges along the diversification of the genus (Ferreira et al., 2021).

While most of the candidate genes revealed here had not been implicated in known instances of adaptive evolution in hares, the presence of the agouti signalling protein gene (*ASIP*) among candidates for local adaptation appears recurrent (Giska et al., 2019a; this study; Jones et al., 2018). In both snowshoe hares and Faroese mountain hares, the variant causing nonwhite winter morphs introgressed from closely related species, that is, from black tailed jackrabbits (*L. californicus*) and Iberian hares (*L. granatensis*), respectively. Here we found no evidence of introgression of the Irish mountain

hare winter-brown variant (Figure 4), which appears distinct from that generating the grey morph in Faroese mountain hares (Figure S9), and suggests adaptation from species-specific standing genetic variation. In the absence of admixture mapping in natural populations (which is difficult given the isolated nature of the concerned populations) or experimental crosses, the suggestion that *ASIP* underlies the winter brown colour morph in Irish mountain hares remains tentative. However, it is striking that diverse evolutionary pathways recruiting the same gene appear to recurrently promote local adaptation in independent hare populations or species, reinforcing the role of this gene as an evolutionary hotspot for adaptive variation in mammals.

Species remain the central units for conservation assessment and management in national and regional plans, including for the International Union of Conservation of Nature (IUCN) Red Lists and action plans. Yet, this emphasis on species for conservation is controversial, because it does not consider the continuum of genetic variation, from populations to species (Coates et al., 2018). It also ignores local adaptation and the unique genetic variation underlying adaptive traits (Teixeira & Huber, 2021). Our work shows that the studied allopatric populations of mountain hares had an independent evolutionary trajectories probably since the Last Glacial Maximum, and that part of the specific gene pool of each population (which may be much larger than our conservative approach detected) underlies adaptation to local conditions. As population genomic studies become common, detecting intraspecific adaptive variation will provide an additional level of information that may be incorporated into conservation planning and practice, contributing to integrate the evolutionary process in conservation (Hoelzel et al., 2019; Mills et al., 2018). Regardless of the taxonomic status of the studied mountain hare populations, which are classified as subspecies (Angerbjörn & Schai-Braun, in press), the identification of unique adaptive variants in these endemisms may be of interest when assessing the conservation status of the species and for the definition of region-specific conservation policies.

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#### AUTHOR CONTRIBUTIONS

José Melo-Ferreira coordinated the study; Iwona Giska, José Melo-Ferreira, Paulo C. Alves, Paulo A. Prodöhl, W. Ian Montgomery, and Neil Reid designed research; Paulo A. Prodöhl, W. Ian Montgomery, Neil Reid, Hannes Jenny, and Klaus Hackländer contributed with sampling; Liliana Farelo generated data; José Melo-Ferreira and Pierre Boursot provided computational resources and support; Iwona Giska, João Pimenta and José Melo-Ferreira analysed data; José Melo-Ferreira and Iwona Giska wrote the study with contribution of João Pimenta; all authors interpreted the results and read, revised, and approved the final version of the manuscript.

#### DATA AVAILABILITY STATEMENT

The reference genomes used in this work have been made available in Dryad (<https://doi.org/10.5061/dryad.x95x69pd8> and <https://doi.org/10.5061/dryad.j0zpc86bk>). Whole genome DNA sequencing data from previous studies are available in the NCBI Sequence Read Archive (BioProjects PRJNA564335, PRJNA420081, PRJNA399194 and PRJNA561582; and BioSamples SAMN12618118, SAMN12618122 and SAMN12640762; details in Tables S1 and S2). The newly obtained RAD-sequencing and whole genome sequencing data is available in NCBI SRA BioProject PRJNA783878. Complete mitochondrial DNA sequences are deposited in Dryad (<https://doi.org/10.5061/dryad.fqz612jtt>). Samples used in this work are archived in CIBIO LagBank (CIBIO Lagomorph Tissue and DNA Bank) and they are accessible for research purposes (permanent accession codes in Table S1).

#### BENEFIT SHARING STATEMENT

Benefits from this research include the availability of data and results on public databases, as described above.

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#### REFERENCES

- Altuna, J. (1970). Hallazgo de una liebre artica (*Lepus timidus*) en el yacimiento prehistorico de Urtiga (Guipuzcoa). *Munibe. Sociedad De Ciencias Naturales Aranzadi (San Sebastian)*, 22, 165–168.
- Alves, P. C., Melo-Ferreira, J., Branco, M., Suchentrunk, F., Ferrand, N., & Harris, D. J. (2008). Evidence for genetic similarity of two allopatric European hares (*Lepus corsicanus* and *L. castroviejoii*) inferred from nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, 46(3), 1191–1197. <https://doi.org/10.1016/j.ympev.2007.11.010>
- Angerbjörn, A. (2018). *Lepus timidus*, Mountain hare. In A. T. Smith, C. H. Johnston, P. C. Alves, & K. Hackländer (Eds.), *Lagomorphs: Pikas, rabbits and hares of the world* (pp. 212–216). John Hopkins University Press.
- Angerbjörn, A., & Flux, J. (1995). *Lepus timidus*. *Mammalian Species*, 495, 1–11. <https://doi.org/10.2307/3504302>
- Angerbjörn, A., & Schai-Braun, S. C. (in press). Mountain hare *Lepus timidus* Linnaeus, 1758. In K. Hackländer, & F. E. Zachos (Eds.), *Handbook of the mammals of Europe*. Springer.
- Arif, A., Terenzi, F., Potdar, A. A., Jia, J., Sacks, J., China, A., Halawani, D., Vasu, K., Li, X., Brown, J. M., Chen, J., Kozma, S. C., Thomas, G., & Fox, P. L. (2017). EPRS is a critical mTORC1–S6K1 effector that influences adiposity in mice. *Nature*, 542(7641), 357–361. <https://doi.org/10.1038/nature21380>
- Barrett, R. D. H., Laurent, S., Mallarino, R., Pfeifer, S. P., Xu, C. C. Y., Foll, M., Wakamatsu, K., Duke-Cohan, J. S., Jensen, J. D., & Hoekstra, H. E. (2019). Linking a mutation to survival in wild mice. *Science*, 363(6426), 499. <https://doi.org/10.1126/science.aav3824>
- Barrett, R. D. H., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in Ecology & Evolution*, 23(1), 38–44. <https://doi.org/10.1016/j.tree.2007.09.008>
- Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>
- Booker, T. R., Yeaman, S., & Whitlock, M. C. (2021). Global adaptation complicates the interpretation of genome scans for local adaptation. *Evolution Letters*, 5(1), 4–15. <https://doi.org/10.1002/evl3.208>
- Bouckaert, R., Vaughan, T. G., Barido-Sottani, J., Duchêne, S., Fourment, M., Gavryushkina, A., Heled, J., Jones, G., Kühnert, D., De Maio, N., Matschiner, M., Mendes, F. K., Müller, N. F., Ogilvie, H. A., du Plessis, L., Poppinga, A., Rambaut, A., Rasmussen, D., Siveroni, I., ... Drummond, A. J. (2019). BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLOS Computational Biology*, 15(4), e1006650. <https://doi.org/10.1371/journal.pcbi.1006650>
- Carneiro, M., Hu, D., Archer, J., Feng, C., Afonso, S., Chen, C., & Andersson, L. (2017). Dwarfism and altered craniofacial development in rabbits is caused by a 12.1 kb deletion at the HMGA2 Locus. *Genetics*, 205(2), 955–965. <https://doi.org/10.1534/genetics.116.196667>
- Carneiro, M., Rubin, C.-J., Di Palma, F., Albert, F. W., Alföldi, J., Barrio, A. M., Pielberg, G., Rafati, N., Sayyab, S., Turner-Maier, J., Younis, S., Afonso, S., Aken, B., Alves, J. M., Barrell, D., Bolet, G., Boucher, S., Burbano, H. A., Campos, R., ... Andersson, L. (2014). Rabbit genome analysis reveals a polygenic basis for phenotypic change during domestication. *Science*, 345(6200), 1074–1079. <https://doi.org/10.1126/science.1253714>

- Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Molecular Ecology*, 22(11), 3124–3140. <https://doi.org/10.1111/mec.12354>
- Chang, C. C., Chow, C. C., Tellier, L. C. A. M., Vattikuti, S., Purcell, S. M., & Lee, J. J. (2015). Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience*, 4, 1–16. <https://doi.org/10.1186/s13742-015-0047-8>
- Coates, D. J., Byrne, M., & Moritz, C. (2018). Genetic diversity and conservation units: Dealing with the species-population continuum in the age of genomics. *Frontiers in Ecology and Evolution*, 6(165), 1–13. <https://doi.org/10.3389/fevo.2018.00165>
- Cruickshank, T. E., & Hahn, M. W. (2014). Reanalysis suggests that genomic islands of speciation are due to reduced diversity, not reduced gene flow. *Molecular Ecology*, 23(13), 3133–3157. <https://doi.org/10.1111/mec.12796>
- Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9(8), 772. <https://doi.org/10.1038/nmeth.2109>
- Dietz, A. J., Wohner, C., & Kuenzer, C. (2012). European snow cover characteristics between 2000 and 2011 derived from improved MODIS daily snow cover products. *Remote Sensing*, 4(8), 2432–2454. <https://doi.org/10.3390/rs4082432>
- Drummond, A. J., Ho, S. Y., Phillips, M. J., & Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biology*, 4(5), e88. <https://doi.org/10.1371/journal.pbio.0040088>
- Durand, E. Y., Patterson, N., Reich, D., & Slatkin, M. (2011). Testing for ancient admixture between closely related populations. *Molecular Biology and Evolution*, 28(8), 2239–2252. <https://doi.org/10.1093/molbev/msr048>
- Ehrich, D., Gaudeul, M., Assefa, A., Koch, M. A., Mummenhoff, K., Nemomissa, S., Consortium, I., & Brochmann, C. (2007). Genetic consequences of Pleistocene range shifts: contrast between the Arctic, the Alps and the East African mountains. *Molecular Ecology*, 16(12), 2542–2559. <https://doi.org/10.1111/j.1365-294X.2007.03299.x>
- Ewing, G., & Hermisson, J. (2010). MSMS: a coalescent simulation program including recombination, demographic structure and selection at a single locus. *Bioinformatics*, 26(16), 2064–2065. <https://doi.org/10.1093/bioinformatics/btq322>
- Excoffier, L., Dupanloup, I., Huerta-Sánchez, E., Sousa, V. C., & Foll, M. (2013). Robust demographic inference from genomic and SNP data. *PLoS Genetics*, 9(10), e1003905. <https://doi.org/10.1371/journal.pgen.1003905>
- Feder, J. L., Xie, X., Rull, J., Velez, S., Forbes, A., Leung, B., Dambroski, H., Filchak, K. E., & Aluja, M. (2005). Mayr, Dobzhansky, and Bush and the complexities of sympatric speciation in *Rhagoletis*. *Proceedings of the National Academy of Sciences USA*, 102(Suppl 1), 6573–6580. <https://doi.org/10.1073/pnas.0502099102>
- Ferreira, M. S., Jones, M. R., Callahan, C. M., Farello, L., Tolesa, Z., Suchentrunk, F., Boursot, P., Mills, L. S., Alves, P. C., Good, J. M., & Melo-Ferreira, J. (2021). The Legacy of Recurrent Introgression during the Radiation of Hares. *Systematic Biology*, 70(3), 593–607. <https://doi.org/10.1093/sysbio/syaa088>
- Fontanesi, L., Forestier, L., Allain, D., Scotti, E., Beretti, F., Deretz-Picoulet, S., Pecchioli, E., Vernesi, C., Robinson, T. J., Malaney, J. L., Russo, V., & Oulmouden, A. (2010). Characterization of the rabbit agouti signaling protein (ASIP) gene: transcripts and phylogenetic analyses and identification of the causative mutation of the nonagouti black coat colour. *Genomics*, 95(3), 166–175. <https://doi.org/10.1016/j.ygeno.2009.11.003>
- Funk, W. C., McKay, J. K., Hohenlohe, P. A., & Allendorf, F. W. (2012). Harnessing genomics for delineating conservation units. *Trends in Ecology & Evolution*, 27(9), 489–496. <https://doi.org/10.1016/j.tree.2012.05.012>
- Gautier, M. (2015). Genome-wide scan for adaptive divergence and association with population-specific covariates. *Genetics*, 201(4), 1555–1579. <https://doi.org/10.1534/genetics.115.181453>
- Giska, I., Farello, L., Pimenta, J., Seixas, F. A., Ferreira, M. S., Marques, J. P., Miranda, I., Letty, J., Jenny, H., Hackländer, K., Magnussen, E., & Melo-Ferreira, J. (2019a). Introgression drives repeated evolution of winter coat color polymorphism in hares. *Proceedings of the National Academy of Sciences USA*, 116(48), 24150–24156. <https://doi.org/10.1073/pnas.1910471116>
- Giska, I., Farello, L., Pimenta, J., Seixas, F. A., Ferreira, M. S., Marques, J. P., Miranda, I., Letty, J., Jenny, H., Hackländer, K., Magnussen, E., & Melo-Ferreira, J. (2019b). Whole-genome sequence data of mountain hares (*Lepus timidus*) from different European populations. NCBI Sequence Read Archive. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA564335> Deposited 6 September 2019
- Giska, I., Pimenta, J., Farello, L., Boursot, P., Hackländer, K., Jenny, H., & Melo-Ferreira, J. (2021a). Data from: The evolutionary pathways for local adaptation in mountain hares. *Dryad*. <https://doi.org/10.5061/dryad.fqz612jtt>. Deposited
- Giska, I., Pimenta, J., Farello, L., Boursot, P., Hackländer, K., Jenny, H., & Melo-Ferreira, J. (2021b). Mountain hare RAD-Sequence and Whole Genome Sequence data. NCBI Sequence Read Archive. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA783878> Deposited
- Green, R. E., Krause, J., Briggs, A. W., Maricic, T., Stenzel, U., Kircher, M., & Paabo, S. (2010). A draft sequence of the Neandertal genome. *Science*, 328(5979), 710–722.
- Gronau, I., Hubisz, M. J., Gulko, B., Danko, C. G., & Siepel, A. (2011). Bayesian inference of ancient human demography from individual genome sequences. *Nature Genetics*, 43(10), 1031–1034. <https://doi.org/10.1038/ng.937>
- Hackländer, K., & Jenny, H. (2011). One man's meat is the other man's poison: How global warming affects *Lepus europaeus* and *Lepus timidus* in the Alps. *Mammalian Biology*, 76S, 10.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.
- Hamala, T., & Savolainen, O. (2019). Genomic patterns of local adaptation under gene flow in *Arabidopsis lyrata*. *Molecular Biology and Evolution*, 36(11), 2557–2571. <https://doi.org/10.1093/molbev/msz149>
- Hamill, R. M., Doyle, D., & Duke, E. J. (2006). Spatial patterns of genetic diversity across European subspecies of the mountain hare, *Lepus timidus* L. *Heredity*, 97(5), 355–365. <https://doi.org/10.1038/sj.hdy.6800880>
- Hendricks, S. A., Schweizer, R. M., & Wayne, R. K. (2019). Conservation genomics illuminates the adaptive uniqueness of North American gray wolves. *Conservation Genetics*, 20(1), 29–43. <https://doi.org/10.1007/s10592-018-1118-z>
- Hivert, V., Leblois, R., Petit, E. J., Gautier, M., & Vitalis, R. (2018). Measuring genetic differentiation from pool-seq data. *Genetics*, 210(1), 315. <https://doi.org/10.1534/genetics.118.300900>
- Hoban, S., Kelley, J. L., Lotterhos, K. E., Antolin, M. F., Bradburd, G., Lowry, D. B., Poss, M. L., Reed, L. K., Storfer, A., & Whitlock, M. C. (2016). Finding the genomic basis of local adaptation: pitfalls, practical solutions, and future directions. *The American Naturalist*, 188(4), 379–397. <https://doi.org/10.1086/688018>
- Hoekstra, H. E. (2006). Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity*, 97(3), 222–234. <https://doi.org/10.1038/sj.hdy.6800861>
- Hoelzel, A. R., Bruford, M. W., & Fleischer, R. C. (2019). Conservation of adaptive potential and functional diversity. *Conservation Genetics*, 20(1), 1–5. <https://doi.org/10.1007/s10592-019-01151-x>
- Jackman, S. D., Vandervalk, B. P., Mohamadi, H., Chu, J., Yeo, S., Hammond, S. A., Jahesh, G., Khan, H., Coombe, L., Warren, R. L., & Birol, I. (2017). ABySS 2.0: resource-efficient assembly of large

- genomes using a Bloom filter. *Genome Research*, 27(5), 768–777. <https://doi.org/10.1101/gr.214346.116>
- Jones, M. R., Mills, L. S., Alves, P. C., Callahan, C. M., Alves, J. M., Lafferty, D. J. R., Jiggins, F. M., Jensen, J. D., Melo-Ferreira, J., & Good, J. M. (2017). *Whole exome and whole genome sequencing of hares and jackrabbits (genus Lepus)*. NCBI Sequence Read Archive. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA420081> Deposited 28 November 2017
- Jones, M. R., Mills, L. S., Alves, P. C., Callahan, C. M., Alves, J. M., Lafferty, D. J. R., Jiggins, F. M., Jensen, J. D., Melo-Ferreira, J., & Good, J. M. (2018). Adaptive introgression underlies polymorphic seasonal camouflage in snowshoe hares. *Science*, 360(6395), 1355–1358. <https://doi.org/10.1126/science.aar5273>
- Jones, P., Chase, K., Martin, A., Davern, P., Ostrander, E. A., & Lark, K. G. (2008). Single-nucleotide-polymorphism-based association mapping of dog stereotypes. *Genetics*, 179(2), 1033–1044. <https://doi.org/10.1534/genetics.108.087866>
- Kerns, J. A., Newton, J., Berryere, T. G., Rubin, E. M., Cheng, J.-F., Schmutz, S. M., & Barsh, G. S. (2004). Characterization of the dog Agouti gene and a nonagouti mutation in German Shepherd Dogs. *Mammalian Genome*, 15(10), 798–808. <https://doi.org/10.1007/s00335-004-2377-1>
- Kofler, R., Orozco-terWengel, P., De Maio, N., Pandey, R. V., Nolte, V., Futschik, A., Kosiol, C., & Schlötterer, C. (2011). PoPoolation: A toolbox for population genetic analysis of next generation sequencing data from pooled individuals. *PLoS One*, 6(1), e15925. <https://doi.org/10.1371/journal.pone.0015925>
- Kofler, R., Pandey, R. V., & Schlötterer, C. (2011). PoPoolation2: Identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). *Bioinformatics*, 27, 3435–3436. <https://doi.org/10.1093/bioinformatics/btr589>
- Korneliussen, T. S., Albrechtsen, A., & Nielsen, R. (2014). ANGSD: Analysis of next generation sequencing data. *BMC Bioinformatics*, 15(1), 356. <https://doi.org/10.1186/s12859-014-0356-4>
- Lefort, V., Desper, R., & Gascuel, O. (2015). FastME 2.0: A comprehensive, accurate, and fast distance-based phylogeny inference program. *Molecular Biology and Evolution*, 32(10), 2798–2800. <https://doi.org/10.1093/molbev/msv150>
- Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, 25(14), 1754–1760. <https://doi.org/10.1093/bioinformatics/btp324>
- Lopez-Martinez, N. (1980). Les lagomorphs (Mammalia) du pléistocène supérieur de Jaurens. *Nouvelles Archives Du Museum D'histoire Naturelle De Lyon*, 18, 5–16.
- Ma, Y., Ding, X., Qanbari, S., Weigend, S., Zhang, Q., & Simianer, H. (2015). Properties of different selection signature statistics and a new strategy for combining them. *Heredity*, 115(5), 426–436. <https://doi.org/10.1038/hdy.2015.42>
- Malinsky, M., Svardal, H., Tyers, A. M., Miska, E. A., Genner, M. J., Turner, G. F., & Durbin, R. (2018). Whole-genome sequences of Malawi cichlids reveal multiple radiations interconnected by gene flow. *Nature Ecology & Evolution*, 2(12), 1940–1955. <https://doi.org/10.1038/s41559-018-0717-x>
- Marboutin, E., & Peroux, R. (1995). Survival pattern of European hare in a decreasing population. *Journal of Applied Ecology*, 32, 809–816. <https://doi.org/10.2307/2404820>
- Marques, J. P., Boursot, P., Alves, P. C., & Melo-Ferreira, J. (2019). *Genomic resources for L. castroviejoi*, BioSample SAMN12640762. NCBI Sequence Read Archive. <https://www.ncbi.nlm.nih.gov/biosample/SAMN12640762> Deposited 27 August 2019
- Marques, J. P., Seixas, F. A., Farello, L., Callahan, C. M., Good, J. M., Montgomery, W. I., Reid, N., Alves, P. C., Boursot, P., & Melo-Ferreira, J. (2019). *The annotated draft genome of the mountain hare (Lepus timidus)*. NCBI Sequence Read Archive. <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA561582> Deposited 22 August 2019
- Marques, J. P., Seixas, F. A., Farello, L., Callahan, C. M., Good, J. M., Montgomery, W. I., Reid, N., Alves, P. C., Boursot, P., & Melo-Ferreira, J. (2020a). An annotated draft genome of the mountain hare (*Lepus timidus*). *Genome Biology and Evolution*, 12(1), 3656–3662. <https://doi.org/10.1093/gbe/evz273>
- Marques, J. P., Seixas, F. A., Farello, L., Callahan, C. M., Good, J. M., Montgomery, W. I., Reid, N., Alves, P. C., Boursot, P., & Melo-Ferreira, J. (2020b). Data from: An annotated draft genome of the mountain hare (*Lepus timidus*). *Dryad*. <https://doi.org/10.5061/dryad.j0zpc86bk>. Deposited 28 October
- Martinkova, N., McDonald, R. A., & Searle, J. B. (2007). Stoats (*Mustela erminea*) provide evidence of natural overland colonization of Ireland. *Proceedings of the Royal Society B: Biological Sciences*, 274(1616), 1387–1393. <https://doi.org/10.1098/rspb.2007.0334>
- Matiu, M., Crespi, A., Bertoldi, G., Carmagnola, C. M., Marty, C., Morin, S., Schöner, W., Cat Berro, D., Chiogna, G., De Gregorio, L., Kotlarski, S., Majone, B., Resch, G., Terzago, S., Valt, M., Beozzo, W., Cianfarra, P., Gouttevin, I., Marcolini, G., ... Weilguni, V. (2021). Observed snow depth trends in the European Alps: 1971 to 2019. *The Cryosphere*, 15(3), 1343–1382. <https://doi.org/10.5194/tc-15-1343-2021>
- McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernysky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., & DePristo, M. A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*, 20(9), 1297–1303. <https://doi.org/10.1101/gr.107524.110>
- Melo-Ferreira, J., Alves, P. C., Freitas, H., Ferrand, N., & Boursot, P. (2009). The genomic legacy from the extinct *Lepus timidus* to the three hare species of Iberia: contrast between mtDNA, sex chromosomes and autosomes. *Molecular Ecology*, 18(12), 2643–2658. <https://doi.org/10.1111/j.1365-294X.2009.04221.x>
- Melo-Ferreira, J., Boursot, P., Carneiro, M., Esteves, P. J., Farello, L., & Alves, P. C. (2012). Recurrent Introgression of Mitochondrial DNA Among Hares (*Lepus* spp.) revealed by Species-tree Inference and Coalescent Simulations. *Systematic Biology*, 61(3), 367–381. <https://doi.org/10.1093/sysbio/syr114>
- Melo-Ferreira, J., Boursot, P., Randi, E., Kryukov, A., Suchentrunk, F., Ferrand, N., & Alves, P. C. (2007). The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. *Molecular Ecology*, 16(3), 605–618. <https://doi.org/10.1111/j.1365-294X.2006.03166.x>
- Melo-Ferreira, J., Seixas, F. A., Cheng, E., Mills, L. S., & Alves, P. C. (2014). The hidden history of the snowshoe hare, *Lepus americanus*: extensive mitochondrial DNA introgression inferred from multilocus genetic variation. *Molecular Ecology*, 23(18), 4617–4630. <https://doi.org/10.1111/mec.12886>
- Mills, L. S., Bragina, E. V., Kumar, A. V., Zimova, M., Lafferty, D. J. R., Feltner, J., Davis, B. M., Hackländer, K., Alves, P. C., Good, J. M., Melo-Ferreira, J., Dietz, A., Abramov, A. V., Lopatina, N., & Fay, K. (2018). Winter color polymorphisms identify global hot spots for evolutionary rescue from climate change. *Science*, 359(6379), 1033–1036. <https://doi.org/10.1126/science.aan8097>
- Miranda, I., Giska, I., Farello, L., Pimenta, J., Zimova, M., Bryk, J., & Melo-Ferreira, J. (2021). Museomics dissects the genetic basis for adaptive seasonal colouration in the least weasel. *Molecular Biology and Evolution*, 38(10), 4388–4402. <https://doi.org/10.1093/molbev/msab177>
- Mitchell-Jones, A., Amori, G., Bogdanowicz, W., Krystufek, B., Reijnders, P., Spitzenberger, F., & Zima, J. (1999). *Atlas of European Mammals*. Academic Press.
- Montgomery, W. I., Provan, J., McCabe, A. M., & Yalden, D. W. (2014). Origin of British and Irish mammals: disparate post-glacial colonisation and species introductions. *Quaternary Science Reviews*, 98, 144–165. <https://doi.org/10.1016/j.quascirev.2014.05.026>
- Muster, C., & Berendonkschm, T. U. (2006). Divergence and diversity: lessons from an arctic-alpine distribution (ehr group,

- Lycosidae). *Molecular Ecology*, 15(10), 2921–2933. <https://doi.org/10.1111/j.1365-294X.2006.02989.x>
- Nogués-Bravo, D., Rodríguez-Sánchez, F., Orsini, L., de Boer, E., Jansson, R., Morlon, H., Fordham, D. A., & Jackson, S. T. (2018). Cracking the Code of Biodiversity Responses to Past Climate Change. *Trends in Ecology & Evolution*, 33(10), 765–776. <https://doi.org/10.1016/j.tree.2018.07.005>
- Olazuaga, L., Loiseau, A., Parrinello, H., Paris, M., Frainout, A., Guedot, C., Diepenbrock, L. M., Kenis, M., Zhang, J., Chen, X., Borowiec, N., Facon, B., Vogt, H., Price, D. K., Vogel, H., Prud'homme, B., Estoup, A., & Gautier, M. (2020). A Whole-Genome Scan for Association with Invasion Success in the Fruit Fly *Drosophila suzukii* Using Contrasts of Allele Frequencies Corrected for Population Structure. *Molecular Biology and Evolution*, 37(8), 2369–2385. <https://doi.org/10.1093/molbev/msaa098>
- Pavlidis, P., Zivkovic, D., Stamatakis, A., & Alachiotis, N. (2013). SweeD: likelihood-based detection of selective sweeps in thousands of genomes. *Molecular Biology and Evolution*, 30(9), 2224–2234. <https://doi.org/10.1093/molbev/mst112>
- Peel, M. C., Finlayson, B. L., & McMahon, T. A. (2007). Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences*, 11(5), 1633–1644. <https://doi.org/10.5194/hess-11-1633-2007>
- Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species. *PLoS One*, 7(5), e37135. <https://doi.org/10.1371/journal.pone.0037135>
- Pickrell, J. K., & Pritchard, J. K. (2012). Inference of population splits and mixtures from genome-wide allele frequency data. *PLoS Genetics*, 8(11), e1002967. <https://doi.org/10.1371/journal.pgen.1002967>
- Plummer, M. S., Best, N., Cowles, K., & Vines, K. (2006). CODA: Convergence diagnosis and output analysis for MCMC. *R News*, 6(1), 7–11.
- Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, 67(5), 901–904. <https://doi.org/10.1093/sysbio/syy032>
- Savolainen, O., Lascoux, M., & Merila, J. (2013). Ecological genomics of local adaptation. *Nature Reviews Genetics*, 14(11), 807–820. <https://doi.org/10.1038/nrg3522>
- Schmitt, T. (2017). Molecular Biogeography of the High Mountain Systems of Europe: An Overview. In J. Catalan, J. M. Ninot, & M. M. Aniz (Eds.), *High mountain conservation in a changing world* (pp. 63–74). Springer International Publishing.
- Seixas, F. A. (2017). *Genome admixture with massive mitochondrial DNA introgression in hares (Lepus spp.): the relative roles of demography and natural selection*. PhD. University of Porto and University of Montpellier.
- Seixas, F. A., Boursot, P., & Melo-Ferreira, J. (2017). The genomic impact of historical hybridization with massive mitochondrial DNA introgression in the Iberian hare (*Lepus granatensis*). *NCBI Sequence Read Archive*, Deposited 21 August 2017 <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA399194>
- Seixas, F. A., Boursot, P., & Melo-Ferreira, J. (2018). The genomic impact of historical hybridization with massive mitochondrial DNA introgression. *Genome Biology*, 19(1), 91. <https://doi.org/10.1186/s13059-018-1471-8>
- Seixas, F. A., Boursot, P., & Melo-Ferreira, J. (2019). Data from: The genomic impact of historical hybridization with massive mitochondrial DNA introgression. *Dryad*. <https://doi.org/10.5061/dryad.x95x69pd8>. Deposited 24 October
- Seixas, F. A., Marques, J. P., Boursot, P., Alves, P. C., & Melo-Ferreira, J. (2019a). Genomic resources for *L. europaeus*, BioSample, SAMN12618118. *NCBI Sequence Read Archive*. <https://www.ncbi.nlm.nih.gov/biosample/SAMN12618118> Deposited 21 August 2019
- Seixas, F. A., Marques, J. P., Boursot, P., Alves, P. C., & Melo-Ferreira, J. (2019b). Genomic resources for *L. europaeus*, BioSample, SAMN12618122. *NCBI Sequence Read Archive*. <https://www.ncbi.nlm.nih.gov/biosample/SAMN12618122> Deposited 21 August 2019
- Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J. M., & Birol, I. (2009). ABySS: a parallel assembler for short read sequence data. *Genome Research*, 19(6), 1117–1123. <https://doi.org/10.1101/gr.089532.108>
- Smith, S., Sandoval-Castellanos, E., Lagerholm, V. K., Napierala, H., Sablin, M., Von Seth, J., Fladerer, F. A., Germonpré, M., Wojtal, P., Miller, R., Stewart, J. R., & Dalén, L. (2017). Nonreceding hare lines: genetic continuity since the Late Pleistocene in European mountain hares (*Lepus timidus*). *Biological Journal of the Linnean Society*, 120(4), 891–908. <https://doi.org/10.1093/biolinnean/blw009>
- Soraggi, S., Wiuf, C., & Albrechtsen, A. (2018). Powerful Inference with the D-Statistic on Low-Coverage Whole-Genome Data. *G3 Genes/genomes/genetics*, 8(2), 551–566. <https://doi.org/10.1534/g3.117.300192>
- Stamatakis, A. (2015). Using RAxML to Infer Phylogenies. *Current Protocols in Bioinformatics* 51(1), 11–16. <https://doi.org/10.1002/0471250953.bi0614s51>
- Stewart, J. R., Lister, A. M., Barnes, I., & Dalen, L. (2010). Refugia revisited: individualistic responses of species in space and time. *Proceedings of the Royal Society B: Biological Sciences*, 277(1682), 661–671. <https://doi.org/10.1098/rspb.2009.1272>
- Teixeira, J. C., & Huber, C. D. (2021). The inflated significance of neutral genetic diversity in conservation genetics. *Proceedings of the National Academy of Sciences USA*, 118(10), e2015096118. <https://doi.org/10.1073/pnas.2015096118>
- Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22), 4673–4680. <https://doi.org/10.1093/nar/22.22.4673>
- Vaughan, T. G., Kühnert, D., Popinga, A., Welch, D., & Drummond, A. J. (2014). Efficient Bayesian inference under the structured coalescent. *Bioinformatics*, 30(16), 2272–2279. <https://doi.org/10.1093/bioinformatics/btu201>
- Verity, R., Collins, C., Card, D. C., Schaal, S. M., Wang, L., & Lotterhos, K. E. (2017). minotaur: A platform for the analysis and visualization of multivariate results from genome scans with R Shiny. *Molecular Ecology Resources*, 17(1), 33–43. <https://doi.org/10.1111/1755-0998.12579>
- Vieira, F. G., Lassalle, F., Korneliussen, T. S., & Fumagalli, M. (2016). Improving the estimation of genetic distances from Next-Generation Sequencing data. *Biological Journal of the Linnean Society*, 117(1), 139–149. <https://doi.org/10.1111/bij.12511>
- Xu, Z., You, W., Zhou, Y., Chen, W., Wang, Y., & Shan, T. (2019). Cold-induced lipid dynamics and transcriptional programs in white adipose tissue. *BMC Biology*, 17(1), 74. <https://doi.org/10.1186/s12915-019-0693-x>
- Yi, X., Liang, Y. U., Huerta-Sanchez, E., Jin, X., Cuo, Z. X. P., Pool, J. E., Xu, X., Jiang, H., Vinckenbosch, N., Korneliussen, T. S., Zheng, H., Liu, T., He, W., Li, K., Luo, R., Nie, X., Wu, H., Zhao, M., Cao, H., ... Wang, J. (2010). Sequencing of 50 Human Exomes Reveals Adaptation to High Altitude. *Science*, 329(5987), 75. <https://doi.org/10.1126/science.1190371>
- Zimova, M., Hackländer, K., Good, J. M., Melo-Ferreira, J., Alves, P. C., & Mills, L. S. (2018). Function and underlying mechanisms of seasonal colour moulting in mammals and birds: what keeps them changing



in a warming world? *Biological Reviews*, 93, 1478–1498. <https://doi.org/10.1111/brv.12405>

Zimova, M., Mills, L. S., & Nowak, J. J. (2016). High fitness costs of climate change-induced camouflage mismatch. *Ecology Letters*, 19(3), 299–307. <https://doi.org/10.1111/ele.12568>

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