

# Non-receding hare lines: genetic continuity since the Late Pleistocene in European mountain hares (*Lepus timidus*)

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1 **ABSTRACT**

2 Throughout time, climate changes have caused substantial rearrangements of habitats which  
3 have alternately promoted and disfavoured different types of taxa. At first glance, the  
4 mountain hare (*Lepus timidus*) shows the typical hallmarks of a cold-adapted species that has  
5 retreated to refugia since the onset of the current Holocene interglacial. In contrary to  
6 expectations, however, the species has a high contemporary genetic diversity with no clear  
7 differentiation between geographically isolated populations. In order to clarify the  
8 phylogeographic history of European mountain hares, we here analysed ancient DNA from  
9 the glacial populations that inhabited the previous midlatitude European tundra region. Our  
10 results reveal that the Ice Age hares had similar levels of genetic variation and lack of  
11 geographic structure as observed today, and the ancient samples were intermingled with  
12 modern individuals throughout the reconstructed evolutionary tree. This suggest a temporal  
13 genetic continuity in Europe, where the mountain hares were able to keep pace with the rapid  
14 changes at the last glacial/interglacial transition, and successfully track their shifting habitat to  
15 northern and alpine regions. Further, the temporal demographic analyses showed that the  
16 species' population size in Europe appear to have been tightly linked with palaeoclimatic  
17 fluctuations, with increases and declines occurring during periods of global cooling and  
18 warming, respectively. Taken together, our results suggest that neither habitat shifts nor  
19 demographic fluctuations have had any substantial impact on the genetic diversity of  
20 European mountain hares. This remarkable resilience, which contrasts to a majority of  
21 previously investigated cold-adapted species, is likely due to its generalist nature which  
22 makes it less vulnerable to environmental changes.

23

24 **Keywords:** Ancient DNA; approximate Bayesian computation; coalescent simulations;  
25 glacial cycles; climate change; phylogeography; range shift

26

## 27 INTRODUCTION

### 28 *Background*

29 Many species currently inhabiting temperate as well as arctic environments are likely to have  
30 a demographic history that includes periods of stability, followed by contraction into suitable  
31 habitat during unfavourable environmental conditions. The timing of such retreats into refugia  
32 and the subsequent mode of expansion has important implications for species' evolutionary  
33 trajectories. These trajectories depend a great deal on the life histories of the various taxa and  
34 particularly to what extent they are adapted to cold or temperate environments (Stewart *et al.*,  
35 2010).

36 Cold-adapted species have generally been more abundant and widespread during glaciations,  
37 and experienced population bottlenecks and range contractions during interglacials (Muster *et*  
38 *al.*, 2009) such as the current Holocene period. These contractions occurred into polar refugia,  
39 as well as to the so-called cryptic southern refugia like the European Alps and Pyrenees  
40 (Stewart *et al.*, 2010), and therefore many cold-adapted taxa today show disjunct  
41 distributional patterns. Further, past local population extinctions and re-colonisations are  
42 expected to have left corresponding signatures of founder events in previously glaciated areas,  
43 which show genetic associations with the source populations. These source populations  
44 usually inhabited the surrounding lowlands, the tundra of periglacial Europe, or further to the  
45 east, as the European high latitude and altitude habitats were largely covered by ice caps  
46 during the Late Pleistocene glacial period (Fig. 1). One important parameter that has affected  
47 the genetic diversity in cold-adapted species is to what extent the glacial populations that  
48 inhabited non-refugial areas were able to track the decreases in habitat that took place at the  
49 Pleistocene/Holocene transition. A lack of such ability has been seen for true lemmings  
50 (*Lemmus* spp.) and arctic foxes (*Vulpes lagopus*), whose southern populations were unable to  
51 follow their shifting habitat during postglacial warming and became locally extinct (Dalén *et*  
52 *al.*, 2007; Lagerholm *et al.*, 2014). Similarly, extant collared lemmings (*Dicrostonyx*  
53 *torquatus*) only retain a small fraction of the genetic diversity observed in Late Pleistocene  
54 samples, due to a series of local extinctions associated with past periods of climate  
55 fluctuations (Palkopoulou *et al.*, 2016; Prost *et al.*, 2010).

56 Taxa adapted to other environments show different patterns and responses to global climate  
57 fluctuations. Temperate species tend to expand their ranges and population sizes in the  
58 warmer interglacial periods (Hewitt, 1999), and during glacials go through range contractions

59 into southern as well as cryptic northern refugia. The latter are restricted areas with favourable  
60 climate that allow the survival of temperate species at higher latitudes than would be  
61 anticipated (Stewart *et al.*, 2010). Modern temperate species are thus expected to show a  
62 distinct and separate population genetic pattern compared to their cold-adapted neighbours,  
63 with strong signals of postglacial expansion from different southern refugia and decreasing  
64 diversity towards the north (Hewitt, 1996). Examples of temperate species with  
65 phylogeographic signals reflecting traditional expansions from southern refugia are  
66 hedgehogs (*Erinaceus* spp.), water voles (*Arvicola terrestris*) and crested newts (*Triturus*  
67 *cristatus*) (Hewitt, 1999).

68 Although species with similar climatic adaptations are generally expected to have similar  
69 demographic histories, it has become increasingly clear that species within each category (*i.e.*  
70 cold-adapted and temperate) also have responded in an individual manner to changes in  
71 habitat availability associated with past climatic fluctuations (Stewart, 2008). Indeed, the  
72 ecological characteristics of each species likely determine the pace of population expansion,  
73 endurance in refugia, and the degree of any associated genetic differentiation (Stewart *et al.*,  
74 2010). For example, moose (*Alces alces*), grey wolf (*Canis lupus*) and brown bear (*Ursus*  
75 *arctos*) all display relatively large distributions today as well as during the Late Pleistocene.  
76 Consequently, these species do not conform to the typical pattern of range expansions and  
77 contractions in concert with the glacial cycles (Niedziałkowska *et al.*, 2014). Moreover, due  
78 to their wide dispersal capabilities, they have experienced significant levels of gene flow and  
79 population admixture and show relatively constant effective population sizes ( $N_e$ 's) through  
80 time.

81 The mountain hare (*Lepus timidus*) is an interesting case amongst the Late Pleistocene fauna.  
82 The glacial distribution of the species (Fig. 1) seems broadly consistent with the Mammoth  
83 steppe biome (Kahlke, 1999), and mountain hare fossil finds are frequently reported from  
84 non-cultural and Palaeolithic sites across Europe (e.g. Koby, 1960; López-Martínez, 1980;  
85 Napierala, 2008; Wojtal *et al.*, 2012). Today, the European range is restricted to the Alps,  
86 Ireland and Scotland, as well as being more continuous throughout Fennoscandia and north-  
87 eastern Europe (Fig. 1). Mountain hares from the Irish population have also in recent times  
88 been introduced to some Scottish islands (Angerbjörn & Flux, 1995). From the fossil remains  
89 and its modern distribution, the mountain hare consequently shows the hallmarks of a cold-  
90 adapted species that retreats to northern and alpine refugia during interglacials. This disjunct  
91 Holocene distribution might be expected to create genetic differentiation between recently

92 isolated populations, as has been observed in other cold-adapted species that inhabit similar  
93 regions in Europe today, such as the rock ptarmigan, *Lagopus muta* (Sahlman, Segelbacher &  
94 Höglund, 2009). However, the phylogeographic relationships between modern-day mountain  
95 hare populations are very confusing. There is little geographical structuring among genetic  
96 lineages, and the overall and regional sequence diversity is high (Melo-Ferreira *et al.*, 2007).  
97 It is difficult to deduce whether this pattern is shaped by contemporary gene flow or is a  
98 consequence of shared ancestral polymorphisms among postglacial colonisers. Hamill *et al.*  
99 (2006) and Melo-Ferreira *et al.* (2007) favour the latter scenario, in which the diversity in  
100 modern arctic and alpine populations is explained by recent colonisation from a large and  
101 continuous glacial mountain hare population. Based on Fu's *F* statistics and mismatch  
102 distributions, Melo-Ferreira *et al.* (2007) also estimate that this ancestral population increased  
103 substantially in size during the cold climate of an earlier glacial period (ca 164,000 years BP).  
104 On the other hand, Walteri & Cook (2005) and Pierpaoli *et al.* (1999) hypothesise that the  
105 lack of modern phylogeographic structure may reflect ongoing high levels of gene flow  
106 throughout Eurasia, but also acknowledge that it may be a result of incomplete lineage  
107 sorting. Further, Pierpaoli *et al.* (1999) dates the ancient population expansion of mountain  
108 hares to the late Middle Pleistocene, just before start of the last warm interglacial period,  
109 some 135,000 years BP. The time of the first fossil appearance of *L. timidus* is much earlier,  
110 at least before 0.4 Myrs (Fostowicz-Frelik & Gasparik, 2006). It is thus plausible that the  
111 species evolved from an Early Pleistocene ancestor, which was adapted to the spreading of  
112 steppe biota triggered by the onset of the Ice age. The competing views by molecular  
113 biologists on the extant species consequently fuel an ongoing debate over the preferred  
114 ecological niche of the mountain hare (Hamill *et al.*, 2006). The division of species into  
115 categories that conform to the traditional expansion/contraction model would suggest that *L.*  
116 *timidus* is a cold-adapted species that is now confined to polar and cryptic southern refugia.  
117 Populations thus remain fragmented with limited dispersal across temperate zones (Hamill *et*  
118 *al.*, 2006). The question remains, however, why the phylogeographic pattern does not indicate  
119 a recent population decline if the species has recently shifted its range into isolated refugia  
120 which were previously glaciated during the Pleistocene. One possible explanation for this  
121 pattern could be that the species managed to quickly and efficiently track suitable habitat as  
122 the climate warmed, thus preserving much of the genetic diversity despite some reduction in  
123 population size. On the other hand, the argument of high contemporary gene flow supposes  
124 that mountain hares have the general phylogeographic history of temperate species, and thus  
125 have expanded into arctic and alpine regions following postglacial climate warming, while also

126 maintaining connectivity with continental populations at lower latitudes and to the east. This  
127 view, however, cannot explain the high diversity observed at the northern extent of the  
128 species' range, which for temperate species should be lower than that at the core of the range  
129 due to genetic founder effects (e.g. Hewitt, 1996). A final alternative may be that a decline in  
130 genetic variation and  $N_e$  did occur but has not been detected, because extinct Pleistocene  
131 lineages cannot be directly observed in modern sequence datasets (Brace *et al.*, 2012).

132 These conflicting interpretations of the mountain hare's phylogeographic history are unlikely  
133 to be reconciled with modern genetic data alone, due to the similar patterns produced by  
134 contemporary gene flow and incomplete lineage sorting. Further, the complex climatic history  
135 of the Pleistocene period gives good reasons to expect that modern arctic/alpine species'  
136 distributions are the result of multiple and varied phases of population expansions and  
137 contractions. Empirical data to resolve biogeographical histories of these taxa, however,  
138 remain very scarce (Muster & Berendonk, 2006; Muster *et al.*, 2009). One solution is to  
139 investigate genetic variation in ancient samples from the final stages of the Late Pleistocene  
140 glaciation. A comparison of past and present diversity makes it possible to detect series of  
141 historical local extinctions and recolonization events (Brace *et al.*, 2012) that possibly have  
142 been associated with the Pleistocene/Holocene transition. This approach is thus of great value  
143 for understanding how habitat and climate changes have differentially affected the  
144 evolutionary history of temperate and cold-adapted species (Hofreiter & Barnes, 2010). More  
145 specifically, ancient sequence data can help resolve whether mountain hare haplotypes have  
146 disappeared in certain areas and been replaced by those carried by recent colonisers, or if the  
147 weak phylogeographic structure observed in modern hares was a feature also of ancient  
148 populations. Further, temporally sampled data makes it possible to infer the demographic  
149 history and to resolve the trajectory of post-glacial expansions or contractions.

150 The aim of this study is to use subfossil remains of mountain hares dating back to the Late  
151 Pleistocene from several palaeontological sites in mainland Europe, to clarify the  
152 phylogeographic history of the species. We use mitochondrial DNA sequencing of the control  
153 region (CR) and Cytochrome B gene (CytB) together with Bayesian phylogenetic analyses  
154 and approximate Bayesian computation (ABC) to examine the historical distribution of  
155 genetic diversity and to determine whether, and if so in what direction, the effective  
156 population size has changed since the Pleistocene/Holocene transition.

157

158 **MATERIAL AND METHODS**

159 *Sampling*

160 We collected ancient mountain hare bones from 45 individuals sampled from 20  
161 palaeontological sites across Europe (Table S1), ranging in age from approximately 44  
162 thousand (k) calendar years before present (BP) to 10 k BP. Samples were selected from  
163 different natural history collections to cover the extent of the modern-day European range of  
164 the mountain hare and also to provide a reasonable representation of the range during the Late  
165 Pleistocene period (see Fig. 1). Additionally, we compiled sequence data from Melo-Ferreira  
166 *et al.* (2007) of portions of the mtDNA control region and Cytochrome B gene from 124  
167 modern mountain hares, originating from 10 different Eurasian countries.

168

169 *DNA extraction and sequencing*

170 DNA extractions from the ancient samples were conducted in the ancient DNA laboratory at  
171 the Swedish Museum of Natural History, Stockholm, according to Brace *et al.* (2012).  
172 Briefly, 50 mg of bone powder was incubated in an EDTA buffer with urea and proteinase K  
173 for 24 hours at 55 °C. The supernatants were then concentrated using Vivaspin filters before  
174 mixing with PB buffer (Qiagen, Germany) and application to Qiaquick spin columns. Spin  
175 columns were washed with PE buffer and then eluted with EB buffer to a final volume of 100  
176 µl.

177 In order to cover the equivalent length fragments of Cytochrome B and control region as in  
178 Melo-Ferreira *et al.* (2007), we designed primers for five overlapping segments of each  
179 mtDNA region (see Table S2). PCRs were performed in 25 µl volumes containing 1 × PCR  
180 buffer, 0.2 µM of each primer, 200 µM dNTPs, 2.5 mM MgCl<sub>2</sub>, 0.1 mg/mL BSA, 0.4 Units  
181 HotStar Taq DNA polymerase (Qiagen, Germany), purified water, and 2 µL of DNA extract.  
182 PCR conditions were 10 min at 95 °C, followed by 55 cycles of 30 s at 94 °C, 30 s at 50 °C or  
183 51 °C (dependent on primer pair specifications), 30 s at 72 °C, and a final extension of 7 min  
184 at 72 °C.

185 Amplicons were purified using Exonuclease I and Shrimp Alkaline Phosphatase and  
186 sequenced on an ABI3130XL at the Konrad Lorenz Institute for Ethology, Vienna.  
187 Sequencing chromatograms were assembled and analysed using Geneious 5.5.4. Throughout  
188 our procedures, protocols to prevent contamination and ensure accurately coded (undamaged)

189 mtDNA were followed, including isolation of work areas, negative controls, reduced fragment  
190 length amplification, and repeated PCRs and sequencing of all samples and sub-amplicons.

191

### 192 *Sequence analysis and network construction*

193 Ancient DNA sequence data were aligned with modern sequences from Melo-Ferreira *et al.*  
194 (2007), and grouped into 5 geographic regions (see Table S3). Three datasets were  
195 constructed; one for cytochrome B (CytB), one for control region (CR), and one consisting of  
196 concatenated CytB and CR sequences. Arlequin v.3.5.2.2 (Excoffier & Lischer, 2010) was  
197 used to calculate standard diversity estimates for the two gene regions, during both the present  
198 and the Late Pleistocene period. Further, temporal statistical parsimony networks were  
199 created with the R-script TempNet v1.4 (Prost & Anderson, 2011) to display the relationships  
200 between all haplotypes found in the two time periods. This was done for the concatenated  
201 CytB+CR dataset, as well as for each mitochondrial region separately.

202

### 203 *Phylogenetic and demographic analyses*

204 We used BEAST v1.8.0 (Drummond *et al.*, 2012) to create a dated phylogeny, based on the  
205 concatenated dataset. The mean of the approximate ages of the Late Pleistocene samples were  
206 used as tip dates, and a strict molecular clock of 7.7 % substitutions per million years (Myr<sup>-1</sup>)  
207 was employed, following the rates used in (Cheng *et al.*, 2014) and the proportion of the two  
208 mitochondrial regions in our dataset. The mutation rate is a sensitive parameter that could bias  
209 demographic analyses if accurate estimates are not employed. In addition, the so called  
210 temporal dependency of the mutation rate, a phenomenon whereby substitution rates are faster  
211 on shorter time scales, can be a source of uncertainty (Ho, 2014). For that reason, we assessed  
212 the robustness of our conclusions by replicating the entire set of analyses with mutation rates  
213 of 15.8 % (following Melo-Ferreira *et al.*, 2007) and 50 % Myr<sup>-1</sup>. We also performed parallel  
214 analyses with both the complete Eurasian dataset, as well as using European sequences only  
215 (*i.e.* the geographic extent of the ancient sampling). The substitution model was set to  
216 HKY+I+G, as recommended by the AIC and hLRT criterion in MrModeltest v2.3 (Nylander,  
217 2004), and a Coalescent Constant size tree prior was used together with a randomly generated  
218 starting tree. The length of the Markov chain Monte Carlo (MCMC) was set to 50 million  
219 generations, with parameters logged to file every 1,000 generations. Two independent runs

220 were made on the same input file and the results were checked in Tracer v1.4.0 (Rambaut &  
221 Drummond, 2007) to ensure effective sampling and convergence on the same posterior  
222 distributions. The trees from two runs were then combined in LogCombiner v1.8.0  
223 (Drummond *et al.*, 2012) and annotated to a maximum clade credibility tree in TreeAnnotator  
224 v1.8.0 (Drummond *et al.*, 2012), using a burnin of 10% and a posterior probability limit of  
225 0.5. Finally, FigTree v1.4.0 (Rambaut, 2012) was used to graphically edit the output.

226 BEAST v1.8.0 (Drummond *et al.*, 2012) was also employed to analyse demographic histories  
227 using the Coalescent Bayesian Skyline approach (Drummond *et al.*, 2005). The analysis was  
228 made both for Eurasia and Europe (again with the concatenated dataset), with a piecewise-  
229 constant Skyline model, 10 groups and a random starting tree, and run for 70 million  
230 generations. The tip dates, clock rates and evolutionary model were the same as in the  
231 previous phylogenetic reconstruction. Log and tree files from 2 replicate runs were combined  
232 in LogCombiner v1.8.0 (Drummond *et al.*, 2012) and then used as input to Tracer v1.4.0  
233 (Rambaut & Drummond, 2007) in which Skyline plots were created.

234

### 235 *Approximate Bayesian computation*

236 We employed approximate Bayesian computation (ABC) to infer the model of demographic  
237 history that best fitted the temporal pattern of genetic diversity in Eurasian mountain hares.  
238 Inferring a demographic history by ABC is usually performed following a model choice  
239 approach, in which a collection of feasible models are simulated and compared via Bayes  
240 factors, which are ratios of their estimated Bayesian likelihoods. Although such an approach  
241 has been successfully applied in numerous studies, concerns have been raised that it is highly  
242 subjective when applied to complex models (Templeton, 2010). Moreover, the hypotheses  
243 regarding the demographic history of *L. timidus* are diverse and debated. For those reasons,  
244 we conducted an analysis that bypassed the problem of defining relevant models. It consisted  
245 of sequentially performing ABC with simulations that were constrained according to the  
246 posterior sample of the previous run, until a demographic history with a recognizable shape  
247 was obtained. This procedure was operated in a “let the data speak” fashion that reduced  
248 subjective choices because the posteriors obtained by ABC are conditioned exclusively to the  
249 observed data, and the initial run itself started from completely random demographic  
250 histories. Afterwards, additional model choice analyses were performed to test simple models  
251 pre-defined on both the “random histories” and on the results obtained with the Coalescent  
252 Bayesian Skyline approach.

253 Our ABC procedure employed coalescent simulations with the concatenated CytB+CR  
254 dataset as the observed data. Following a dedicated analysis, we selected a working set of 15  
255 summary statistics that typically are sensitive to demographic change, including three  
256 multivariate statistics (mismatch distributions, Table S4). The simulation of “random  
257 histories” was carried out by randomly sampling: the number of demographic changes  
258 (exponential prior,  $\lambda=1.0$ ); the time to those changes (uniform prior in 0-200 k BP); and the  
259  $N_e$  values from each time period (exponential prior,  $\lambda=100,000$ ). Exponential priors were  
260 employed in the initial round because they sample uniformly in logarithmic scales, which is  
261 optimal for screening large parametric spaces, but these were later substituted by uniform  
262 priors or fixed values. Changes in population size were sudden, and the populations remained  
263 constant between changes. The sequential process included five ABC analyses with 20  
264 million simulations each, retaining 0.005% of the simulations for further analysis. The  
265 performance of our sequential procedure was assessed by posterior predictive checks, in  
266 which the observed summary statistics were compared with their posterior predictive  
267 distributions (Csillery *et al.*, 2010).

268 We then performed three separate model-choice analyses henceforth called “old-change test”,  
269 “growth test”, and “all-models test”; the first two were intended to obtain simple tendencies  
270 while the last one aimed to infer the historical demography with improved resolution. They  
271 included models that were suggested by the “random histories” and the Bayesian Skyline  
272 analyses, as well as alternative models that were based on expectations from previous  
273 research or associations with climatic records (Fig. 2). The “old-change test” compared three  
274 models of a single, sudden demographic change (increase, decrease and constant) occurring  
275 70-140 k BP. The “growth test” compared recent scenarios of positive versus negative  
276 exponential growth (uniformly sampled in -0.005 to 0.005) around the last glacial/interglacial  
277 transition, starting at 9-15 k BP and continuing until the present. The “all-models test”  
278 included five models differing in two recent demographic changes, one occurring at 9-15 k  
279 BP and the other occurring between the first one and 40 k BP. Also, the “all-models test”  
280 included a palaeoclimate records model (PCRM) and its inverse version (PCRM<sup>-1</sup>) in which  
281  $N_e$  had a perfect correlation (positive for PCRM and negative for PCRM<sup>-1</sup>) with past  
282 temperature records, averaged at stadial/interstadial level. Palaeoclimate records were taken  
283 from the Eemian interglacial, ca 130 k BP, to the present following Blockley *et al.* (2014).  $N_e$   
284 values were sampled uniformly in the interval 10,000-2,000,000. The mean ages of the  
285 ancient samples were simulated from normal distributions to account for uncertainties in  
286 stratigraphic inferences. We employed a generation time of two years and mutation rates of

287 7.7, 15.8 and 50% Myr<sup>-1</sup>, as in the phylogenetic analysis, but focused on the first two after  
288 obtaining poor fitting with 50% Myr<sup>-1</sup>. Model likelihoods were estimated with three methods:  
289 *i*) a direct approach; *ii*) a weighted method in which the model likelihoods were adjusted by  
290 the Epanechnikov kernel evaluated on the distances to the observed data, as implemented in  
291 the software BaySICS (Sandoval-Castellanos, Palkopoulou & Dalén, 2014); and *iii*) a logistic  
292 regression (Fagundes *et al.*, 2007). One million simulations were run for each model and 20  
293 different acceptance proportions were employed to assess consistency of the estimates  
294 (0.0025% to 0.05%).

295 We performed additional analyses to adjust the methodology as well as to test relevant  
296 hypotheses. They included: *i*) pilot essays for adjusting simulation scenarios, priors, and  
297 summary statistics; *ii*) an analysis for assessing the effect of contemporary genetic  
298 differentiation (initiated during the Holocene) following the geographic structure reported by  
299 Hamill *et al.* (2006); and *iii*) a procedure for assessing the “temporal signal” of our sample,  
300 following Firth *et al.* (2010). In the last analysis, 10,000 temporally random datasets were  
301 used to build null distributions that were employed for testing the significance of our  
302 parameter estimates.

303 Finally, we validated our model choice methodology using pseudo observed datasets (PODs),  
304 which are simulated datasets that are used as real data to obtain rates of correct and incorrect  
305 model choices. One complete PODs analysis included the iteration of 500 model choice  
306 procedures, treating one POD at the time as the real data. As we needed to know the rates of  
307 right/wrong choices for every model, it was necessary to carry out a complete PODs analysis  
308 (with its 500 replicates) with every model that was considered in a specific analysis. Then we  
309 replicated that procedure for the three different model choice analyses (“old-change test”,  
310 “growth test”, and “all-models test”), and again for the entire set of analyses of both the  
311 Eurasian and European datasets, as well as for mutation rates of 7.7 % and 15.8 % Myr<sup>-1</sup>. Due  
312 to the massive amount of computational time required for all those analyses, it was necessary  
313 to reduce the amount of simulations analysed in each run (from 1 million in the main model  
314 choice analyses to 250,000) and to include only 500 PODs per analysis, and to skip the  
315 analyses of the 50% mutation rate since it was a poor fit with the data and was also left out of  
316 the model choice analyses anyway.

317 Simulations were performed in the software BaySICS (Sandoval-Castellanos *et al.*, 2014).  
318 The “random histories” analyses as well as the “temporal signal test” were programed in a  
319 custom software (available on request) that employed the source code of BaySICS, using the

320 platform provided by Microsoft Visual Studio Professional 2011 and Intel Visual Fortran  
321 Composer XE 2013. Minor estimations such as transition/transversion bias and the gamma  
322 parameter were made with the software MEGA 6 (Tamura *et al.*, 2013).

323

## 324 **RESULTS**

### 325 *Sequencing success rate*

326 We successfully obtained consensus sequences from 18 ancient samples (between 10 k and 23  
327 k BP in age) originating from 10 palaeontological sites, for a 266 bp part of the mitochondrial  
328 cytochrome B (CytB) region. For the control region (CR), 226 bp was obtained from 17  
329 ancient samples (covering the same temporal span as above), originating from 9  
330 palaeontological sites. Further, we built a concatenated 542 bp dataset for the 17 individuals  
331 that produced sequences from both gene regions, containing 316 bp CytB (*i.e.* an additional  
332 49 bp to the strictly CytB dataset) and 226 bp CR (see Table S3 for an overview). All datasets  
333 were aligned with modern Eurasian sequences from Melo-Ferreira *et al.* (2007).

334 The most southern ancient sample from Loutra Almopias Cave in northern Greece did not  
335 provide DNA for all sub-amplicons, and could therefore not be included in the phylogenetic  
336 analysis. It did however produce enough sequence data (424 bp in total) to be confirmed as *L.*  
337 *timidus* from GenBank Blast searches, indicating that the mountain hare may have had a more  
338 southern Late Pleistocene range limit than previously thought. Conversely, one bone sample  
339 from the Belgian site Caverne Marie-Jeanne (ca 44 k BP in age) was genetically determined  
340 as *L. europaeus*. While such a northern glacial distribution may seem surprising at first  
341 glance, we propose that this could be explained by a northwards expansion of brown hares  
342 during one of the warm interstadials during Marine Isotope Stage 3.

343

### 344 *mtDNA diversity and population structure*

345 Within the entire mountain hare dataset (ancient and modern combined), there were 93  
346 control region and 27 CytB haplotypes. In the control region for modern samples, gene ( $H$ )  
347 and nucleotide ( $\pi$ ) diversities were 0.988 ( $\pm 0.003$ ) and 0.075 ( $\pm 0.037$ ) respectively. For the  
348 ancient samples, gene and nucleotide diversities were slightly lower ( $H = 0.956 \pm 0.044$ ;  $\pi =$   
349  $0.052 \pm 0.028$ ). For CytB, the diversity in the modern samples ( $H = 0.786 \pm 0.033$ ;  $\pi = 0.0067$

350  $\pm 0.004$ ) was slightly lower than in the ancient samples ( $H = 0.889 \pm 0.053$ ;  $\pi = 0.010 \pm$   
351  $0.006$ ). It could be noted that within the ancient data set, there were no general tendencies for  
352 changed diversity over time, with  $\sim 20$  k BP old samples having slightly higher CR diversity  
353 but lower CytB diversity than samples with an age of around 10 k BP.

354 The temporal haplotype network for the modern and ancient CytB sequences is shown in Fig.  
355 3. Three haplotypes are shared among the time periods, and the network pattern is similar in  
356 the modern and ancient datasets: We find a common main clade with star-shaped topology  
357 and a secondary clade connected to the main one only three base pairs distant, but there is no  
358 clear geographic structure. There are a number of missing haplotypes in the ancient network  
359 that could be interpreted either as historically absent or as a result of reduced sample numbers  
360 and sampling sites for this dataset. The overall pattern for this gene region, however, has not  
361 changed markedly since the final stages of the Late Pleistocene. In the control region network  
362 (Fig. 3), there are no shared haplotypes between the two time periods and the pattern reflects  
363 high levels of diversity and historic admixture. The ancient haplotypes, although different  
364 from the modern ones, still appear within and in proximity to the contemporary types.

365

#### 366 *Phylogenetic and demographic analyses*

367 The Bayesian phylogeny constructed from the concatenated CytB and CR dataset (Fig. 4)  
368 further illustrates the lack of clear geographic structure among the analysed Eurasian  
369 mountain hares. Also, the Late Pleistocene individuals are generally not positioned in close  
370 proximity to the modern hares from the same region. A Bayesian skyline plot (BSP) was  
371 initially made on European individuals only, in line with the software assumption of a single  
372 population over time (Drummond *et al.*, 2005). However, since the phylogeny indicated a  
373 general lack of phylogeographic structure across Eurasia (see Fig. 4), we also performed the  
374 BSP on the complete Eurasian dataset. The results for the two geographic regions were very  
375 similar, revealing a fairly constant population size up until ca 150-100 k BP when the  
376 numbers increased (ca 4,5 and 2,5 fold, respectively, in Eurasia and Europe). Then, following  
377 a longer period of stability, the populations experience a rapid decline after ca 25 k BP of less  
378 than 8 fold (Fig. 5, Eurasia and Fig S1, Europe).

379

#### 380 *Approximate Bayesian computation*

381 *i) Random demographic histories*

382 Five sequential rounds of ABC analyses were necessary to obtain a final posterior sample of  
383 random histories with a recognizable shape for all mutation rates and both geographic datasets  
384 (Figs. S2-S6). Attempts to further refine the obtained shape failed as disparate histories  
385 obtained similar support. The demographic histories in the final posterior sample displayed a  
386 population increase occurring at some time between 70 and 140 k BP (supported by >95% of  
387 the posterior simulations), and a set of demographic changes occurring after 40 k BP, where a  
388 bottleneck was the most frequent pattern (supported by >50% of posterior simulations). The  
389 posterior distributions of the parameters from the final analysis yielded estimations of  $N_e$   
390 broadly agreeing with those obtained by the BSP, while the time to the demographic changes  
391 were informative only for the oldest change (Figs. S2-S6). Interestingly, the mode of that  
392 parameter occurred around 110-125 k BP for most combinations of datasets and mutation  
393 rates, which roughly corresponds with the end of the last interglacial (the distribution has a  
394 large variance however). The fit of the simulations to the observed data was very good with  
395 the intervals of 95 % of HPD capturing 100% of the observed values, despite some being well  
396 outside the intervals in the initial rounds. Furthermore, the 95% HPD intervals were up to one  
397 order of magnitude narrower in the final analysis than in the initial run, indicating that our  
398 procedure indeed generated random histories that matched the observed data (Figs. S2-S11).

399

400 *ii) Model choice analyses*

401 The “old change test” was a model choice aimed to assess the support for the demographic  
402 increase observed in the random histories and BSP at 70-140 k BP, when tested against a  
403 model with constant population size and a model with a reduction. The increase received  
404 ample and consistent support across methods, mutation rates, and datasets (Table S5).

405 The “growth test” was a model choice comparing a continuous exponential decline in  $N_e$  with  
406 an exponential growth starting around the onset of the Holocene (9-15 k BP), to support or  
407 contradict the pattern of reduction observed in the BSP. In agreement with the BSP, the  
408 results supported a demographic decline in Europe. However, only the logistic regression  
409 supported this scenario in the Eurasian dataset (Table S5).

410 As the "random histories" failed to provide a clear picture of the demographic history of *L.*  
411 *timidus* after the LGM, and the most common pattern (a bottleneck) contrasted with the pure

412 decline observed in the BSP, we performed a third model choice analysis, “all-models test”,  
413 aimed to infer the post-LGM demographic history at higher resolution. For the European  
414 dataset, this analysis indeed improved our reconstruction of demographic history as it  
415 supported the inverse palaeoclimate records model (PCRM<sup>1</sup>), consistently across methods  
416 and mutation rates (Table S5). However, with the Eurasian dataset the logistic regression  
417 supported the PCRM<sup>1</sup> as the best model (for all mutation rates) while the direct and the  
418 weighted methods supported the bottleneck model. This result is probably not surprising since  
419 the employed palaeoclimate records were modelled only for Europe, and Eurasia has a very  
420 large area with a more heterogeneous climate record.

421 Since the presence of population genetic structure could mimic demographic change (for  
422 instance by promoting deep genealogical branching which also occurs with demographic  
423 growth), we performed a model choice analysis between a model with structure (growth being  
424 flexible) and a model with a panmictic population since the start of Holocene. However, our  
425 model choice with population structure failed to obtain more support than a model without  
426 population structure (Bayes factors = 0.6-1.2), thus rejecting the genetic structure as a factor  
427 affecting our inference of demographic history.

428

### 429 *iii) PODs analyses and temporal signal*

430 The statistical power for the models of the “old-change test” was moderate but rose to high  
431 levels when a Bayes factor of 2.0 was used as a threshold criterion (Table S6). Such high  
432 power is interesting because in those specific models the demographic change is very old and  
433 a reduced power was expected. However, the increased power could be due to two factors: the  
434 large population sizes of mountain hares which stretch the coalescent genealogies and widen  
435 the time window of detectable change; and (2) the ancient samples that gave additional depth  
436 to the genealogies. The statistical power for differentiating a continuous demographic growth  
437 from a decline during the Holocene (“growth test”; Table S6) was in general high for the  
438 growth, but a little lower for the decline, which could explain our lack of consistent support  
439 for the decline in the Eurasian dataset. Finally, the statistical power for detecting changes in  
440 the “all-models test” (Table S6) was very disparate and frequently low, also for the best  
441 supported model (PCRM<sup>1</sup>). The reason seems to be some overlap between the models that  
442 can produce similar scenarios despite being nominally different. The moderate power of the  
443 model choice could therefore be attributed to the limitations of the technique and sampling.

444 However, we believe that our power was substantially improved by assessing the consistency  
445 of the results among different types of tests, mutations rates, datasets, acceptance thresholds,  
446 and methods.

447 In addition, the temporal signal test showed that our parameter estimations were highly  
448 significant in respect to their null distributions, indicating that our temporal sampling  
449 significantly contributed to the inference ( $p < 0.001$ ).

450

## 451 **DISCUSSION**

### 452 *Contrast between hares and other arctic/alpine species*

453 Our analyses of mtDNA sequence data derived from subfossil mountain hare remains, and  
454 comparisons with modern data, can provide answers for many of the questions relating to the  
455 species' phylogeographic history. Despite the very complicated pattern in the historical  
456 haplotype network, we can start to resolve the ambiguity about the driving forces with some  
457 certainty. The sharing of haplotypes between modern regions (at least for CytB) is not a  
458 recent phenomenon, and extensive phases of dispersal from the east appear to have occurred  
459 well before the most recent glaciation. This is a scenario already suggested by Melo-Ferreira  
460 *et al.* (2007) based on modern mtDNA phylogenies, but also by others with the inclusion of  
461 nuclear DNA (Hamill *et al.*, 2006; Suchentrunk *et al.*, 1999). However, the historically high  
462 levels of diversity revealed in our analyses suggest that the mountain hare has maintained  
463 diversity over time unlike many classic arctic/alpine specialists.

464 Both the Bayesian Skyline Plot (BSP) and Approximate Bayesian Computation (ABC)  
465 analyses show clear signals of an expansion that started approximately at the onset of the last  
466 glaciation, which may have continued throughout much of the Late Pleistocene, although the  
467 actual starting time of the population growth has a degree of uncertainty. The BSP then shows  
468 a decline in  $N_e$  after the LGM which could coincide with the start of the Holocene warming  
469 (Fig. 5; Suppl. Fig. S1). The ABC model choice of "growth test" agrees with the post-LGM  
470 decline identified in the BSP, but its support is consistent only with the European dataset.  
471 Further, the results from the "all-models test" and the "random histories" analysis weakly  
472 suggested a "true" bottleneck (reduction and recovery) and the inverse palaeoclimate records  
473 model (PCRM<sup>-1</sup>) received the highest support, at least in Europe (note that the PCRM<sup>-1</sup> has a  
474 bottleneck around the Pleistocene-Holocene transition during the warm Bølling-Allerød

475 interstadial immediately prior to the Younger Dryas cold stadial). It seems reasonable that the  
476 varying levels of support in these analyses is mostly due to a limited statistical power, and  
477 does not rule out demographic dynamics between Europe and Asia as well as the presence of  
478 more complex models than the tested ones. However, we consider it unlikely that the  
479 consistent support for the PCRM<sup>1</sup> is due to a random fit to a fluctuating climate pattern  
480 (which would also support the PCRM), or to a long term noisy pattern (which would also  
481 support the constant size model). Further, the support for the PCRM<sup>1</sup> is not only provided by  
482 the most recent demographic changes, since both the bottleneck and the anti-bottleneck  
483 models failed to obtain much support in the analyses of the European dataset. Combined,  
484 these results support the notion that the PCRM<sup>1</sup> is the most parsimonious explanation for our  
485 data and that a negative correlation between palaeoclimate temperature records and the  
486 demographic history of *L. timidus* is likely real rather than a methodological artefact. This  
487 implies that *L. timidus* populations not only grew and decreased following the glacial-  
488 interglacial cycles (expand during glacials, decline during interglacials), but also followed the  
489 finer scale temperature changes at the stadial-interstadial level.

490 One important question to address is why the BSP only identified demographic changes  
491 associated with the glacial-interglacial cycles, whereas the ABC analyses also revealed much  
492 finer millennial-scale fluctuations. The answer could be that BSP inference includes the use of  
493 a smoothing function that tends to buffer sudden changes provided by short coalescent  
494 intervals, in order to prevent the noisy pattern observed in early versions of skyline plots (Ho  
495 & Shapiro, 2011). But in doing so, it may absorb some of the finer scale recent demographic  
496 changes and therefore fail to identify complicated patterns of  $N_e$  shifts. Thus, sudden  
497 demographic peaks or short bottlenecks, as those predicted by a demographic history linked to  
498 stadial-interstadial cycling, are usually absent from BSPs.

499 Similarly, violation of the assumption of panmixia in BSP estimation combined with an  
500 unbalanced sampling strategy can lead to a false signal of population decline (Heller, Chikhi  
501 & Siegismund, 2013). Our sampling design was by its very nature unbalanced, as we were  
502 reliant upon those few locations where bone fragments were discovered and adequately  
503 preserved to allow retrieval of DNA. Further, despite the complicated pattern of the haplotype  
504 network, it is clear from the current species distribution that gene flow is restricted across the  
505 contemporary range, and has likely been restricted at many points historically for the species.  
506 However, the incorporation of population structure failed to improve support in the model  
507 choice analyses across the Eurasian dataset. Future studies would thus benefit from larger

508 sample sizes as well as analysis of older samples, in order to allow further testing of the  
509 conclusions and hypotheses presented in this paper.

510 In summary, our analyses lend some support for signatures of population contraction and  
511 expansion in and out of interglacial refugia, but also intriguing signals of more complex  
512 changes likely correlated with millennial-scale events of interstadial warming as well as  
513 stadial cooling. However, as apparent in the comparisons of glacial and modern genetic  
514 variation, as well as in the haplotype network, there seems to have been no major losses of  
515 genetic diversity across the Pleistocene/Holocene transition. Consequently, the pattern of  
516 temporal genetic change in the mountain hare does not resemble that seen in other cold-  
517 adapted species such as collared lemmings (Palkopoulou *et al.*, 2016; Prost *et al.*, 2010), true  
518 lemmings (Lagerholm *et al.*, 2014) and arctic foxes (Dalén *et al.*, 2007), which experienced  
519 local extinctions and large declines in genetic diversity following Holocene warming. The  
520 mountain hare instead seems to have been able to keep pace with the rapid changes at the  
521 Pleistocene/Holocene transition, and successfully track their contracting habitat to northern  
522 and alpine regions as their former areas became unsuitable. The classification of the mountain  
523 hare as a typical cold-adapted species under the expansion/contraction model framework is  
524 thus complicated as its generalist nature appears to offer certain resilience to climate effects,  
525 which may allow the mountain hare to buffer sudden demographic impacts and maintain  
526 genetic diversity.

527 There are aspects of the mountain hare's ecology and evolutionary history that have  
528 similarities with those of temperate species. For example, its current distribution covers large  
529 temperate areas in Europe and north-western Asia, similar in some respects to red squirrels  
530 and water voles. It also displays high levels of diversity in the core of its range similar to  
531 these and other rodents but, unlike those species, the mountain hare shows no reduction in  
532 diversity towards the northern margins of its range (Alves *et al.*, 2008; Waltari & Cook,  
533 2005). This feature of its phylogeography seems to place the mountain hare in a separate  
534 category compared to many modern Eurasian mammals in that it can fulfil the roles of both  
535 temperate and cold-adapted species. Over long time scales, the expansion/contraction model  
536 predicts that both cold-adapted and temperate species will have a reduced level of genetic  
537 diversity due to bottlenecks, either during interglacials or glaciations (*i.e.* the effect of  
538 refugia). The mountain hare appears to be an exception to this pattern, and could thus be one  
539 of the few species whose mtDNA diversity is more or less in mutation/drift equilibrium. The  
540 high diversity and lack of structure in the ancient concatenated networks and phylogeny (Fig.

541 3 and 4) suggests large populations that were widely connected throughout the Pleistocene,  
542 including earlier warm interglacials. Indeed, the present-day diversity shows that the  
543 mountain hare maintains similar levels of variation and haplotype sharing across a wide  
544 geographic range during the current interglacial period.

545 To find similar phylogeographic arrangements, one has to look beyond the classic  
546 arctic/alpine and temperate species. A resemblance to the pattern we observe in mountain  
547 hares can be seen in modern habitat generalists such as the red fox and grey wolf. Despite  
548 recent population crashes due to persecution (Jansson *et al.*, 2012) and the proliferation since  
549 the Pleistocene of a previously rare haplogroup (Pilot *et al.*, 2010), grey wolves show a  
550 relatively stable phylogeographic history throughout Europe (Vilà *et al.*, 1999). Similar to the  
551 mountain hare haplotype network, a network constructed from ancient and modern Eurasian  
552 wolf samples shows weak geographic structuring and high levels of diversity (Germonpré *et*  
553 *al.*, 2009). Hunting of wolves has acted to reduce diversity somewhat in modern times, but  
554 their high dispersal ability and generalist habitat requirements have acted to maintain a large  
555 effective population size (Flagstad *et al.*, 2003; Pilot *et al.*, 2010). Red foxes also show little  
556 genetic structuring in both ancient and modern populations and a relatively stable effective  
557 population size since the Pleistocene (Teacher, Thomas & Barnes, 2011). The reason for this  
558 pattern is also hypothesised to relate to the high dispersal ability and adaptability of red foxes  
559 which has allowed them to thrive throughout climate oscillations and in diverse habitat zones.  
560 These ancient DNA studies have indicated that dispersal and the ability of species to adapt  
561 quickly are important features in shaping evolutionary histories, which together with  
562 interspecific interactions create the observed phylogeographic patterns.

563 A further aspect to consider when studying phylogeography is the dynamics of co-existing  
564 species. Predation and inter-specific competition are likely to have played significant roles in  
565 shaping the realised niche of the mountain hare. In particular, interactions with the European  
566 brown hare (*Lepus europaeus*) have strongly influenced the distribution of modern mountain  
567 hare populations in Europe (Alves *et al.*, 2006; Kasapidis *et al.*, 2005). The brown hare is  
568 common throughout western and central Europe whereas the mountain hare is found in  
569 northern and north-eastern Europe, as well as in the Alps and British Isles (see Fig. 1). There  
570 is a range overlap in Finland, Sweden and Eastern Europe, where recent expansions of the  
571 brown hare are replacing resident populations of mountain hares (Thulin & Tegelstrom,  
572 2001). Historically, *L. timidus* populations in the Iberian Peninsula were also displaced by  
573 brown hares and their close relatives *L. granatensis* and *L. castroviejo* (Melo-Ferreira *et al.*,

574 2007). Our data showing a historical continuity of high gene flow and genetic diversity,  
575 combined with the modern distribution of the mountain hare relative to the brown hare,  
576 indicates that climate is not the only factor influencing the more recent phylogeographic  
577 pattern of *L. timidus*. Instead, an additional, yet related impact is likely to be competitive  
578 exclusion by brown hares during the Holocene.

579 Although the close historical contact between *Lepus* species in the Iberian Peninsula is still  
580 evident from the modern-day persistence of introgressed *L. timidus* mtDNA in their relatives  
581 (Alves *et al.*, 2008; Melo-Ferreira *et al.*, 2005), results from northern Europe show that  
582 acquired mountain hare mtDNA subsequently disappears from brown hare populations when  
583 there is no continuous interspecific gene flow (Thulin & Tegelström, 2002). The bones  
584 collected for this study were morphologically identified as *L. timidus*, and since the mountain  
585 hare and the brown hare mainly had allopatric European distributions during the Late  
586 Pleistocene period (Averianov, 2001) we find it highly unlikely that some of our analysed  
587 ancient sequences are derived from hybrids with introgressed mountain hare mtDNA. Also,  
588 the high number of haplotypes detected in our dataset means that a potential inclusion of an  
589 admixed individual would have little influence on the overall results of our analyses.

590 Despite some recent population declines (Jennings *et al.*, 2006; Marboutin *et al.*, 2003),  
591 brown hares have thrived in landscapes modified for agriculture (Tapper & Barnes, 1986).  
592 They have better body condition and reduced mortality in high intensity arable landscape  
593 types, compared to marginal upland and pastoral areas (Smith, Vaughan Jennings & Harris,  
594 2005). Farmland is the primary habitat for brown hares, and as the extent of farmlands has  
595 increased with human expansion, so too has the range and abundance of the brown hare. The  
596 corresponding retreat of the mountain hare from much of central and western Europe is thus  
597 likely affected by decreases in forest cover and competitive exclusion, rather than being solely  
598 a temperature mediated process. Based on that assumption, we would expect that *L. timidus*  
599 had a wider distribution in Europe and connections with Asia during previous interglacials,  
600 when forest cover was more extensive than today (Birks & Birks, 2004). This hypothesis is  
601 supported by our finding that the mountain hare has a less dramatic demographic history than  
602 other cold-adapted species that conform to the classic expansion/contraction model.

603 Our results indicate that the mountain hare has shown a remarkable resilience throughout the  
604 Quaternary climate changes, likely due to its capacity for occupying habitats that range from  
605 temperate steppe to Alpine landscapes. Its effective population size in Europe seems to have  
606 been tightly coupled with palaeoclimatic fluctuations, with growth and declines occurring in

607 line with temperature cooling and warming, respectively. However, although the range has  
608 shifted substantially in space there is no apparent decrease in genetic variation following the  
609 last glacial-interglacial transition. This is probably due to the high dispersal capability in the  
610 species, which may have enabled it to track shifts in habitat availability as well as led to long  
611 term gene flow among regions. The latter would have resulted in weak population structure,  
612 which may have acted as an additional buffer against major declines in diversity during range  
613 shifts, since local extinctions would not have led to any losses of locally distributed clades or  
614 haplogroups. However, the mountain hare's modern day distribution may have been  
615 additionally influenced by competitive interactions with the brown hare, which has expanded  
616 due to anthropogenic landscape changes during the Holocene.

617

618

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**Figure 1.** Extant (A) and Late Pleistocene (B) distribution of the mountain hare (*Lepus timidus*) in Europe, with locations indicated for the palaeontological sites that yielded ancient *L. timidus* DNA. Also shown are the extended coastlines resulting from lowered sea levels during the last glacial maximum (LGM), as well as the extent of the LGM ice sheets. Further isolated peaks and regions in the Mediterranean area, the Carpathians and Anatolia were glaciated at different times throughout the Pleistocene (Hughes & Woodward, 2008), but are not shown due to restrictions of scale and the fact that they are diachronous and not necessarily dated to the LGM. Modern European localities with available sequence data are taken from Melo-Ferreira *et al.* (2007). Palaeontological sites are 1: Trou Al'Wesse; 2: Kesslerloch; 3: Luegloch; 4: Nixloch and Gamssulzenhöhle; 5: Smrcka Lorenz-Abris; 6: Krucza Skala; 7: Deszczowa Cave; 8: Oblazowa Cave; 9: Kostenki 1; 10: Loutra Almopias Cave. See Table S1 for a complete list of all Late Pleistocene sites from which bone samples were retrieved.

**Figure 2.** Schematic view of the models compared in the three ABC model choice analyses. The vertical axis shows time and to the bottom left the ancient hare samples are represented by filled red circles, with the vertical position indicating their ages. The black shapes represent populations and the width represents their relative size over time. The curve to the bottom right shows the variations in  $\delta^{18}\text{O}$  levels over the last 130 thousand years (Blockley *et al.*, 2014), which is the temperature proxy that our two palaeoclimate models are directly based upon. The PCR<sub>M</sub> (palaeoclimate records model) predicts population growth during warm periods, while in the PCR<sub>M</sub><sup>-1</sup> (the inverse of PCR<sub>M</sub>) populations increase in size during periods of cold climate.

**Figure 3.** Temporal networks of cytochrome B (A) and control region (B) haplotypes found in modern and Late Pleistocene mountain hares (upper and lower layer, respectively). Shared haplotypes among the two temporal layers are connected by vertical lines, while empty circles illustrate haplotypes that are missing during one time period but present in the other. The number of individuals sharing a haplotype is reflected by its size, and more than one mutational difference between these is shown as a black dot.

**Figure 4.** Bayesian phylogeny of modern and Late Pleistocene mountain hares, based on the Eurasian concatenated CytB and CR dataset and a mutation rate of  $7.7\% \text{ Myr}^{-1}$ . The time scale is in calendar years before present, and asterix (\*) denotes internal nodes with posterior probabilities above 0.8. Sequences obtained from ancient samples are indicated with filled ovals.

**Figure 5.** Effective population size ( $N_e$ ) over time, obtained from the Bayesian Skyline Plot (upper part) and the best performing model in the “random histories” ABC analysis (middle part). The  $N_e$  scale is shown to the right and left of each graph, respectively, while time (in thousands of calendar years before the present) is found below. Both analyses are based on the European dataset and a mutation rate of  $7.7\% \text{ Myr}^{-1}$ . The small stepwise graphs at the bottom show the shape of  $N_e$  in the two palaeoclimate records models (PCRM and PCRM<sup>-1</sup>) employed in the ABC model choice analyses, which are based on the average  $\delta^{18}\text{O}$  records (black curve) (Blockley *et al.*, 2014). The light blue and orange bands show the placement of the cold Younger Dryas (YD) and Last Glacial Maximum (LGM), and the warm Eemian interglacial, as a time reference.