

1 **Large-scale mitochondrial DNA analysis reveals new light on the phylogeography of**  
2 **Central and Eastern-European Brown hare (*Lepus europaeus* Pallas, 1778)**

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29 Short title: Phylogeography of Central-, Eastern-European Brown hare

30

## 31 **Abstract**

32 European brown hare, *Lepus europaeus*, from Central and Eastern European countries  
33 (Hungary, Poland, Serbia, Lithuania, Romania, Georgia and Italy) were sampled, and  
34 phylogenetic analyses were carried out on two datasets: 1.) 137 sequences (358 bp) of control  
35 region mtDNA; and 2.) 105 sequences of a concatenated fragment (916 bp), including the  
36 cytochrome b, tRNA-Thr, tRNA-Pro and control region mitochondrial DNA. Our sequences  
37 were aligned with additional brown hare sequences from GenBank. A total of 52 and 51  
38 haplotypes were detected within the two datasets, respectively, and assigned to two previously  
39 described major lineages: Anatolian/Middle Eastern (AME) and European (EUR).  
40 Furthermore, the European lineage was divided into two subclades including South Eastern  
41 European (SEE) and Central European (CE). Sympatric distribution of the lineages of the  
42 brown hare in South-Eastern and Eastern Europe revealed contact zones there. BAPS analysis  
43 assigned sequences from *L. europaeus* to five genetic clusters, whereas CE individuals were  
44 assigned to only one cluster, and AME and SEE sequences were each assigned to two  
45 clusters. Our findings uncover numerous novel haplotypes of Anatolian/Middle Eastern  
46 brown hare outside their main range, as evidence for the combined influence of Late  
47 Pleistocene climatic fluctuations and anthropogenic activities in shaping the phylogeographic  
48 structure of the species. Our results support the hypothesis of a postglacial brown hare  
49 expansion from Anatolia and the Balkan Peninsula to Central and Eastern Europe, and  
50 suggest some slight introgression of individual haplotypes from *L. timidus* to *L. europaeus*.

51

52 **Keywords:** Central-, Eastern Europe; contact zones; genetic structure; glacial refugia;  
53 phylogeography; *Lepus europaeus*

54

## 55 **Introduction**

56 The brown hare (*Lepus europaeus* Pallas, 1778) is a native species to Northern, Central,  
57 Western Europe and the Western part of Asia, and it was introduced as a game into several  
58 countries (Argentina, Australia, Barbados, Brazil, Canada, Chile, Falkland Islands, New  
59 Zealand, R union and the United States; [1]).

60 The effect of translocation on hare genome was proved by previous genetic studies and they  
61 suggested that the brown hare and the Cape hare (*Lepus capensis*) are the same species [2].  
62 However, later the same authors performed mitochondrial DNA (mtDNA) analysis and found  
63 a significant divergence between them, and therefore they are currently considered to be two  
64 different species [3]. Pierpaoli et al. [4] showed that Italian and European hares did not share  
65 any mitochondrial haplotypes, indicating the lack of interspecific gene flow between the two  
66 species due to reproductive isolation in the course of their long separate evolutionary history.  
67 They identified two main groups of Eurasian and African hare haplotypes: Clade A (*L.*  
68 *granatensis*, *L. corsicanus*, *L. timidus*) and Clade B (*L. c. mediterraneus*, *L. habessinicus*, *L.*  
69 *starcki*, *L. europaeus*). These results suggest that the three species belonging to Clade A, with  
70 a common ancestor, would have colonized Europe independently of *L. europaeus* and would  
71 have originated by isolation during the Pleistocene glaciations in the southern or northern  
72 areas of refuge.

73 It is strongly argued that the current geographical distribution of temperate species and  
74 genetic relationships among their populations have been influenced by the climatic  
75 oscillations during the Late Quaternary [5, 6]. Specifically, different lineages represent  
76 populations repeatedly isolated into distinct glacial refugia such as the Iberian, the Apennine,  
77 the Balkan Peninsulas and Turkey [5, 7-10]. Furthermore, different human activities,  
78 competition for food or breeding and hybridization between species also led to a higher  
79 diversity in the southern refugial areas and the present genetic diversity of the hares [11-13].

80 There is evidence for human-mediated translocations that is well documented in the southern  
81 part of Europe [14].

82 Previous studies based on mitochondrial DNA (mtDNA) analysis on extant brown hare  
83 populations has revealed a relatively high degree of geographic partitioning [6, 15-18]. These  
84 studies distinguished two major geographically distinct lineages, the European (EUR) and the  
85 Anatolian/Middle Eastern (AME) clade. The EUR lineage is further subdivided into two  
86 subclades: the Central European (CE) and the South-Eastern European (SEE) [6]. The CE  
87 subclade includes individuals from across North-Central Europe, whereas the SEE comprises  
88 hares living in South-Eastern Europe. The second lineage, AME, includes individuals from  
89 Anatolia, South-Eastern Europe and the eastern Mediterranean Islands [17].

90 A recent study [18] found that there were three major haplogroups including Anatolia/Middle  
91 East (AMh), Balkans (BLh), and central Europe (cEUh) among brown hare populations  
92 worldwide. Additionally, three subgroups were revealed within the BLh haplogroup including  
93 South-Eastern Balkans (SEB), Southern Balkans (SB) and Greek islands excluding those  
94 harboring A-lineages (GI-B) off the Anatolian coast. Moreover, the South-Eastern and Central  
95 Balkans (SEB), comprising northeastern Greece, south and North-Western as well as South-  
96 Central Bulgaria, north-eastern part of Republic of Northern Macedonia, South-Eeastern and  
97 South-Western Serbia, was identified as the primary source region for most other Balkan  
98 brown hare populations [18].

99 On the other hand, Anatolian/Middle Eastern haplotypes have not been observed in South,  
100 Central and North-Western Greece and the rest of Europe, with the exception of one Serbian  
101 haplotype [18]. Also, European haplotypes have not been reported across the entire species  
102 range in the Middle East [6, 15, 19]. Further, the existence of a contact zone between the  
103 European and Anatolian/Middle Eastern lineages was detected in Bulgaria and North-Eastern  
104 Greece [6, 10, 15].

105 Detection of brown hare lineages is mostly based on the mtDNA control region (CR), and is  
106 usually well supported by cytochrome b (cyt b). It proves that mtDNA genomic data are  
107 useful in determining phylogenetic relationships between closely related species and within  
108 species [20-21] and for understanding the extent and nature of contact zones [10].

109 Overall, despite a relatively large number of genetic studies on brown hares, their  
110 phylogenetic relationships still remain challenging. Only several broad-range studies of  
111 phylogeography of brown hares have been done, relying on mtDNA control region sequences  
112 from Serbian, Greek and Bulgarian hares [6, 15, 18, 22-26]. Using wide-range geographic  
113 sampling over seven countries, we aimed to study (i) the extent of mitochondrial genetic  
114 variability and diversity of the brown hare in Central and Eastern Europe; (ii) the  
115 phylogeographic relationships of the studied populations, and furthermore (iii) to provide  
116 comprehensive information on the genetic characteristics of brown hares for conservation  
117 programs and management plans.

118

119

## 120 **Materials and methods**

### 121 Sample collection

122 A total of 137 legally hunted, unprotected adult brown hares were sampled in seven countries  
123 (Hungary, Poland, Serbia, Lithuania, Romania, Georgia, Italy; Fig 1, and see S1 Table)  
124 between 2010 and 2015. Also, three mountain hares have been accidentally collected along  
125 with our samples. No animals were killed for the purposes of this research.

126

127 **Fig 1. Spatial distribution of the European hares' maternal lineages, based on the 358-**  
128 **bp mtDNA control region, resulting when combining sequence data from GenBank (S1**  
129 **Table) and the present study.** Squares and polygons indicate the Central European and

130 South-East European subclades, respectively, in the European lineage. Circles and triangles  
131 indicate the Anatolian/Middle Eastern lineage and Mountain hare (*L. timidus*), respectively.  
132 Ellipses depict the two discovered contact zone areas between brown hare lineages in South-  
133 Eastern and North-Eastern Europe. Filled geometric shapes indicate the geographic location  
134 of the sampling sites in this study. Colours of the geometric shapes are in accord with  
135 clades/lineages; light green: Central European, dark green: South-East European, red:  
136 Anatolian/Middle Eastern, blue: Mountain hare.

137

138

139 All tissue samples were stored in 96% ethanol at -4°C. Hair follicles samples were kept in  
140 individually registered nylon or paper bags and stored at -4°C until the laboratory analysis.  
141 Total DNA was extracted using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek, USA), the  
142 High Pure PCR Template Preparation Kit (Roche, USA) and standard FAO protocol. DNA  
143 concentrations were evaluated spectrophotometrically and visually by standard agarose gel  
144 electrophoresis.

145 Different regions of the mitochondrial DNA were amplified. PCR protocols and primers  
146 (Le.H-Dloop\_F, Le.L-Dloop\_R [15] for the control region (CR) and LepCyb2L\_F,  
147 LepD2H\_R [4] for cytochrome b (cyt b) + tRNA-Thr + tRNA-Pro + control region) were  
148 used to the amplification. PCRs were carried out in a total volume of 25 µl, using the  
149 following sequence of steps: denaturation at 94 °C for 5 minutes, followed by 35 cycles of  
150 amplification 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, and a final step  
151 at 72 °C for 5 minutes. The forward sequencing reaction was performed by MacroGen Europe  
152 (The Netherlands).

153 In addition, previously published sequences of the species were downloaded from the  
154 GenBank (S1 and S2 Tables).

155

156 Ethics statement

157 Animals were not shot for the purpose of this study. The study did not involve the collection  
158 of samples from live animals. An ethics statement was not required. Samples from the  
159 different countries were obtained from licensed collaborators and licensed hunters who took  
160 samples following their regulations in brown hare management.

161

162 Sequence analysis

163 Two datasets were created from the sequences. The first dataset comprised 137 CR mtDNA  
164 sequences with a total length of 358 bp. The second dataset comprised 105 concatenated  
165 sequences cyt b + tRNA-Thr + tRNA-Pro + CR, with a total length of 916 bp after alignment.

166 Alignment was performed using Seqscape 2.6 (Applied Biosystems) and ClustalW in MEGA  
167 6 [27], respectively. The aligned sequences were deposited in GenBank with the Accession  
168 numbers: MG865671-MG865724 for CR and MG841060- MG841112 for the cyt b + tRNA-  
169 Thr + tRNA-Pro + CR region (S1 and S2 Tables). The European Rabbit (*Oryctolagus*  
170 *cuniculus*) (GenBank: AJ001588) [28] was used as an outgroup for the phylogenetic analyses.  
171 DAMBE 6 [29] was used to analyze substitution saturation.

172 The number of polymorphic sites, haplotype diversity, nucleotide diversity, average number  
173 of nucleotide differences for each location and number of haplotypes were estimated with  
174 DnaSP 5.10 [30]. The best-fitting partitioning scheme and nucleotide substitution model were  
175 selected using the Bayesian information criterion (BIC) and the corrected Akaike Information  
176 Criterion (AICc) implemented in PartitionFinder 2.1.1 [31].

177 Bayesian inference (BI) was performed using BEAST v2.3 [32] with 40,000,000 generations  
178 of Monte Carlo Markov chains (MCMC), sampling every 100 generations. Maximum  
179 likelihood (ML) analyses were implemented in IQ-TREE 1.6 [33] with 10,000 bootstrap  
180 steps. Additionally, MEGA 6 [27] was used to construct a neighbour-joining (NJ)



181 phylogenetic tree, applying the pairwise distance data and p-distance model with 10,000  
182 bootstrap replications. Furthermore, median-joining networks [34] among haplotypes were  
183 inferred using PopART 1.7 [35].

184 Fu's FS [36] and Tajima's D [37], performed in Arlequin 3.5 [38], were employed to assess  
185 the demographic history and to examine hypotheses of selective neutrality [39]. The  
186 significance of these tests was calculated using 10,000 permutations. The hierarchical analysis  
187 of molecular variance (AMOVA) and fixation index were implemented with 10,000 iterations  
188 using Arlequin 3.5 [38] to evaluate levels of population structure. The aim of the AMOVA  
189 analysis was to examine whether genetic variation was significantly structured among  
190 different haplogroups.  $\Phi_{ST}$  can provide an estimate of the genetic differentiation among  
191 populations in order to make inferences of past demographic changes.

192 To estimate the presence of genetic clusters (populations) within the sequences of *L.*  
193 *europaeus* and *L. timidus* (or introgressed individuals), we used Bayesian Analysis of  
194 Population Structure (BAPS) v6 [40-41] implementing the method of "clustering for linked  
195 loci" with two independent runs and  $K = 10$  repetitions. To assess introgression occurring  
196 within the populations of these two species, we performed the method of "admixture based on  
197 mixture clustering" implemented in BAPS. To provide a correct assessment of population  
198 genetic structure, it is recommended to use the admixture models, because these models are  
199 robust to an absence of admixture in the sample; reciprocally, models without admixture are  
200 not robust to the inclusion of admixed individuals in the sample [42].

201

## 202 **Results**

203 MtDNA control region sequences (358 bp)

204

205 The substitution saturation test based on both datasets (916 bp and 358 bp sequences)  
206 revealed that the base substitutions did not reach saturation, and these datasets were suitable  
207 for phylogenetic analyses.

208 For the 358 bp control region, 137 samples were sequenced from Central-Eastern Europe (S1  
209 Table). Additional sequences from Europe and the Middle East published in GenBank were  
210 included in the analyses, yielding a dataset comprising a total of 447 sequences and 259  
211 haplotypes (S1 Table). A total of 52 haplotypes were identified among the 137 new  
212 sequences, including 40 novel haplotypes and 12 previously reported haplotypes.

213 The phylogenetic analyses (BI, ML, and NJ trees) yielded relatively identical topologies,  
214 indicating that among 137 selected haplotypes from the dataset (447 individuals) two lineages  
215 were identified (Fig 2).

216

217

218 **Fig 2. Phylogenetic relationships of brown hare from Central-Eastern Europe with other**  
219 **brown hares, based on the 358-bp mtDNA control region sequences and rooted with**  
220 ***Oryctolagus cuniculus* (AJ001588).** The numbers on the branches are posterior probabilities  
221 in the Bayesian inference and bootstrap support in maximum likelihood and neighbour-  
222 joining. Colored ovals represent haplotypes identified in the current study. The branches  
223 within blue rectangular include mountain hare sequences or introgressed haplotypes of this  
224 species in other hare species. For detailed information on haplotypes see S1 Table.

225

226 The MJ network analysis (Fig 3) also supported the clusters distinguished in the phylogenetic  
227 trees. The first lineage, European (EUR), was divided into two phylogeographically distinct  
228 subclades: Central European (CE) and South-East European (SEE).

229

230

231 **Fig 3. Median joining network of brown hare from Central-Eastern Europe and other**  
232 **brown hares, based on the 358-bp mtDNA control region.** The numbers on the haplotypes  
233 (1-259) are the same haplotype codes (CR1-CR259) as in Fig 2 and S1 Table. Dark circles are  
234 connecting nodes (i.e. putative undetected haplotypes). Blue circles include mountain hare  
235 sequences or introgressed haplotypes of this species in other hare species.

236

237

238 The subclade CE was mostly distributed across various regions of Central Europe, Scotland,  
239 England, the Netherlands, France, Germany, Italy, Austria, Switzerland, Poland, Lithuania,  
240 Hungary and Northern Serbia (Fig 1). However, two individuals belonging to the subclade  
241 were found in Eastern Romania and Southern Serbia. Also, one brown hare from Cyprus  
242 (Cyprus 4 in S1 Table) clustered within CE (falling into haplotype CR40, S1 Table).  
243 Haplotype CR40 along with haplotypes CR1 and CR10 was the most common haplotype in  
244 the subclade CE and was shown to inhabit more than one region in Europe (Fig 3). Haplotype  
245 CR40 was identified as the most abundant (38 individuals) and central haplotype in the  
246 subclade, and was observed across Northern Europe, from Lithuania to Poland, Germany,  
247 France, England, and Scotland. Haplotype CR1 was observed in Poland, Hungary, Romania,  
248 Serbia, and Italy, whereas haplotype CR10 was observed in Lithuania, Poland, Hungary,  
249 Serbia, Austria, Italy and France. The subclade SEE predominantly occurred in South-Eastern  
250 Europe including Bulgaria, Greece, Republic of Northern Macedonia and Serbia (Fig 1).  
251 However, individuals belonging to this subclade were also present in Hungary, Poland,  
252 Central Italy and France (Corsica Island) (Figs 1 and 2, S1 Table). Haplotypes in SEE were  
253 mostly specific to relatively limited spatial distributions (Fig 3). However, three haplotypes  
254 belonging to this subclade were recorded over a larger geographical range: CR8 (Hungary and

255 Italy), CR32 (Serbia and Italy) and CR62 (Italy and Poland). Phylogenetic analyses revealed  
256 no shared haplotype between the subclades in this lineage.

257 The second cluster, the Anatolian/Middle Eastern lineage (AME) was predominantly present  
258 in Georgia, Turkey and the Middle East, and was also found in Lithuania, Poland, Romania,  
259 North-Eastern Greece, Republic of Northern Macedonia, Italy and France (Corsica Island)  
260 (Fig 1). Haplotypes in this lineage were mostly restricted to small geographic ranges.  
261 However, within AME, haplotypes CR52, CR53, and CR54 were recorded both in Romania  
262 and Italy, but haplotypes CR57 (Italy and Republic of Northern Macedonia) and CR200  
263 (Turkey and Cyprus) were also found in distant localities (Figs 1, 2 and 3).

264

265 MtDNA cytochrome b, tRNA-Thr, tRNA-Pro and control region (916 bp)

266 Phylogenetic analyses of the control region revealed two major lineages in Central-Eastern  
267 Europe, with contact zones discovered in the geographic range (Fig 1). To obtain a better  
268 assessment of phylogeographic structure, we sequenced the additional fragments cyt b (426  
269 bp), tRNA-Thr (66 bp) and tRNA-Pro (66 bp) of 105 brown hares from Italy, Hungary,  
270 Serbia, Georgia, Romania, Poland and Lithuania (S2 Table). Sixteen additional sequences  
271 belonging to brown hares from Germany, Sweden, Poland, Greece, Turkey and Cyprus  
272 available in GenBank were also added to the alignment (S2 Table). Finally, a total dataset  
273 comprising 124 sequences (916 bp fragment of mtDNA), corresponding to a total of 62  
274 haplotypes was used for phylogenetic analysis. According to this longer fragment, and in  
275 accordance with control region sequences, the brown hare population in Central-Eastern  
276 Europe is divided into the same two distinct phylogeographic lineages (EUR and AME) (Figs  
277 4 and 5).

278

279 **Fig 4. Phylogenetic relationships of brown hare from Central-Eastern Europe with other**  
 280 **brown hares, based on the 916-bp mtDNA sequences (cyt b + tRNA-Thr + tRNA-Pro +**  
 281 **control region) and rooted with *Oryctolagus cuniculus* (AJ001588).** The numbers on the  
 282 branches are posterior probabilities in the Bayesian inference and bootstrap support in  
 283 maximum likelihood and neighbour-joining. Colored ovals represent haplotypes identified in  
 284 the current study. For detailed information on haplotypes see S2 Table.

285

286 **Fig 5. Median joining network of brown hare from Central-Eastern Europe and other**  
 287 **brown hares, based on the 916-bp mtDNA sequences (cyt b + tRNA-Thr + tRNA-Pro +**  
 288 **control region).** For detailed information on haplotypes see Fig 4 and S2 Table. Dark circles  
 289 are connecting nodes (i.e. putative undetected haplotypes).

290

291 Furthermore, brown hares belonging to the lineage EUR fall into two subclades, the same CE  
 292 and SEE as in the first dataset. The contact zones among all lineages and subclades were  
 293 identified in the same geographic ranges as in Fig 1.

294 A total of 51 haplotypes was found throughout Central-Eastern Europe. Moreover, 50 novel  
 295 haplotypes and only one previously reported haplotype were detected among them. The  
 296 genetic statistics for the sequenced brown hares in this study are displayed in Table 1.

297

298 **Table 1. Comparison of genetic statistics for the brown hares sequenced in this study,**  
 299 **originating from Central-Eastern Europe, based on the 916-bp mtDNA sequences (cyt b**  
 300 **+ tRNA-Thr + tRNA-Pro + control region)**

<b>Group</b>	<b>n</b>	<b>h</b>	<b>Hd (SD)</b>	<b>Pi (SD)</b>	<b>K</b>	<b>P</b>	<b>Fu's FS</b>	<b>Tajima's D</b>
Central European	83	32	0.927(0.019)	0.0051(0.0003)	4.71	41	-	-1.455*

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							15.340**	
South-East	14	12	0.978(0.035)	0.0153(0.0021)	14.14	52	-1.567	-0.593
European								
Anatolian/Middle	8	7	0.964(0.077)	0.0198(0.0029)	18.32	40	-0.607	0.623
Eastern								

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301 n, number of individuals; h, number of haplotypes; Hd, haplotype diversity; SD, Standard  
302 Deviation; Pi, nucleotide diversity (per site); K, average number of nucleotide differences; P,  
303 variable (polymorphic) sites. Statistical significance: \*p<0.05, Statistical high significance:  
304 \*\*p<0.01.

305

306

307

308 High haplotype diversity values and relatively low-moderate nucleotide diversity were  
309 obtained for brown hares of the study populations. The lineage AME (only for Fu's FS) and  
310 both the subclades of lineage EUR presented negative values for Tajima's and Fu's neutrality  
311 tests, but only the outcome for the Central European subclade was found significant (D = -  
312 1.455, P = 0.045; FS = -15.34, P = 0.00) (Table 1). Thus, this subclade is likely to have  
313 undergone a recent population expansion. Results of the AMOVA revealed that the among-  
314 haplogroups component of variance (67.59%) was higher than the variation within  
315 haplogroups (32.41%) (Table 2). According to the fixation index a significant genetic  
316 structure among all haplogroups was also observed ( $\Phi_{ST} = 0.676$ , P = 0.00) (Table 2).

317

318 **Table 2. AMOVA results for three major haplogroups (AME, SEE and CE) of brown**  
319 **hare originating from Central-Eastern Europe, based on the 916-bp mtDNA sequences**  
320 **(cyt b + tRNA-Thr + tRNA-Pro + control region).**

Source of variation	d.f.	Percentage of variation	Fixation index ( $\Phi_{ST}$ )	p-value
Among haplogroups	2	67.59	0.676	p<0.000
Within haplogroups	101	32.41		
Total	103			

321

322

323 The analysis performed with BAPS v6 separated *L. europaeus* and *L. timidus* (and  
324 introgressed mountain hare in other hare species) with  $K = 6$  ( $\ln(P) = -8954.5009$ ). This  
325 analysis assigned sequences from *L. europaeus* to five genetic clusters, and *L. timidus* to only  
326 one cluster (Fig 6). Within *L. europaeus*, sequences belonging to lineage AME and subclade  
327 SEE (lineage EUR) were each assigned to two clusters, whereas individuals belonging to  
328 subclade CE (lineage EUR) fell into one cluster.

329

330 **Fig 6. Bayesian clustering analysis of 358-bp mtDNA control region sequences from**  
331 **brown hares (*L. europaeus*) and mountain hares (*L. timidus* and introgressed haplotypes**  
332 **of this species in other hares) as implemented in BAPS v6, resulting in  $K = 6$ .** We  
333 detected 5 clusters within major lineages of *L. europaeus*; 2 and 3 clusters within lineages  
334 AME and EUR (SEE = 2 clusters; CE = 1 cluster), respectively. Also, *L. timidus* and  
335 introgressed individuals were assigned to one cluster. Numbers 1 to 20 are the localities of  
336 sequence data from our study and others (see S1 Table): 1. Georgia; 2. Middle East; 3.  
337 Cyprus; 4. Turkey; 5. Greece; 6. Bulgaria; 7. Romania; 8. Republic of Northern Macedonia;  
338 9. Serbia; 10. Hungary; 11. Austria; 12. Switzerland; 13. Italy; 14. France; 15. Poland; 16.  
339 Lithuania; 17. Sweden; 18. Germany; 19. The Netherlands, England and Scotland; 20. Iberian  
340 Peninsula.

341

## 342 **Discussion**

343 Previous studies estimated phylogenetic relationships among brown hare populations in  
344 Europe and the Middle East, where insufficient sampling left a relatively large gap in several  
345 geographic ranges, especially in Central-Eastern Europe. This information gap has prevented  
346 the delineation of a comprehensive picture of genetic diversity and phylogeographic structure  
347 of the species. European brown hares have been classified to two major lineages, European  
348 (EUR) and Anatolian/Middle Eastern (AME) [6, 15, 17-18] that co-exist in Republic of  
349 Northern Macedonia, North-Eastern Greece and Bulgaria [6, 10, 15]. In this study, we  
350 presented a relatively comprehensive dataset on mtDNA cytochrome b, tRNA-Thr, tRNA-Pro  
351 and control region fragments (a total of 916 bp) of brown hares in Central-Eastern Europe,  
352 where two datasets were used in the genetic analyses; the first dataset included a 358-bp  
353 control region sequence, whereas the second dataset covered a concatenated sequence of  
354 mtDNA fragments (the 916-bp sequence).

355 Our findings revealed a high genetic diversity within the 916-bp mtDNA sequence (105 new  
356 sequences, 51 haplotypes) of brown hares from Central-Eastern Europe, where 50 haplotypes  
357 were reported for the first time (Table 1). Phylogenetic analyses revealed two major lineages  
358 of brown hare in the study area, based on a combination of our sequences and previously  
359 published sequences (S1 and S2 Tables) for both datasets: (i) AME, which comprises  
360 individuals from Georgia, Anatolia, the Middle East and also includes some hares living in  
361 South-Eastern, North-Eastern and Central Europe, and (ii) EUR, which includes hares from  
362 Central, South-Eastern, Eastern and Northern Europe. In accordance with others [6, 15], the  
363 EUR lineage is subdivided into two well-supported subclades, Central European (CE) and  
364 South-East European (SEE).



365 The significant genetic structure among brown hare haplogroups from Central-Eastern Europe  
366 was well supported by  $\Phi_{ST}$  and AMOVA (Table 2). The fixation index is a standard measure,  
367 which gives an estimate of the degree of genetic differentiation among and within  
368 populations/haplogroups [43]. In fact, the analyses demonstrated that partitioning into the  
369 major haplogroups explains 67.59% of the overall mtDNA variability and corresponds to a  
370 highly significant fixation index ( $p < 0.000$ ). The female philopatry of brown hares [16, 44]  
371 could have resulted in the formation of multigenerational matrilineal assemblages that are  
372 geographically structured [45].

373 The population structure determined by BAPS v6 partially described diversity allocation  
374 between clusters based on the control region mtDNA sequences. BAPS is known to be  
375 relatively highly efficient in identifying hidden population structures [46]. The analysis  
376 revealed five genetic clusters within the populations of *L. europaeus* and only one cluster  
377 within *L. timidus* (and introgressed) sequences. Within *L. europaeus*, individuals belonging to  
378 the major lineage AME were assigned to two clusters: (i) cluster 1, which includes brown  
379 hares from Georgia, Turkey, Cyprus, Bulgaria, Romania, Republic of Northern Macedonia,  
380 Central Italy, France (Corsica Island), Poland and Lithuania; (ii) cluster 2, which comprises  
381 brown hares living in the Middle East, Georgia, Turkey, Greece, Republic of Northern  
382 Macedonia, Central Italy and France (Corsica Island). Sequences belonging to subclade SEE  
383 (lineage EUR), within *L. europaeus*, were divided to two clusters: (i) cluster 1, including the  
384 sequences from Greece, Republic of Northern Macedonia, Serbia, Hungary, Central Italy,  
385 France (Corsica Island), Germany and Poland; (ii) cluster 2, which includes individuals from  
386 Greece, Bulgaria, Republic of Northern Macedonia, Serbia, Central Italy and France (Corsica  
387 Island). It is interesting that all genetic clusters of brown hare are present in Central Italy and  
388 France (Corsica Island) (Fig 6).

389 Our findings revealed some slight introgression of individual haplotypes from *L. timidus* into  
390 *L. europaeus* only in one sample (GER4 in S1 Table) from Germany (Fig 6). Extensive  
391 introgression mtDNA and nuclear genes of mountain hare into other hares has been reported  
392 in previous studies (e.g., [47-48]). The introgression of individual genotypes among  
393 populations potentially could have resulted from recent genetic hybridization or incomplete  
394 lineage sorting of ancestral variation.

395 The contact zones among the two major lineages (and two subclades belonging to lineage  
396 EUR), interestingly, were discovered in two large areas in Central-Eastern Europe,  
397 encompassing South-Eastern (Republic of Northern Macedonia, North-Eastern Greece,  
398 Bulgaria and Romania) and North-Eastern (Lithuania and North-Eastern Poland) Europe (Fig  
399 1). While the sympatric distribution of haplotypes of lineages EUR and AME in Republic of  
400 Northern Macedonia, North-Eastern Greece and Bulgaria had already been shown by others  
401 [6, 10, 15], other overlapping distributions are characterized here for the first time. However,  
402 the region comprising Thrace and Bulgaria, which probably extends into Turkish Thrace and  
403 maybe into Anatolia is a well-known hybrid zone of Europe [5] for species that were  
404 restricted to refuge areas in the Southern Balkans and Anatolia during the Pleistocene cold  
405 stages [15].

406 Based on the combined analyses of our sequences and those of others [15; Strzala et al.  
407 unpublished, direct submission to GenBank), Polish brown hares harbour haplotypes of both  
408 lineages (and the two EUR subclades). Whereas lineage EUR (mostly the subclade CE) is  
409 widespread and predominant in Poland, another lineage is only found in the eastern part of the  
410 country. Brown hares living in Western Romania fall into the European lineage (subclade  
411 CE), whereas individuals from Eastern Romania also show haplotypes of lineage AME.  
412 Overall, our data reveal overlapping EUR and AME haplotypes both in Romania and  
413 Lithuania.

414 Brown hares inhabiting Georgia exhibited high genetic diversity (dataset 1: 7 individuals, 6  
415 novel haplotypes; and dataset 2: 4 individuals, 3 new haplotypes), but only within the lineage  
416 AME. Thus, based on our data, extending the contact zone to Georgia and the Middle East, as  
417 speculated by others [6, 15] is not justified. It is interesting that among the sequences  
418 previously reported from Cyprus [15, 17], one brown hare (CYP4, listed in S1 and S2 Tables;  
419 published by [17]) shared a common haplotype (CR40 that distributes across Northern  
420 Europe; see Fig 3 and S1 Table for detailed information) of European lineage origin (subclade  
421 CE). However, the haplotype was found outside the range of Northern Europe only in Cyprus.  
422 We consider human-mediated translocations for these introgressions, as has been widely  
423 confirmed for both recent and historic times [15, 49-50]. However, more extensive samplings,  
424 especially in Eastern Europe, Balkans, north of the Black Sea and Anatolia, may reveal  
425 important phylogeographic signatures.

426 Our data confirm the presence of both subclades (CE and SEE) belonging to the lineage EUR  
427 in Hungary and Serbia. Whereas haplotypes belonging to SEE are predominant in Southern  
428 and Central Serbia, the unique sequences of CE are predominantly found in Hungary and  
429 Northern Serbia. Moreover, a recent study reported one haplotype belonging to AME among  
430 brown hares from Northern Serbia as a possible consequence of human-mediated  
431 translocations [18].

432 According to the combined analysis of our sequences and those of others [51], haplotypes  
433 belonging to lineages EUR (both subclades CE and SEE) and AME are present in Italy.  
434 Nevertheless, haplotypes belonging to CE are predominant in this country. The European  
435 brown hare is a major game species in Europe [52], and different populations of the species  
436 have been introduced in different areas, mostly for hunting. Thus, this presence of AME is  
437 also probably due to human-mediated translocations, as reported in other studies (e.g., [51]).  
438 Furthermore, the occurrence of *L. europaeus* in Corsica is recent and artificial, as it is known

439 that different species of hares have been introduced in the region up to this day [53]. Overall,  
440 the presence of both major lineages (and the European subclades) of brown hare in Corsica  
441 could be the result of several human-mediated introduction events from different origins [54].  
442 Likewise, a contact zone between mountain hares (*L. timidus*) and brown hares can be  
443 observed in Lithuania, as recorded in different populations of brown hares [9, 48,55-57].  
444 The network result, in accordance with Stamatis et al. [6], showed that there are relatively  
445 close relationships between some haplotypes belonging to CE and several haplotypes from  
446 SEE (Fig 3). This finding indicates that one haplotype of the first subclade is only connected  
447 by one, so far undetected, haplotype, to another haplotype from the second subclade.  
448 However, the network analysis based on the longer sequence (916 bp) (Fig 5) does not  
449 provide strong support for this hypothesis. Overall, the close phylogenetic relationships  
450 between the two subclades SEE and CE in large geographic ranges of Europe support the idea  
451 that the brown hare colonized the current spatial ranges, when ecological conditions in these  
452 areas became suitable for the species after the Last Glacial Maximum [6, 58]. Also, the  
453 presence of a large number of unique haplotypes in South-Eastern Europe (the Balkans) and  
454 Anatolia is evidence for maintenance of a high proportion of the pre-glacial brown hare  
455 diversity in these areas during at least the last glacial period. Other studies have demonstrated  
456 the high intraspecies diversity of brown hare in these areas [6, 15, 18].  
457 We discovered large contact zones for brown hares in several countries of Central-Eastern  
458 Europe. These findings support the existence of probable glacial refugia during the LGM in  
459 some of these areas (especially in Southern Europe), where the refugial populations probably  
460 underwent genetic differentiation [8], resulting in a number of genetic clusters. Following the  
461 retreat of the glaciers, the genetically isolated populations colonized Europe and formed  
462 secondary contact zones [59]. Our findings are in accordance with others [6-8, 15] who  
463 suggest the post-glacial population expansion scenario from southern refugia (such as Iberia,

464 Italy and the Balkans, as well as Asia Minor and the Caspian/Caucasus region). Other studies  
465 [18] provide evidence for the hypothesis of an Anatolian population range expansion of the  
466 brown hare into south-eastern and south-central areas of the Balkans, which has likely acted  
467 as a potentially important source in the pattern of gene flow to southern, central and northern  
468 areas of the Balkan Peninsula. Furthermore, it is suggested that colonization of the central and  
469 western parts of Europe by brown hares started from the Northern Balkans in a postglacial  
470 expansion. However, the Balkans were the most important source of European populations,  
471 due to the lack of major geographical barriers limiting a northward expansion, compared to  
472 the Alps and the Pyrenees for the Italian and the Iberian refugia, respectively [7]. Several  
473 authors described the existence of introgression of Anatolian mtDNA in Bulgarian brown  
474 hares which most likely result of hunting management practices in recent time [6, 15, 18, 49].  
475 The colonization pattern of Central and Northern Europe from the Balkan Peninsula has also  
476 been proposed for other species such as the marbled white butterfly (*Melanargia galathea*)  
477 [60] and the wild boar (*Sus scrofa*) [61].

478 Our data, in combination with additional ones [6, 17, 48], indicate phylogenetically close  
479 relationships among brown hares throughout Central and Northern Europe, where a common  
480 haplotype (CR40 in Fig 3 and S1 Table) was identified. Furthermore, other shared haplotypes  
481 (e.g., CR1 and CR10) were found from the east (Lithuania, Romania, Serbia) to central  
482 (Poland, Hungary, Austria) and west (Italy and France) across Europe. The findings suggest  
483 that source regions for the origin of northern, western, and central populations of brown hare  
484 are probably situated in Eastern or Southern Europe. High variability of mtDNA in these  
485 probable sources support the hypothesis of gene flow in a northward and westward expansion  
486 of the identified contact zones, as Stamatis et al. [6] proposed the gene flow from north-  
487 western populations of Greece into Central Italy via a land bridge between the Balkans and  
488 the Italian peninsula at the end of the Pleistocene and the Holocene. Also, Stamatis et al. [6]

489 suggested the probable pattern of gene flow northward from Italy to Switzerland and Austria,  
490 after the retreat of the southern alpine glaciers. Several studies suggested the postglacial  
491 colonization of Central and North-Western continental Europe by the brown hare of the  
492 Balkans [6, 15, 18]. Others [62] supported the postglacial recolonization of Central Europe by  
493 the Italian populations.

494 The existence of AME haplotypes in South-Eastern Europe support a sudden expansion of  
495 this lineage to Europe during the late Pleistocene via the Bosphorus land bridge that  
496 disappeared only ca. 8000 years ago with the rise of the sea level [18, 63] or some Greek  
497 islands when they were still connected to Anatolia in the late Pleistocene [15]. On the other  
498 hand, the presence of a genetic break at the border between Anatolia and the surrounding  
499 regions has been reported in different species [64].

500 Also, our data confirm the presence of AME haplotypes in North-Eastern Europe, indicating  
501 the gene flow from Anatolian/Middle Eastern brown hares into Eastern and North-Eastern  
502 Europe via west of the Black Sea or other post-glacial colonization routes, especially east of  
503 the Black Sea. Alternatively, the existence of some haplotypes out of their lineage's original  
504 homeland may be due to recent translocations. Indeed, Kasapidis et al. [15] described that the  
505 brown hares living in some Eastern Mediterranean islands (such as Crete and Cyprus) have  
506 probably been introduced by humans because these islands were cut off from the mainland  
507 more than 2.5 million years ago.

508 Neutrality tests were negative for the lineages and subclades (except in AME for the value of  
509 Tajima's D), but only the subclade CE showed a significant negative value, indicating a  
510 significant excess of rare haplotypes suggesting that the population is not under mutation-drift  
511 equilibrium due to sudden expansion [45, 65]. Also, the star-like connection pattern of  
512 haplotypes (CR1, 10, 27, 36, 40, 57, and 167 in Fig 3; and H3, 8 and 38 in Fig 5) gives  
513 support to the hypothesis of population expansion [66]. Some of these haplotypes are the

514 central and most abundant ones and are widely distributed in the study area. Thus, it is highly  
515 likely that the common and central haplotypes are ancestral haplotypes. Moreover, the  
516 patterns of high haplotype diversity along with relatively low nucleotide diversity (as found in  
517 this study) indicate sudden demographic expansion from a restricted area or a small effective  
518 population size in the recent past [65, 67]. In other words, this pattern suggests that the  
519 populations originate from closely related haplotypes.

520

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530

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745 **Supporting information**

746 **S1 Table. Details of sequences used in the phylogenetic analyses of brown hares based on**  
747 **the 358 bp mtDNA control region.**

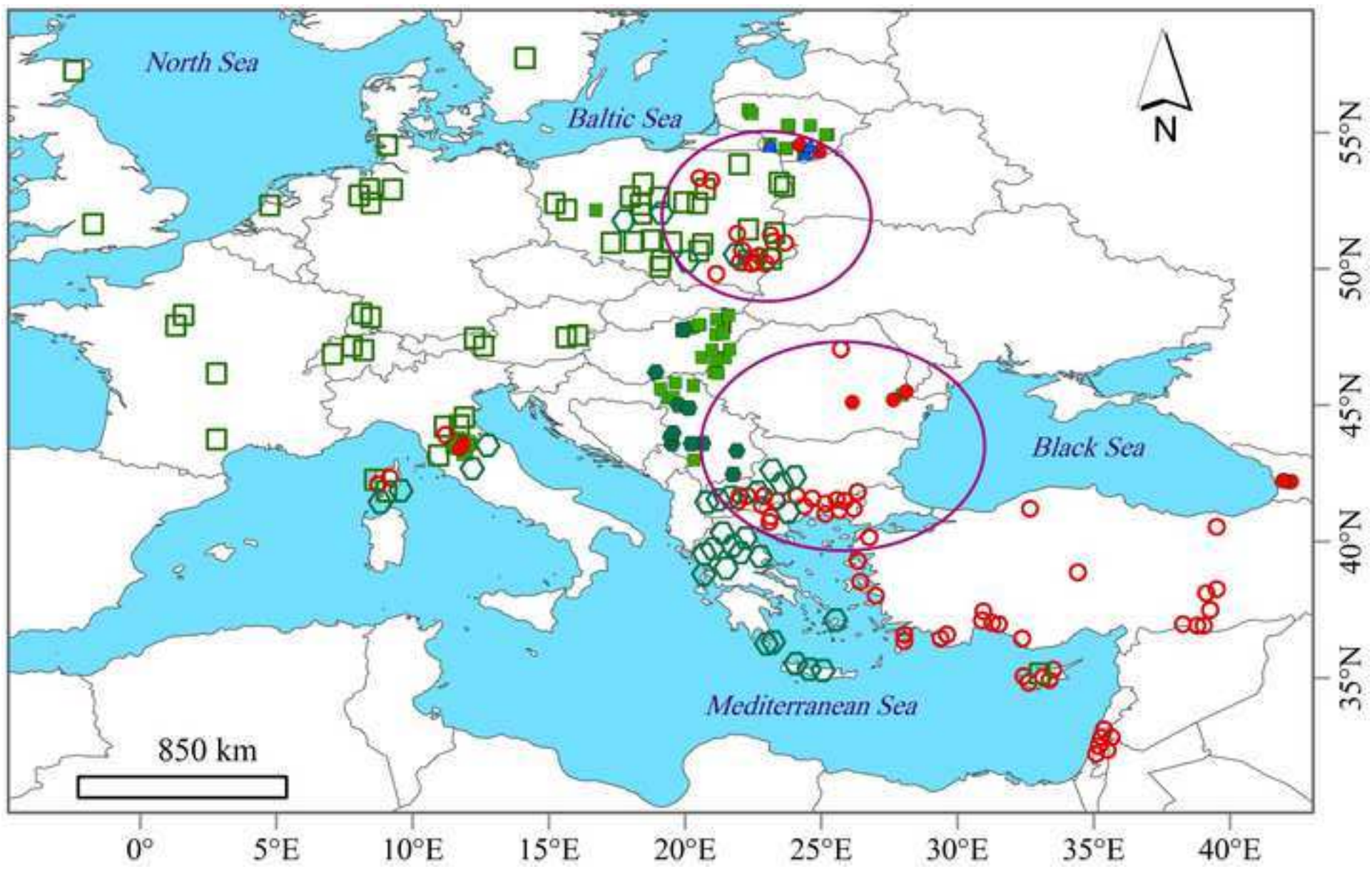
748

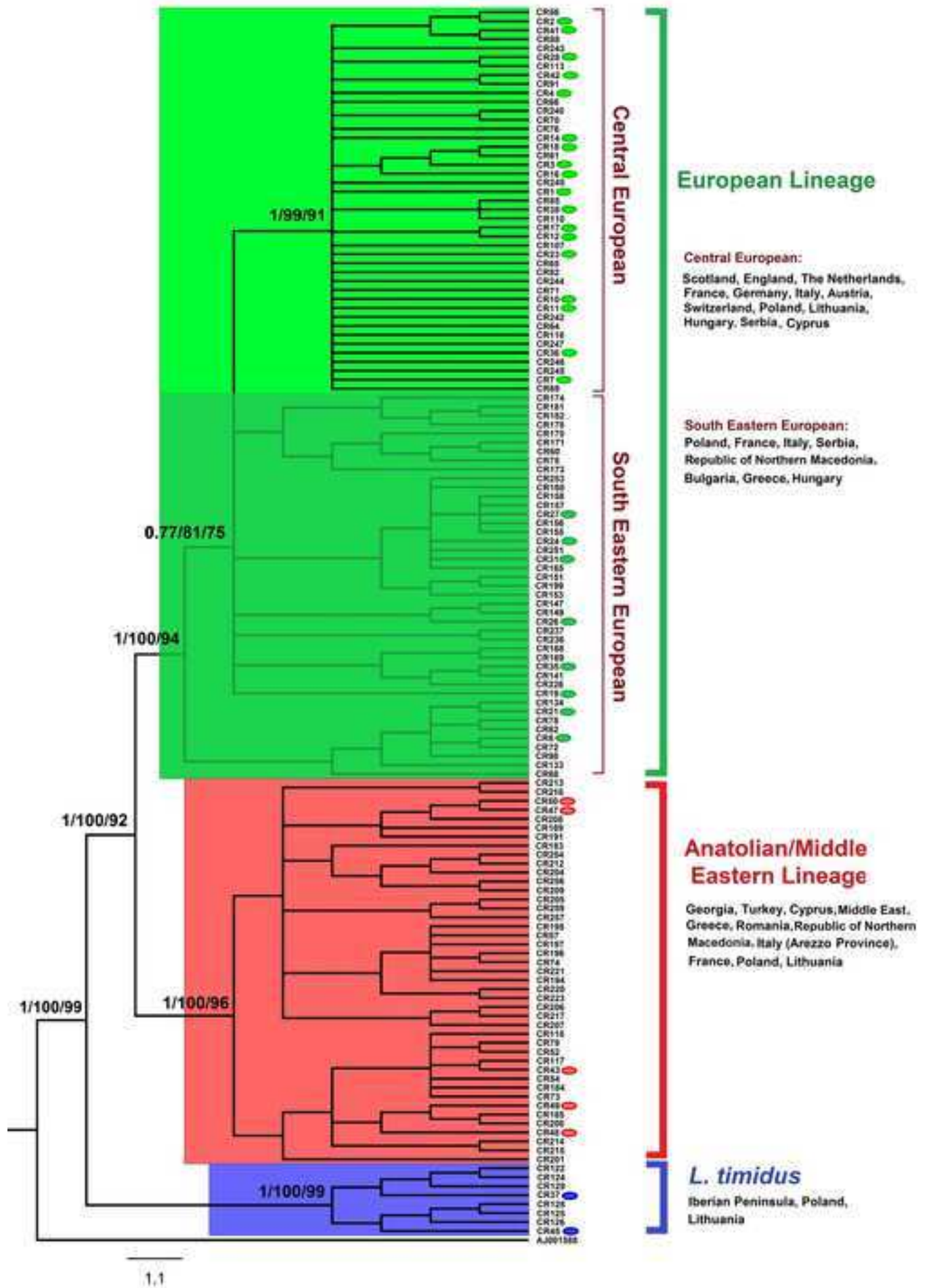
749 **S2 Table. Details of sequences used in the phylogenetic analyses of brown hares based on**  
750 **the 916 bp mtDNA sequences (cytochrome b, tRNA-Thr, tRNA-Pro and control**  
751 **region).**

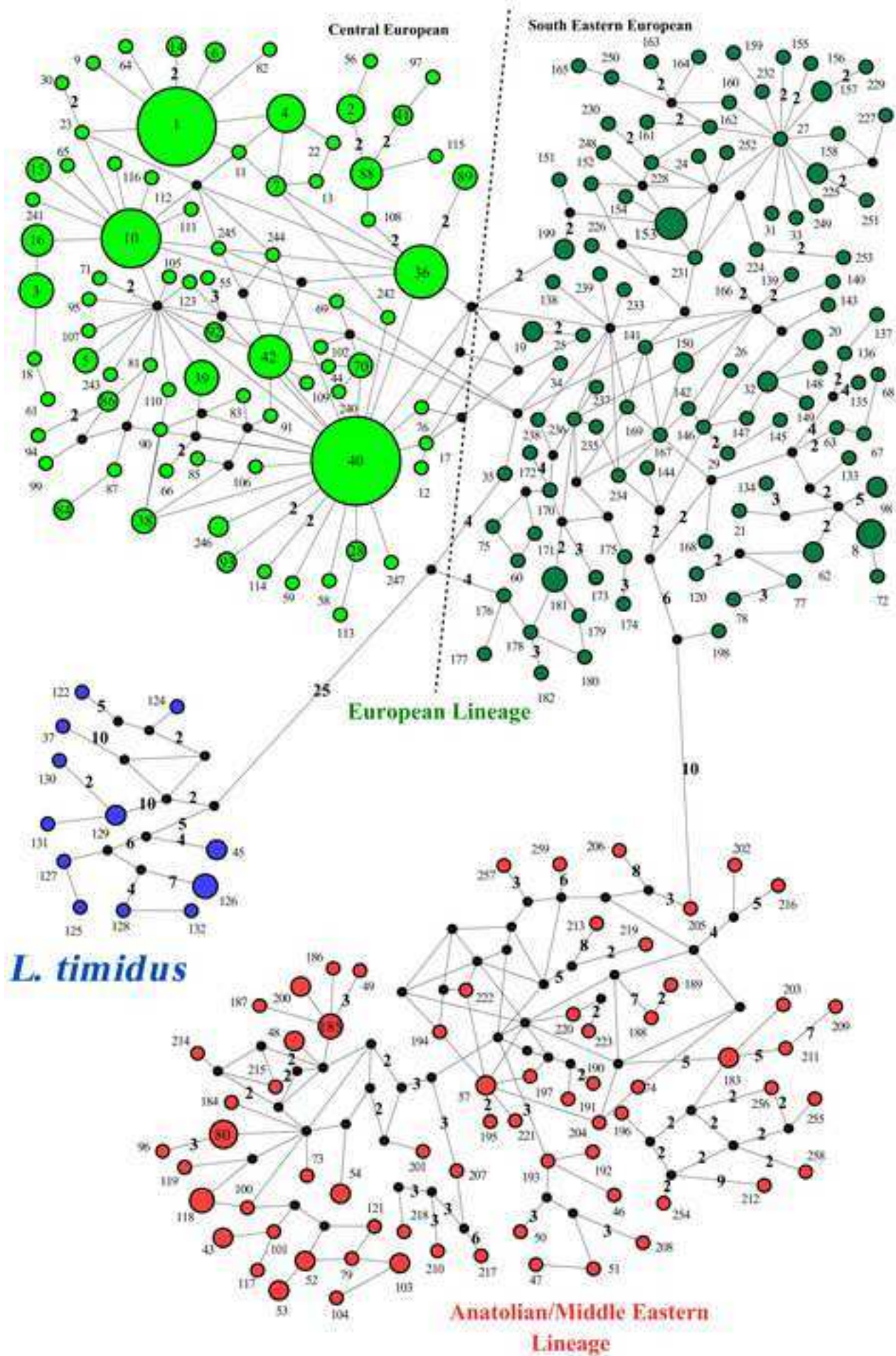
752

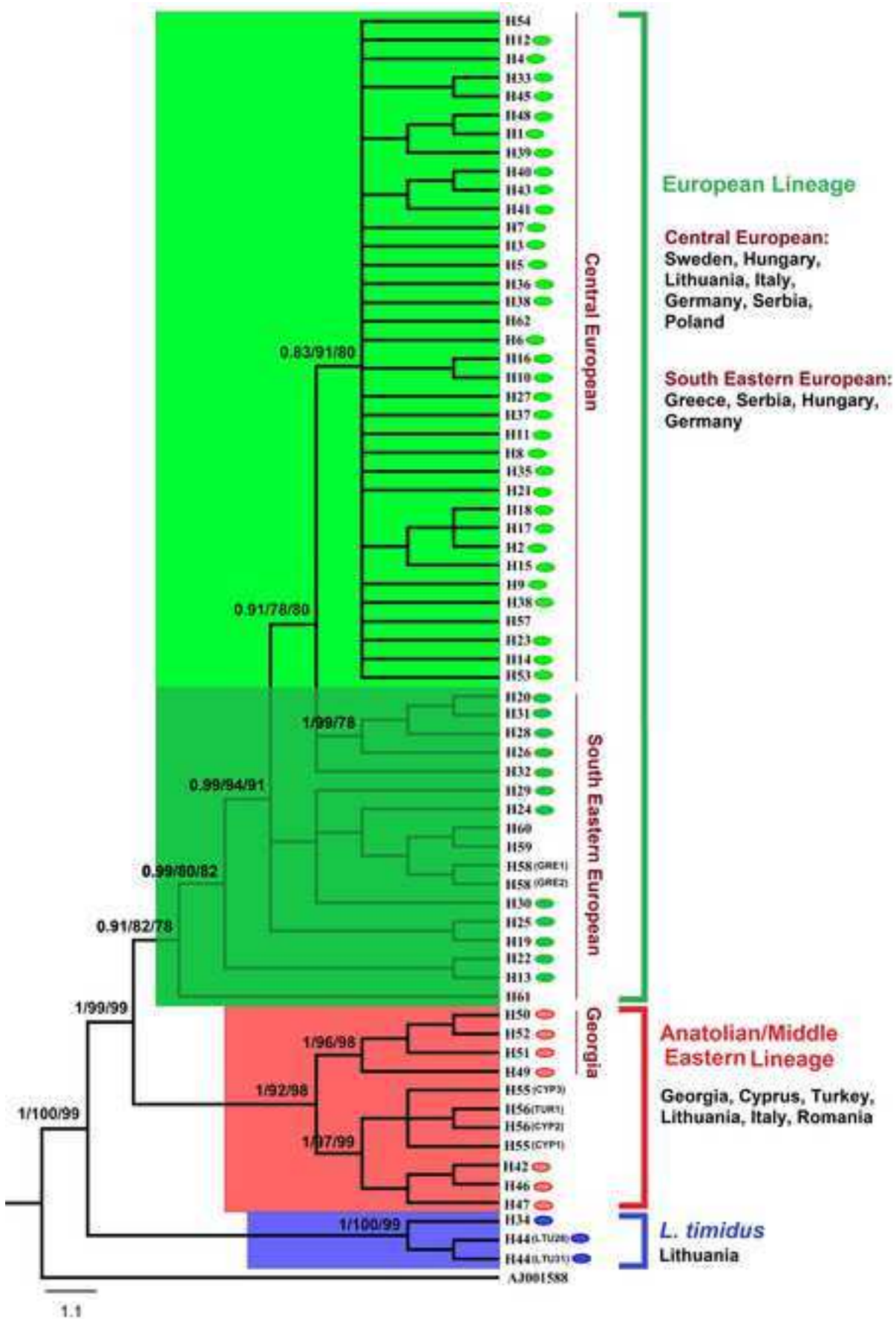
Figure 1

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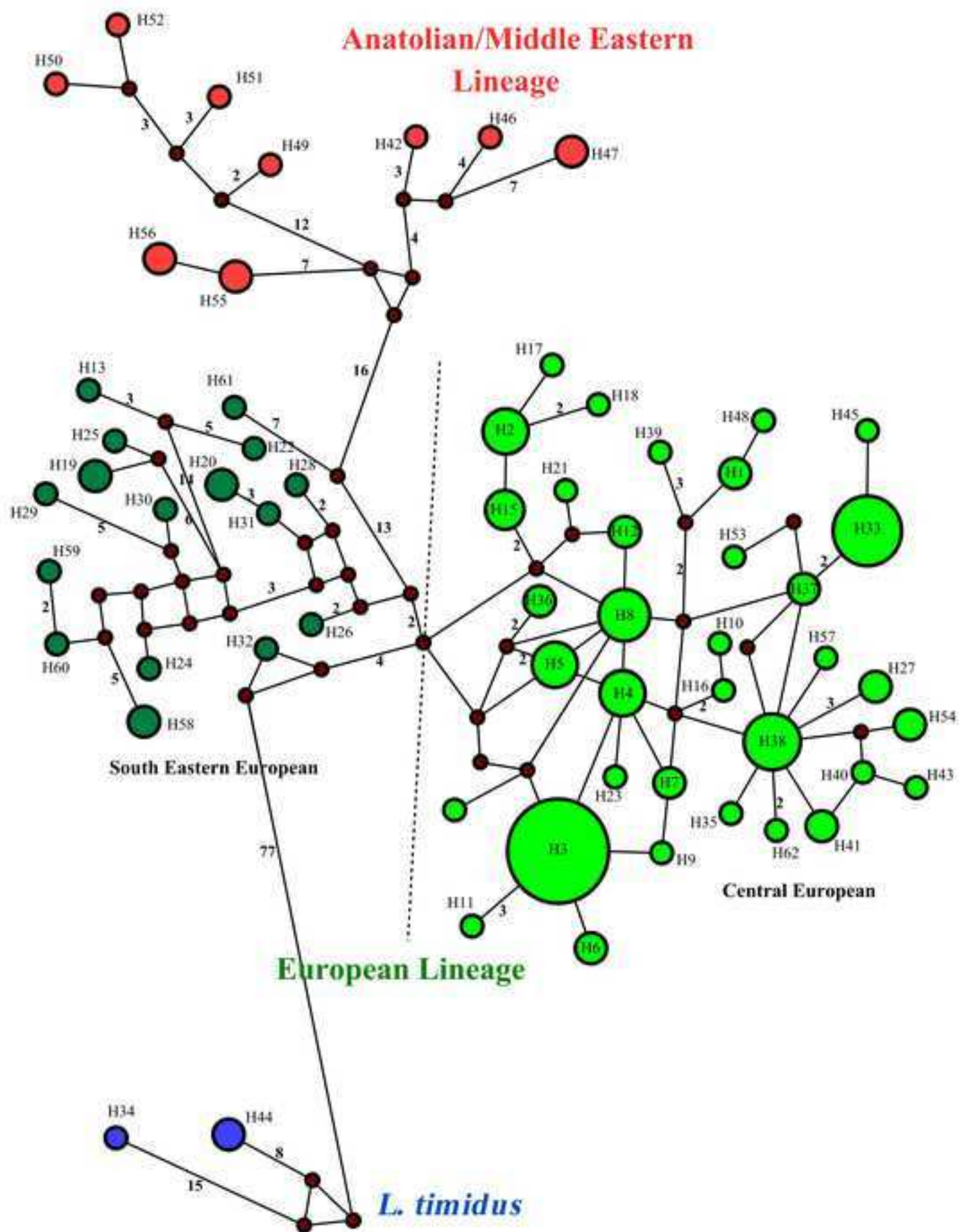
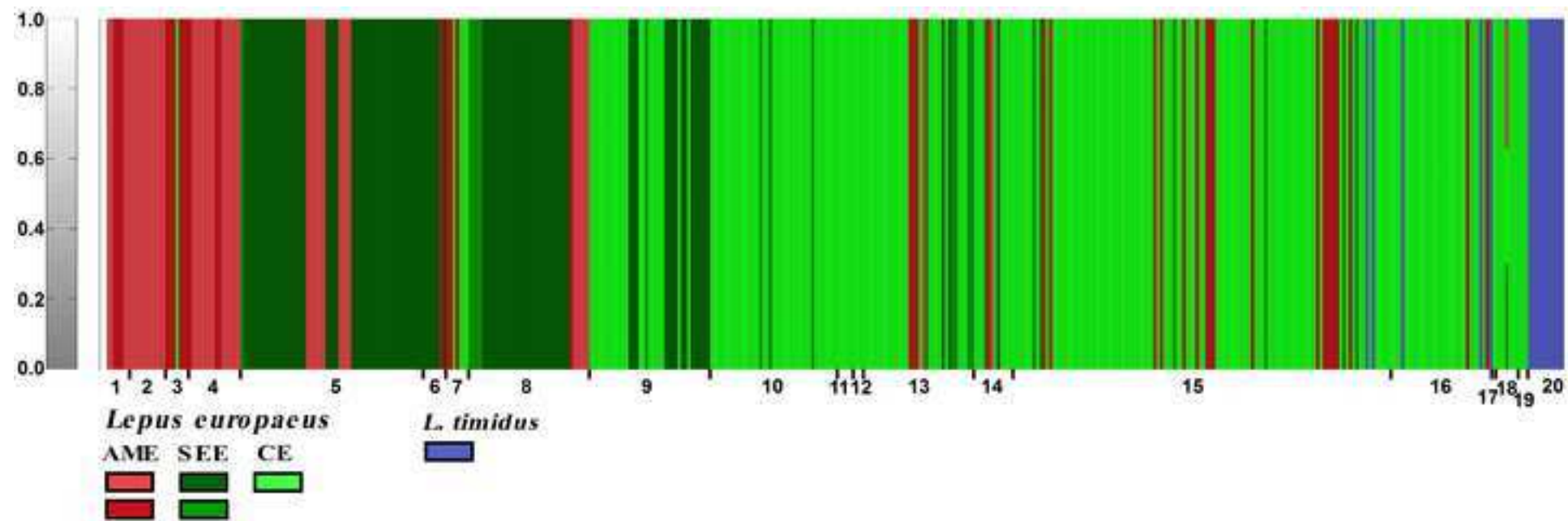


Figure 6

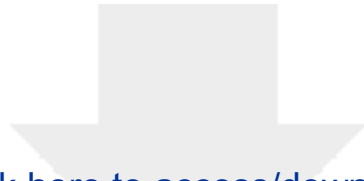




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Table S1 (358bp)\_rev.xlsx







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**Supporting Information**  
Table S2 (916bp)\_rev.xlsx



1 **Large-scale mitochondrial DNA analysis reveals new light on the phylogeography of**  
2 **Central and Eastern-European Brown hare (*Lepus europaeus* Pallas, 1778)**

3  
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28

29 Short title: Phylogeography of Central-, Eastern-European Brown hare

30

31 **Abstract**

32 European brown hare, *Lepus europaeus*, from Central and Eastern European countries  
33 (Hungary, Poland, Serbia, Lithuania, Romania, Georgia and Italy) were sampled, and  
34 phylogenetic analyses were carried out on two datasets: 1.) 137 sequences (358 bp) of control  
35 region mtDNA; and 2.) 105 sequences of a concatenated fragment (916 bp), including the  
36 cytochrome b, tRNA-Thr, tRNA-Pro and control region mitochondrial DNA. Our sequences  
37 were aligned with additional brown hare sequences from GenBank. A total of 52 and 51  
38 haplotypes were detected within the two datasets, respectively, and assigned to two previously  
39 described major lineages: Anatolian/Middle Eastern (AME) and European (EUR).  
40 Furthermore, the European lineage was divided into two subclades including South Eastern  
41 European (SEE) and Central European (CE). Sympatric distribution of the lineages of the  
42 brown hare in South-Eastern and Eastern Europe revealed contact zones there. BAPS  
43 analysis assigned sequences from *L. europaeus* to five genetic clusters, whereas CE  
44 individuals were assigned to only one cluster, and AME and SEE sequences were each  
45 assigned to two clusters. Our findings uncover numerous novel haplotypes of  
46 Anatolian/Middle Eastern brown hare outside their main range, as evidence for the combined  
47 influence of Late Pleistocene climatic fluctuations and anthropogenic activities in shaping the  
48 phylogeographic structure of the species. Our results support the hypothesis of a postglacial  
49 brown hare expansion from Anatolia and the Balkan Peninsula to Central and Eastern Europe,  
50 and suggest some slight introgression of individual haplotypes from *L. timidus* to *L.*  
51 *europaeus*.

52  
53 **Keywords:** Central-, Eastern Europe; contact zones; genetic structure; glacial refugia;  
54 phylogeography; *Lepus europaeus*

55

## 56 **Introduction**

57 The brown hare (*Lepus europaeus* Pallas, 1778) is a native species to Northern, Central,  
58 Western Europe and the Western part of Asia, and it was introduced as a game into several  
59 countries (Argentina, Australia, Barbados, Brazil, Canada, Chile, Falkland Islands, New  
60 Zealand, R union and the United States; [1]).

61 The effect of translocation on hare genome was proved by previous genetic studies and they  
62 suggested that the brown hare and the Cape hare (*Lepus capensis*) are the same species [2].  
63 However, later the same authors performed mitochondrial DNA (mtDNA) analysis and found  
64 a significant divergence between them, and therefore they are currently considered to be two  
65 different species [3]. Pierpaoli et al. [4] showed that Italian and European hares did not share  
66 any mitochondrial haplotypes, indicating the lack of interspecific gene flow between the two  
67 species due to reproductive isolation in the course of their long separate evolutionary history.  
68 They identified two main groups of Eurasian and African hare haplotypes: Clade A (*L.*  
69 *granatensis*, *L. corsicanus*, *L. timidus*) and Clade B (*L. c. mediterraneus*, *L. habessinicus*, *L.*  
70 *starcki*, *L. europaeus*). These results suggest that the three species belonging to Clade A, with  
71 a common ancestor, would have colonized Europe independently of *L. europaeus* and would  
72 have originated by isolation during the Pleistocene glaciations in the southern or northern  
73 areas of refuge.

74 It is strongly argued that the current geographical distribution of temperate species and  
75 genetic relationships among their populations have been influenced by the climatic  
76 oscillations during the Late Quaternary [5, 6]. Specifically, different lineages represent  
77 populations repeatedly isolated into distinct glacial refugia such as the Iberian, the Apennine,  
78 the Balkan Peninsulas and Turkey [5, 7-10]. Furthermore, different human activities,  
79 competition for food or breeding and hybridization between species also led to a higher  
80 diversity in the southern refugial areas and the present genetic diversity of the hares [11-13].

81 There is evidence for human-mediated translocations that is well documented in the southern  
82 part of Europe [14].

83 Previous studies ~~that were~~ based on mitochondrial DNA (mtDNA) analysis on extant brown  
84 hare populations has revealed a relatively high degree of geographic partitioning [6, 15-18].  
85 These studies distinguished two major geographically distinct lineages, the European (EUR)  
86 and the Anatolian/Middle Eastern (AME) clade. The EUR lineage is further subdivided into  
87 two subclades: the Central European (CE) and the South-East~~ern~~ European (SEE) [6]. The CE  
88 subclade includes individuals from across North-Central Europe, whereas the SEE comprises  
89 hares living in South-Eastern Europe. The second lineage, AME, includes individuals from  
90 Anatolia, South-Eastern Europe and the eastern Mediterranean Islands [17].

91 A recent study [18] found that there were three major haplogroups including Anatolia/Middle  
92 East (AMh), Balkans (BLh), and central Europe (cEUh) among brown hare populations  
93 worldwide. Additionally, three subgroups were revealed within the BLh haplogroup including  
94 South-~~E~~eastern Balkans (SEB), Southern Balkans (SB) and Greek islands excluding those  
95 harboring A-lineages (GI-B) off the Anatolian coast. Moreover, the ~~S~~south-~~E~~eastern and  
96 ~~C~~entral Balkans (SEB), comprising northeastern Greece, south and ~~N~~north-~~W~~western as well  
97 as ~~s~~South-~~C~~entral Bulgaria, north-eastern part of Republic of Northern Macedonia, ~~S~~south-  
98 ~~E~~eastern and ~~S~~south-~~W~~western Serbia, was identified as the primary source region for most  
99 other Balkan brown hare populations [18].

100 On the other hand, ~~no~~ Anatolian/Middle Eastern haplotypes have not been observed in South,  
101 Central and North-Western Greece and the rest of Europe, with the exception of one Serbian  
102 haplotype [18]. Also, ~~no~~ European haplotypes have not been reported across the entire  
103 species range in the Middle East [6, 15, 19]. Further, the existence of a contact zone between  
104 the European and Anatolian/Middle Eastern lineages was detected in Bulgaria and North-  
105 Eastern Greece [6, 10, 15].

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106 ~~D~~The detection of brown hare lineages is mostly based on the mtDNA control region (CR),  
107 and ~~it~~ is usually well supported by cytochrome b (cyt b). It ~~has been proven~~ proves that  
108 mtDNA genomic data are useful in determining phylogenetic relationships between closely  
109 related species and within species [20-21] and for understanding the extent and nature of  
110 contact zones [10].

111 Overall, despite a relatively large number of genetic studies on brown hares, their  
112 phylogenetic relationships still remain challenging. Only several broad-~~range~~ studies of  
113 phylogeography of brown hares have been done, relying on mtDNA control region sequences  
114 from Serbian, Greek and Bulgarian hares [6, 15, 18, 22-26]. Using wide-range geographic  
115 sampling over seven countries, we aimed to study (i) the extent of mitochondrial genetic  
116 variability and diversity of the brown hare in Central and Eastern Europe; (ii) the  
117 phylogeographic relationships of the studied populations, and furthermore (iii) to provide  
118 comprehensive information on the genetic characteristics of brown hares for conservation  
119 programs and management plans.

## 122 **Materials and methods**

### 123 Sample collection

124 A total of 137 legally hunted, unprotected adult brown hares were sampled in seven countries  
125 (Hungary, Poland, Serbia, Lithuania, Romania, Georgia, Italy; Fig 1, and see S1 Table)  
126 between 2010 and 2015. Also, three mountain hares have been accidentally collected along  
127 with our samples. No animals were killed for the purposes of this research.

129 **Fig 1. Spatial distribution of the European hares' maternal lineages, based on the 358-**  
130 **bp mtDNA control region, resulting when combining sequence data from GenBank (S1**

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131 **Table) and the present study.** Squares and polygons indicate the Central European and  
132 South-East European subclades, respectively, in the European lineage. Circles and triangles  
133 indicate the Anatolian/Middle Eastern lineage and Mountain hare (*L. timidus*), respectively.  
134 Ellipses depict the two discovered contact zone areas between brown hare lineages in South-  
135 Eastern and North-Eastern Europe. Filled geometric shapes indicate the geographic location  
136 of the sampling sites in this study. Colours of the geometric shapes are in accord with  
137 clades/lineages; light green: Central-European, dark green: South-East European, red:  
138 Anatolian/Middle Eastern, blue: Mountain hare.

139  
140

141 All tissue samples were stored in 96% ethanol at -4°C. ~~and Hair~~ hair follicles samples were kept  
142 in individually registered ~~in~~ nylon or paper bags and stored ~~on~~ at -4°C until the laboratory  
143 analysis. Total DNA was extracted using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek,  
144 USA), the High Pure PCR Template Preparation Kit (Roche, USA) and standard FAO  
145 protocol. DNA concentrations were evaluated spectrophotometrically and visually by  
146 standard agarose gel electrophoresis.

147 Different regions of the mitochondrial DNA were amplified. PCR protocols and primers  
148 (Le.H-Dloop\_F ~~and~~ Le.L-Dloop\_R [15] for the control region (CR) and LepCyb2L\_F ~~and~~  
149 LepD2H\_R [4] for cytochrome b (cyt b) + tRNA-Thr + tRNA-Pro + control region) were  
150 used ~~for to the~~ amplification. PCRs were carried out in a total volume of 25 µl, using the  
151 following sequence of steps: denaturation at 94 °C for 5 minutes, followed by 35 cycles of  
152 amplification 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, and a final step  
153 at 72 °C for 5 minutes. The forward sequencing reaction was performed by Macrogen Europe  
154 (The Netherlands).

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155 In addition, previously published sequences of the species were downloaded from the  
156 GenBank (S1 and S2 Tables).

157

#### 158 Ethics statement

159 Animals were not shot for the purpose of this study. The study did not involve the collection  
160 of samples from live animals. An ethics statement was not required. Samples from the  
161 different countries were obtained from licensed collaborators and licensed hunters who took  
162 samples following their regulations in brown hare management.

163

#### 164 Sequence analysis

165 Two datasets were created from the sequences. The first dataset comprised 137 CR mtDNA  
166 sequences with a total length of 358 bp. The second dataset comprised 105 concatenated  
167 sequences cyt b + tRNA-Thr + tRNA-Pro + CR, with a total length of 916 bp after alignment.

168 Alignment was performed using Seqscape 2.6 (Applied Biosystems) and ClustalW in MEGA  
169 6 [27], respectively. The aligned sequences were deposited in GenBank with the Accession  
170 numbers: MG865671-MG865724 for CR and MG841060- MG841112 for the cyt b + tRNA-  
171 Thr + tRNA-Pro + CR region (S1 and S2 Tables). The European Rabbit (*Oryctolagus*  
172 *cuniculus*) (GenBank: AJ001588) [28] was used as an outgroup for the phylogenetic analyses.

173 DAMBE 6 [29] was used to analyze substitution saturation.

174 The number of polymorphic sites, haplotype diversity, nucleotide diversity, average number  
175 of nucleotide differences for each location and number of haplotypes were estimated with  
176 DnaSP 5.10 [30]. The best-fitting partitioning scheme and nucleotide substitution model were  
177 selected using the Bayesian information criterion (BIC) and the corrected Akaike Information  
178 Criterion (AICc) implemented in PartitionFinder 2.1.1 [31].

179 Bayesian inference (BI) was performed using BEAST v2.3 [32] with 40,000,000 generations  
180 of Monte Carlo Markov chains (MCMC), sampling every 100 generations. Maximum

181 likelihood (ML) analyses were implemented in IQ-TREE 1.6 [33] with 10,000 bootstrap  
182 steps. Additionally, MEGA 6 [27] was used to construct a neighbour-joining (NJ)  
183 phylogenetic tree, applying the pairwise distance data and p-distance model with 10,000  
184 bootstrap replications. Furthermore, median-joining networks [34] among haplotypes were  
185 inferred using PopART 1.7 [35].

186 Fu's FS [36] and Tajima's D [37], performed in Arlequin 3.5 [38], were employed to assess  
187 the demographic history and to examine hypotheses of selective neutrality [39]. The  
188 significance of these tests was calculated using 10,000 permutations. The hierarchical analysis  
189 of molecular variance (AMOVA) and fixation index were implemented with 10,000 iterations  
190 using Arlequin 3.5 [38] to evaluate levels of population structure. The aim of the AMOVA  
191 [analysis](#) was to examine whether genetic variation was significantly structured among  
192 different haplogroups.  $\Phi_{ST}$  can provide an estimate of the genetic differentiation among  
193 populations in order to make inferences of past demographic changes.

194 To estimate the presence of genetic clusters (populations) within the sequences of *L.*  
195 *europaeus* and *L. timidus* (or introgressed individuals), we used Bayesian Analysis of  
196 Population Structure (BAPS) v6 [40-41] implementing the method of "clustering for linked  
197 loci" with two independent runs and  $K = 10$  repetitions. To assess introgression occurring  
198 within the populations of these two species, we performed the method of "admixture based on  
199 mixture clustering" implemented in BAPS. To provide a correct assessment of population  
200 genetic structure, it is recommended to use the admixture models, because these models are  
201 robust to an absence of admixture in the sample; reciprocally, models without admixture are  
202 not robust to the inclusion of admixed individuals in the sample [42].

203

## 204 **Results**

205 MtDNA control region sequences (358 bp)

206

207 The substitution saturation test based on both datasets (916 bp and 358 bp sequences)  
208 revealed that the base substitutions did not reach saturation, and these datasets were suitable  
209 for phylogenetic analyses.

210 For the 358 bp control region, 137 samples were sequenced from Central-Eastern Europe (S1  
211 Table). Additional sequences from Europe and the Middle East published in GenBank were  
212 included in the analyses, yielding a dataset comprising a total of 447 sequences and 259  
213 haplotypes (S1 Table). A total of 52 haplotypes were identified among the 137 new  
214 sequences, including 40 novel haplotypes and 12 previously reported haplotypes.

215 The phylogenetic analyses (BI, ML, and NJ trees) yielded relatively identical topologies,  
216 indicating that among 137 selected haplotypes from the dataset (447 individuals) two lineages  
217 were identified (Fig 2).

218

219

220 **Fig 2. Phylogenetic relationships of brown hare from Central-Eastern Europe with other**  
221 **brown hares, based on the 358-bp mtDNA control region sequences and rooted with**  
222 ***Oryctolagus cuniculus* (AJ001588).** The numbers on the branches are posterior probabilities  
223 in the Bayesian inference and bootstrap support in maximum likelihood and neighbour-  
224 joining. Colored ovals represent haplotypes identified in the current study. The branches  
225 within blue rectangular include mountain hare sequences or introgressed haplotypes of this  
226 species in other hare species. For detailed information on haplotypes see S1 Table.

227

228 The MJ network analysis (Fig 3) also supported the clusters distinguished in the phylogenetic  
229 trees. The first lineage, European (EUR), was divided into two phylogeographically distinct  
230 subclades: Central European (CE) and South-East European (SEE).

231

232

233 **Fig 3. Median joining network of brown hare from Central-Eastern Europe and other**  
234 **brown hares, based on the 358-bp mtDNA control region.** The numbers on the haplotypes  
235 (1-259) are the same haplotype codes (CR1-CR259) as in Fig 2 and S1 Table. Dark circles are  
236 connecting nodes (i.e. putative undetected haplotypes). Blue circles include mountain hare  
237 sequences or introgressed haplotypes of this species in other hare species.

238

239

240 The subclade CE was mostly distributed across various regions of Central Europe, Scotland,  
241 England, the Netherlands, France, Germany, Italy, Austria, Switzerland, Poland, Lithuania,  
242 Hungary and Northern Serbia (Fig 1). However, two individuals belonging to the subclade  
243 were found in Eastern Romania and Southern Serbia. Also, one brown hare from Cyprus  
244 (Cyprus 4 in S1 Table) clustered within CE (falling into haplotype CR40, S1 Table).  
245 Haplotype CR40 along with haplotypes CR1 and CR10 was the most common haplotype in  
246 the subclade CE and was shown to inhabit more than one region in Europe (Fig 3). Haplotype  
247 CR40 was identified as the most abundant (38 individuals) and central haplotype in the  
248 subclade, and was observed across Northern Europe, from Lithuania to Poland, Germany,  
249 France, England, and Scotland. Haplotype CR1 was observed in Poland, Hungary, Romania,  
250 Serbia, and Italy, whereas haplotype CR10 was observed in Lithuania, Poland, Hungary,  
251 Serbia, Austria, Italy and France. The subclade SEE predominantly occurred in South-Eastern  
252 Europe including Bulgaria, Greece, Republic of Northern Macedonia and Serbia (Fig 1).

253 However, individuals belonging to this subclade were also present in Hungary, Poland,  
254 Central Italy and France (Corsica Island) (Figs 1 and 2, S1 Table). Haplotypes in SEE were  
255 mostly specific to relatively limited spatial distributions (Fig 3). However, three haplotypes  
256 belonging to this subclade were recorded over a larger geographical range: CR8 (Hungary and  
257 Italy), CR32 (Serbia and Italy) and CR62 (Italy and Poland). Phylogenetic analyses revealed  
258 no shared haplotype between the subclades in this lineage.

259 The second cluster, the Anatolian/Middle Eastern lineage (AME) was predominantly present  
260 in Georgia, Turkey and the Middle East, and was also found in Lithuania, Poland, Romania,  
261 North-Eastern Greece, Republic of Northern Macedonia, Italy and France (Corsica Island)  
262 (Fig 1). Haplotypes in this lineage were mostly restricted to small geographic ranges.  
263 However, within AME, haplotypes CR52, CR53, and CR54 were recorded both in Romania  
264 and Italy, but haplotypes CR57 (Italy and Republic of Northern Macedonia) and CR200  
265 (Turkey and Cyprus) were also found in distant localities (Figs 1, 2 and 3).

266

267 MtDNA cytochrome b, tRNA-Thr, tRNA-Pro and control region (916 bp)

268 Phylogenetic analyses of the control region revealed two major lineages in Central-Eastern  
269 Europe, with contact zones discovered in the geographic range (Fig 1). To obtain a better  
270 assessment of phylogeographic structure, we sequenced the additional fragments cyt b (426  
271 bp), tRNA-Thr (66 bp) and tRNA-Pro (66 bp) of 105 brown hares from Italy, Hungary,  
272 Serbia, Georgia, Romania, Poland and Lithuania (S2 Table). Sixteen additional sequences  
273 belonging to brown hares from Germany, Sweden, Poland, Greece, Turkey and Cyprus  
274 available in GenBank were also added to the alignment (S2 Table). Finally, a total dataset  
275 comprising 124 sequences (916 bp fragment of mtDNA), corresponding to a total of 62  
276 haplotypes was used for phylogenetic analysis. According to this longer fragment, and in  
277 accordance with control region sequences, the brown hare population in Central-Eastern

278 Europe is divided into the same two distinct phylogeographic lineages (EUR and AME) (Figs  
279 4 and 5).

280

281 **Fig 4. Phylogenetic relationships of brown hare from Central-Eastern Europe with other**  
282 **brown hares, based on the 916-bp mtDNA sequences (cyt b + tRNA-Thr + tRNA-Pro +**  
283 **control region) and rooted with *Oryctolagus cuniculus* (AJ001588).** The numbers on the  
284 branches are posterior probabilities in the Bayesian inference and bootstrap support in  
285 maximum likelihood and neighbour-joining. Colored ovals represent haplotypes identified in  
286 the current study. For detailed information on haplotypes see S2 Table.

287

288 **Fig 5. Median joining network of brown hare from Central-Eastern Europe and other**  
289 **brown hares, based on the 916-bp mtDNA sequences (cyt b + tRNA-Thr + tRNA-Pro +**  
290 **control region).** For detailed information on haplotypes see Fig 4 and S2 Table. Dark circles  
291 are connecting nodes (i.e. putative undetected haplotypes).

292

293 Furthermore, brown hares belonging to the lineage EUR fall into two subclades, the same CE  
294 and SEE as in the first dataset. The contact zones among all lineages and subclades were  
295 identified in the same geographic ranges as in Fig 1.

296 A total of 51 haplotypes was found throughout Central-Eastern Europe. Moreover, 50 novel  
297 haplotypes and only one previously reported haplotype were detected among them. The  
298 genetic statistics for the sequenced brown hares in this study are displayed in Table 1.

299

300 **Table 1. Comparison of genetic statistics for the brown hares sequenced in this study,**  
301 **originating from Central-Eastern Europe, based on the 916-bp mtDNA sequences (cyt b**  
302 **+ tRNA-Thr + tRNA-Pro + control region)**

Group	n	h	Hd (SD)	Pi (SD)	K	P	Fu's FS	Tajima's D
Central European	83	32	0.927(0.019)	0.0051(0.0003)	4.71	41	-	-1.455*
							15.340**	
South-East European	14	12	0.978(0.035)	0.0153(0.0021)	14.14	52	-1.567	-0.593
Anatolian/Middle Eastern	8	7	0.964(0.077)	0.0198(0.0029)	18.32	40	-0.607	0.623

303 n, number of individuals; h, number of haplotypes; Hd, haplotype diversity; SD, Standard  
304 Deviation; Pi, nucleotide diversity (per site); K, average number of nucleotide differences; P,  
305 variable (polymorphic) sites. Statistical significance: \*p<0.05, Statistical high significance:  
306 \*\*p<0.01.

307  
308  
309  
310 High haplotype diversity values and relatively low-moderate nucleotide diversity were  
311 obtained for brown hares of the study populations. The lineage AME (only for Fu's FS) and  
312 both the subclades of lineage EUR presented negative values for Tajima's and Fu's neutrality  
313 tests, but only the outcome for the Central European subclade was found significant (D = -  
314 1.455, P = 0.045; FS = -15.34, P = 0.00) (Table 1). Thus, this subclade is likely to have  
315 undergone a recent population expansion. Results of the AMOVA revealed that the among-  
316 haplogroups component of variance (67.59%) was higher than the variation within  
317 haplogroups (32.41%) (Table 2). According to the fixation index a significant genetic  
318 structure among all haplogroups was also observed ( $\Phi_{ST} = 0.676$ , P = 0.00) (Table 2).

319

320 **Table 2. AMOVA results for three major haplogroups (AME, SEE and CE) of brown**  
 321 **hare originating from Central-Eastern Europe, based on the 916-bp mtDNA sequences**  
 322 **(cyt b + tRNA-Thr + tRNA-Pro + control region).**

Source of variation	d.f.	Percentage of variation	Fixation index ( $\Phi_{ST}$ )	p-value
Among haplogroups	2	67.59	0.676	p<0.000
Within haplogroups	101	32.41		
Total	103			

323  
 324  
 325 The analysis performed with BAPS v6 separated *L. europaeus* and *L. timidus* (and  
 326 introgressed mountain hare in other hare species) with  $K = 6$  ( $\ln(P) = -8954.5009$ ). This  
 327 analysis assigned sequences from *L. europaeus* to five genetic clusters, and *L. timidus* to only  
 328 one cluster (Fig 6). Within *L. europaeus*, sequences belonging to lineage AME and subclade  
 329 SEE (lineage EUR) were each assigned to two clusters, whereas individuals belonging to  
 330 subclade CE (lineage EUR) fell into one cluster.

331  
 332 **Fig 6. Bayesian clustering analysis of 358-bp mtDNA control region sequences from**  
 333 **brown hares (*L. europaeus*) and mountain hares (*L. timidus* and introgressed haplotypes**  
 334 **of this species in other hares) as implemented in BAPS v6, resulting in  $K = 6$ .** We  
 335 detected 5 clusters within major lineages of *L. europaeus*; 2 and 3 clusters within lineages  
 336 AME and EUR (SEE = 2 clusters; CE = 1 cluster), respectively. Also, *L. timidus* and  
 337 introgressed individuals were assigned to one cluster. Numbers 1 to 20 are the localities of  
 338 sequence data from our study and others (see S1 Table): 1. Georgia; 2. Middle East; 3.  
 339 Cyprus; 4. Turkey; 5. Greece; 6. Bulgaria; 7. Romania; 8. Republic of Northern Macedonia;



340 9. Serbia; 10. Hungary; 11. Austria; 12. Switzerland; 13. Italy; 14. France; 15. Poland; 16.  
341 Lithuania; 17. Sweden; 18. Germany; 19. The Netherlands, England and Scotland; 20. Iberian  
342 Peninsula.

343

## 344 **Discussion**

345 Previous studies estimated phylogenetic relationships among brown hare populations in  
346 Europe and the Middle East, where insufficient sampling left a relatively large gap in several  
347 geographic ranges, especially in Central-Eastern Europe. This information gap has prevented  
348 the delineation of a comprehensive picture of genetic diversity and phylogeographic structure  
349 of the species. European brown hares have been classified to two major lineages, European  
350 (EUR) and Anatolian/Middle Eastern (AME) [6, 15, 17-18] that co-exist in Republic of  
351 Northern Macedonia, ~~North-Eastern~~ Greece and Bulgaria [6, 10, 15]. In this study, we  
352 presented a relatively comprehensive dataset on mtDNA cytochrome b, tRNA-Thr, tRNA-Pro  
353 and control region fragments (a total of 916 bp) of brown hares in Central-Eastern Europe,  
354 where two datasets were used in the genetic analyses; the first dataset included a 358-bp  
355 control region sequence, whereas the second dataset covered a concatenated sequence of  
356 mtDNA fragments (the 916-bp sequence).

357 Our findings revealed a high genetic diversity within the 916-bp mtDNA sequence (105 new  
358 sequences, 51 haplotypes) of brown hares from Central-Eastern Europe, where 50 haplotypes  
359 were reported for the first time (Table 1). Phylogenetic analyses revealed two major lineages  
360 of brown hare in the study area, based on a combination of our sequences and previously  
361 published sequences (S1 and S2 Tables) for both datasets: (i) AME, which comprises  
362 individuals from Georgia, Anatolia, the Middle East and also includes some hares living in  
363 South-Eastern, North-Eastern and Central Europe, and (ii) EUR, which includes hares from  
364 Central, South-Eastern, Eastern and Northern Europe. In accordance with others [6, 15], the

365 EUR lineage is subdivided into two well-supported subclades, Central European (CE) and  
366 South-East European (SEE).

367 The significant genetic structure among brown hare haplogroups from Central-Eastern Europe  
368 was well supported by  $\Phi_{ST}$  and AMOVA (Table 2). The fixation index is a standard measure,  
369 which gives an estimate of the degree of genetic differentiation among and within  
370 populations/haplogroups [43]. In fact, the analyses demonstrated that partitioning into the  
371 major haplogroups explains 67.59% of the overall mtDNA variability and corresponds to a  
372 highly significant fixation index ( $p < 0.000$ ). The female philopatry of brown hares [16, 44]  
373 could have resulted in the formation of multigenerational matrilineal assemblages that are  
374 geographically structured [45].

375 The population structure determined by BAPS v6 partially described diversity allocation  
376 between clusters based on the control region mtDNA sequences. BAPS is known to be  
377 relatively highly efficient in identifying hidden population structures [46]. The analysis  
378 revealed five genetic clusters within the populations of *L. europaeus* and only one cluster  
379 within *L. timidus* (and introgressed) sequences. Within *L. europaeus*, individuals belonging to  
380 the major lineage AME were assigned to two clusters: (i) cluster 1, which includes brown  
381 hares from Georgia, Turkey, Cyprus, Bulgaria, Romania, Republic of Northern Macedonia,  
382 Central Italy, France (Corsica Island), Poland and Lithuania; (ii) cluster 2, which comprises  
383 brown hares living in the Middle East, Georgia, Turkey, Greece, Republic of Northern  
384 Macedonia, Central Italy and France (Corsica Island). Sequences belonging to subclade SEE  
385 (lineage EUR), within *L. europaeus*, were divided to two clusters: (i) cluster 1, including the  
386 sequences from Greece, Republic of Northern Macedonia, Serbia, Hungary, Central Italy,  
387 France (Corsica Island), Germany and Poland; (ii) cluster 2, which includes individuals from  
388 Greece, Bulgaria, Republic of Northern Macedonia, Serbia, Central Italy and France (Corsica

389 Island). It is interesting that all genetic clusters of brown hare are present in Central Italy and  
390 France (Corsica Island) (Fig 6).

391 Our findings revealed some slight introgression of individual haplotypes from *L. timidus* into  
392 *L. europaeus* only in one sample (GER4 in S1 Table) from Germany (Fig 6). Extensive  
393 introgression mtDNA and nuclear genes of mountain hare into other hares has been reported  
394 in previous studies (e.g., [47-48]). The introgression of individual genotypes among  
395 populations potentially could have resulted from recent genetic hybridization or incomplete  
396 lineage sorting of ancestral variation.

397 The contact zones among the two major lineages (and two subclades belonging to lineage  
398 EUR), interestingly, were discovered in two large areas in Central-Eastern Europe,  
399 encompassing South-Eastern (Republic of Northern Macedonia, North-Eastern Greece,  
400 Bulgaria and Romania) and North-Eastern (Lithuania and North-Eastern Poland) Europe (Fig  
401 1). While the sympatric distribution of haplotypes of lineages EUR and AME in Republic of  
402 Northern Macedonia, North-Eastern Greece and Bulgaria had already been shown by others  
403 [6, 10, 15], other overlapping distributions are characterized here for the first time. However,  
404 the region comprising Thrace and Bulgaria, which probably extends into Turkish Thrace and  
405 maybe into Anatolia is a well-known hybrid zone of Europe [5] for species that were  
406 restricted to refuge areas in the Southern Balkans and Anatolia during the Pleistocene cold  
407 stages [15].

408 Based on the combined analyses of our sequences and those of others [15; Strzala et al.  
409 unpublished, direct submission to GenBank), Polish brown hares harbour haplotypes of both  
410 lineages (and the two EUR subclades). Whereas lineage EUR (mostly the subclade CE) is  
411 widespread and predominant in Poland, another lineage is only found in the eastern part of the  
412 country. Brown hares living in Western Romania fall into the European lineage (subclade  
413 CE), whereas individuals from Eastern Romania also show haplotypes of lineage AME.

414 Overall, our data reveal overlapping EUR and AME haplotypes both in Romania and  
415 Lithuania.

416 Brown hares inhabiting Georgia exhibited high genetic diversity (dataset 1: 7 individuals, 6  
417 novel haplotypes; and dataset 2: 4 individuals, 3 new haplotypes), but only within the lineage  
418 AME. Thus, based on our data, extending the contact zone to Georgia and the Middle East, as  
419 speculated by others [6, 15] is not justified. It is interesting that among the sequences  
420 previously reported from Cyprus [15, 17], one brown hare (CYP4, listed in S1 and S2 Tables;  
421 published by [17]) shared a common haplotype (CR40 that distributes across Northern  
422 Europe; see Fig 3 and S1 Table for detailed information) of European lineage origin (subclade  
423 CE). However, the haplotype was found outside the range of Northern Europe only in Cyprus.  
424 We consider human-mediated translocations for these introgressions, as has been widely  
425 confirmed for both recent and historic times [15, 49-50]. However, more extensive samplings,  
426 especially in Eastern Europe, Balkans, north of the Black Sea and Anatolia, may reveal  
427 important phylogeographic signatures.

428 Our data confirm the presence of both subclades (CE and SEE) belonging to the lineage EUR  
429 in Hungary and Serbia. Whereas haplotypes belonging to SEE are predominant in Southern  
430 and Central Serbia, the unique sequences of CE are predominantly found in Hungary and  
431 Northern Serbia. Moreover, a recent study reported one haplotype belonging to AME among  
432 brown hares from Northern Serbia as a possible consequence of human-mediated  
433 translocations [18].

434 According to the combined analysis of our sequences and those of others [51], haplotypes  
435 belonging to lineages EUR (both subclades CE and SEE) and AME are present in Italy.  
436 Nevertheless, haplotypes belonging to CE are predominant in this country. The European  
437 brown hare is a major game species in Europe [52], and different populations of the species  
438 have been introduced in different areas, mostly for hunting. Thus, this presence of AME is

439 also probably due to human-mediated translocations, as reported in other studies (e.g., [51].  
440 Furthermore, the occurrence of *L. europaeus* in Corsica is recent and artificial, as it is known  
441 that different species of hares have been introduced in the region up to this day [53]. Overall,  
442 the presence of both major lineages (and the European subclades) of brown hare in Corsica  
443 could be the result of several human-mediated introduction events from different origins [54].  
444 Likewise, a contact zone between mountain hares (*L. timidus*) and brown hares can be  
445 observed in Lithuania, as recorded in different populations of brown hares [9, 48,55-57].  
446 The network result, in accordance with Stamatis et al. [6], showed that there are relatively  
447 close relationships between some haplotypes belonging to CE and several haplotypes from  
448 SEE (Fig 3). This finding indicates that one haplotype of the first subclade is only connected  
449 by one, so far undetected, haplotype, to another haplotype from the second subclade.  
450 However, the network analysis based on the longer sequence (916 bp) (Fig 5) does not  
451 provide strong support for this hypothesis. Overall, the close phylogenetic relationships  
452 between the two subclades SEE and CE in large geographic ranges of Europe support the idea  
453 that the brown hare colonized the current spatial ranges, when ecological conditions in these  
454 areas became suitable for the species after the Last Glacial Maximum [6, 58]. Also, the  
455 presence of a large number of unique haplotypes in South-Eastern Europe (the Balkans) and  
456 Anatolia is evidence for maintenance of a high proportion of the pre-glacial brown hare  
457 diversity in these areas during at least the last glacial period. Other studies have demonstrated  
458 the high intraspecies diversity of brown hare in these areas [6, 15, 18].  
459 We discovered large contact zones for brown hares in several countries of Central-Eastern  
460 Europe. These findings support the existence of probable glacial refugia during the LGM in  
461 some of these areas (especially in Southern Europe), where the refugial populations probably  
462 underwent genetic differentiation [8], resulting in a number of genetic clusters. Following the  
463 retreat of the glaciers, the genetically isolated populations colonized Europe and formed

464 secondary contact zones [59]. Our findings are in accordance with others [6-8, 15] who  
465 suggest the post-glacial population expansion scenario from southern refugia (such as Iberia,  
466 Italy and the Balkans, as well as Asia Minor and the Caspian/Caucasus region). Other studies  
467 [18] provide evidence for the hypothesis of an Anatolian population range expansion of the  
468 brown hare into south-eastern and south-central areas of the Balkans, which has likely acted  
469 as a potentially important source in the pattern of gene flow to southern, central and northern  
470 areas of the Balkan Peninsula. Furthermore, it is suggested that colonization of the central and  
471 western parts of Europe by brown hares started from the Northern Balkans in a postglacial  
472 expansion. However, the Balkans were the most important source of European populations,  
473 due to the lack of major geographical barriers limiting a northward expansion, compared to  
474 the Alps and the Pyrenees for the Italian and the Iberian refugia, respectively [7]. Several  
475 authors described the existence of introgression of Anatolian mtDNA in Bulgarian brown  
476 hares which most likely result of hunting management practices in recent time [6, 15, 18, 49].  
477 The colonization pattern of Central and Northern Europe from the Balkan Peninsula has also  
478 been proposed for other species such as the marbled white butterfly (*Melanargia galathea*)  
479 [60] and the wild boar (*Sus scrofa*) [61].

480 Our data, in combination with additional ones [6, 17, 48], indicate phylogenetically close  
481 relationships among brown hares throughout Central and Northern Europe, where a common  
482 haplotype (CR40 in Fig 3 and S1 Table) was identified. Furthermore, other shared haplotypes  
483 (e.g., CR1 and CR10) were found from the east (Lithuania, Romania, Serbia) to central  
484 (Poland, Hungary, Austria) and west (Italy and France) across Europe. The findings suggest  
485 that source regions for the origin of northern, western, and central populations of brown hare  
486 are probably situated in Eastern or Southern Europe. High variability of mtDNA in these  
487 probable sources support the hypothesis of gene flow in a northward and westward expansion  
488 of the identified contact zones, as Stamatis et al. [6] proposed the gene flow from north-

489 western populations of Greece into **C**entral Italy via a land bridge between the Balkans and  
490 the Italian peninsula at the end of the Pleistocene and the Holocene. Also, Stamatis et al. [6]  
491 suggested the probable pattern of gene flow northward from Italy to Switzerland and Austria,  
492 after the retreat of the southern alpine glaciers. Several studies suggested the postglacial  
493 colonization of Central and North-Western continental Europe by the brown hare of the  
494 Balkans [6, 15, 18]. Others [62] supported the postglacial recolonization of Central Europe by  
495 the Italian populations.

496 The existence of AME haplotypes in South-Eastern Europe support a sudden expansion of  
497 this lineage to Europe during the late Pleistocene via the Bosphorus land bridge that  
498 disappeared only ca. 8000 years ago with the rise of the sea level [18, 63] or some Greek  
499 islands when they were still connected to Anatolia in the late Pleistocene [15]. On the other  
500 hand, the presence of a genetic break at the border between Anatolia and the surrounding  
501 regions has been reported in different species [64].

502 Also, our data confirm the presence of AME haplotypes in North-Eastern Europe, indicating  
503 the gene flow from Anatolian/Middle Eastern brown hares into Eastern and North-Eastern  
504 Europe via west of the Black Sea or other post-glacial colonization routes, especially east of  
505 the Black Sea. Alternatively, the existence of some haplotypes out of their lineage's original  
506 homeland may be due to recent translocations. Indeed, Kasapidis et al. [15] described that the  
507 brown hares living in some Eastern Mediterranean islands (such as Crete and Cyprus) have  
508 probably been introduced by humans because these islands were cut off from the mainland  
509 more than 2.5 million years ago.

510 Neutrality tests were negative for the lineages and subclades (except in AME for the value of  
511 Tajima's D), but only the subclade CE showed a significant negative value, indicating a  
512 significant excess of rare haplotypes suggesting that the population is not under mutation-drift  
513 equilibrium due to sudden expansion [45, 65]. Also, the star-like connection pattern of

514 haplotypes (CR1, 10, 27, 36, 40, 57, and 167 in Fig 3; and H3, 8 and 38 in Fig 5) gives  
515 support to the hypothesis of population expansion [66]. Some of these haplotypes are the  
516 central and most abundant ones and are widely distributed in the study area. Thus, it is highly  
517 likely that the common and central haplotypes are ancestral haplotypes. Moreover, the  
518 patterns of high haplotype diversity along with relatively low nucleotide diversity (as found in  
519 this study) indicate sudden demographic expansion from a restricted area or a small effective  
520 population size in the recent past [65, 67]. In other words, this pattern suggests that the  
521 populations originate from closely related haplotypes.

522

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532

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747 **Supporting information**

748 **S1 Table. Details of sequences used in the phylogenetic analyses of brown hares based on**  
749 **the 358 bp mtDNA control region.**

750

751 **S2 Table. Details of sequences used in the phylogenetic analyses of brown hares based on**  
752 **the 916 bp mtDNA sequences (cytochrome b, tRNA-Thr, tRNA-Pro and control**  
753 **region).**

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