Published by Associazione Teriologica Italiana
Hystrix, the Italian Journal of Mammalogy
Volume 28 (2): 186–193, 2017
OPEN ACCESS
Available online at:
http://www.italian-journal-of-mammalogy.it
doi:10.4404/hystrix-28.2-12202

Research Article

Brown hares (*Lepus europaeus* Pallas, 1778) from the Balkans: a refined phylogeographic model

Mihajla Djan[1], Milomir Stefanović[1,*], Nevena Veličković[1], Vukan Lavadinović[2], Paulo C. Alves[3], Franz Suchentrunk[4]

[1]*Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia*
[2]*Faculty of Forestry, University of Belgrade, Kneza Višeslava 1, 11000 Belgrade, Serbia*
[3]*CIBIO/InBIO – Research Center in Biodiversity and Genetic Resources & Faculty of Sciences, University of Porto, Campus de Vairão 4485-661 Vairão, Portugal*
[4]*Research Institute of Wildlife Ecology, Department of Integrative Biology and Evolution, University of Veterinary Medicine Vienna, Savoyenstrase 1, A-1160 Vienna, Austria*

Keywords:
brown hares
Balkans
phylogeography
refugia
mtDNA
Lepus europaeus

Article history:
Received: 18 Nov 2016
Accepted: 13 Feb 2017

Acknowledgements
We would like to thank the hunters from the studied region for collecting samples. The Austrian Agency for International Cooperation in Education and Research (ÖeAD-GmbH, Vienna) provided financial support for MS, financed by the Austrian Federal Ministry of Science, Research and Economy. The Ministry of Education, Science and Technological Development of Republic of Serbia provided financial support by a post-doc grant for MD. A STSM mobility grant for MD was enabled by the European Cooperation in Science and Technology (COST) Action TD1101 "A Collaborative European Network on Rabbit Genome Biology (RGB-Net)." This work was also partially financially supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Grant No. 43002.

Abstract

The contemporary geographical distribution and genetic structure of temperate species have been strongly influenced by the climatic oscillations during the Late Quaternary. As spatial genetic reconstructions are often markedly affected by geographically meaningful sample distributions, we focused in our study on the analyses of mtDNA control region sequences of brown hares from different regions in northern, central and south-central Balkans that have so far not been covered, with the aim to delineate the most likely glacial refugia wherefrom the postglacial northward expansion into central Europe has originated. Three major haplogroups ("Anatolia/Middle East", "the Balkans", and "central Europe") were revealed with apparent south-north gradual decrease in molecular diversity indices. Moreover, phylogenetic and demographic history analyses identified the southeastern central Balkans as the putative origin for most populations from the southern and northern Balkans, while populations from central and northwestern Europe have originated from the northern Balkans.

Introduction

Brown hares from the Balkans have traditionally been assigned to several subspecies, based on fur coloration and patterns, body size, external body measurements, skull and tooth characteristics (De Beaufort, 1991). Most of the conclusions, however, rely on very limited sample sizes and on traditional data analysis. Recent molecular data revealed high differentiation in the mitochondrial DNA (mtDNA) for some regions in the Balkans, whereas nuclear DNA (nDNA) data indicate rather little overall gene pool differentiation across the whole Balkans and tentatively a decrease of genetic diversity from the southeastern Balkans towards central Europe (Hartl et al., 1993; Fickel et al., 1999, 2005, 2008; Mamuris et al., 2001, 2002; Suchentrunk et al., 2000, 2003, 2008; Kasapidis et al., 2005; Ben Slimen et al., 2005; Djan et al., 2006; Vapa et al., 2007; Stamatis et al., 2009). Genetic diversity is apparently also lower in hares from southeastern Europe compared to that in Anatolian brown hares (Sert et al., 2005, 2009). MtRFLPs and mtDNA sequences reveal a tendency towards regional substructuring in the southern Balkans (i.e., Greece) as well as ancient intraspecific introgression of Anatolian lineages in northeastern Greece and Turkish Thrace (Kasapidis et al., 2005; Sert et al., 2009; Zhelev, 2015). Introgression of Anatolian mtDNA lineages is also present in Bulgarian hares, but it is not clear to what extent this introgression is natural in Bulgaria or has been expanded artificially across the whole country by anthropogenic translocations in recent times, due to hunting management practices (Mamuris et al., 2001; Kasapidis et al., 2005; Stamatis et al., 2009; Zhelev, 2015). The latter might be true to some extent, considering the predominant presence of Anatolian lineages east of the Nestos River in the northeastern Greece, which does not appear to represent a substantial barrier to gene flow (Antoniou et al., 2013). On the other hand, data on hares from the Balkans, predominantly from Greece and Bulgaria, as compared to those of hares from other parts of Europe, strongly suggest that the postglacial recolonization of central and northwestern continental Europe by the brown hare has started from the Balkans (Kasapidis et al., 2005; Stamatis et al., 2009). However, Fickel et al. (2008) argued that postglacial colonization of central Europe has started from northern Italy and indicated the importance of the Apennine Peninsula in brown hare phylogeography.

The contemporary geographical distribution and genetic structure of temperate species have been strongly influenced by the climatic oscillations during the Late Quaternary (Hewitt, 1999). Three major refugia have been proposed for the majority of terrestrial mammals: the Iberian, the Apennine, and the Balkan Peninsulas. The Balkan Peninsula represents the main source region for postglacial recolonization of central Europe by various species, due to the lack of profound geographical barriers, in contrast to the presence of large mountain chains such as the Alps and the Pyrenees for glacial refugia on the Apennine and Iberian Peninsulas (e.g., Hewitt, 1999). A northward expansion out of the Balkans was also strongly suggested for brown hares (Kasapidis et al., 2005; Stamatis et al., 2009), but a lack of data from several parts of the Balkans did not allow detailed inferences. So far, no molecular evidence for other possible source populations (such as southwestern Ukraine) for the postglacial recolonization of central Europe is available, despite the fact that brown hares have roamed e.g. the Crimean Peninsula (together with mountain hares, *Lepus timidus*, Lopez-Martinez, 2008). Presence of mountain hares in the Balkans

*Corresponding author
Email address: milomir.stefanovic@dbe.uns.ac.rs (Milomir Stefanović)

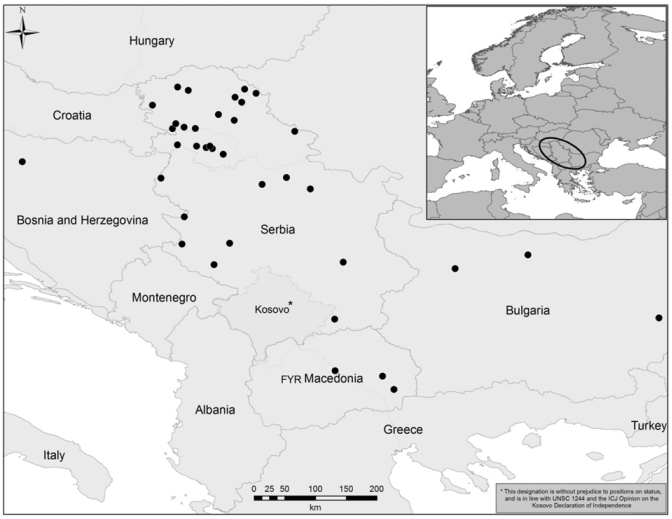

Figure 1 – Locations of brown hare samples collected in the Balkan Peninsula.

has been documented by late Pleistocene fossil records and its late Pleistocene presence in the northern Balkans is also suggested by one species-specific isozyme allele (Sdh-300) in local populations Vapa et al. (2007).

So far, mtDNA data of brown hares identified parts of the southeastern Balkans (Bulgaria and northeastern Greece) as the region with the highest nucleotide diversity, mainly due to the introgression of Anatolian lineages, but also to some extent due to the occurrence of lineages that otherwise have been found only in the northern Balkans and further north (Kasapidis et al., 2005; Stamatis et al., 2009). The published data suggest a late glacial refuge population in the central Balkans as source for the northward range expansion during the latest Pleistocene and early Holocene. However, due to limited data, no geographic delineation could be given, and the question of whether any additional source populations could have existed in the Balkans during the late Pleistocene was not addressed.

The present study focuses on the geographical distribution of mtDNA control region (CR-I) sequence variation in brown hares from different regions in the northern, central, and southern-central Balkans (Serbia, Bosnia & Herzegovina, FYROM). Moreover, we assessed their phylogenetic relationships and demographic history, integrating published CR-I sequences from other parts of the Balkans, as well as from Anatolia and central Europe. As spatial genetic reconstructions are often strongly affected by geographically meaningful sample distribution, our comprehensive sampling in previously unexplored areas should now provide the most likely phylogeographic scenario for hares from the Balkans. Following Kasapidis et al. (2005), Stamatis et al. (2009), and Antoniou et al. (2013), we expect that Anatolian lineages are largely absent in the south-central, central, and northern Balkans, whereas putative indigenous lineages from the southeastern and southern Balkans may not occur further north. That should allow us to delineate one or more late glacial refugia wherefrom the post glacial northward expansion into central Europe has originated. We also inferred demographic histories of phylogeographic units using Bayesian coalescence theory-based models to better understand spatial patterns of population history. Finally, our comprehensive sampling will also allow to assess whether ancient introgression of mountain hare (*L. timidus*)-type mtDNA into brown hares from the Balkans has happened when mountain hares roamed the Balkans during the late Pleistocene as far south as northern Greece (Smith et al., 2017).

Material and methods

Muscle tissue samples of 243 brown hares (*Lepus europaeus* Pallas, 1778) from 37 sampling localities (Fig. 1) across the Balkans were collected during regular hunts and stored at −20 °C. Total DNA was extracted using a slightly modified approach as published by Kocher et al. (1989). The entire first hypervariable region of the mitochondrial control region (CR-1) was amplified following Kasapidis et al. (2005) with the primer pair Le.H-Dloop and Le.L-Dloop. Approximately 100 ng of genomic DNA were amplified in a total volume of 50 µL containing 0.2 mM dNTP, 0.5 µM of each primer, 3 mM $MgCl_2$, 0.5 U Taq polymerase and 1× reaction buffer. PCR amplification conditions were as follows: initial step of denaturation at 94 °C for 5 min, followed by 35 cycles of amplification — each cycle being 94 °C for 60 s, 50 °C for 45 s and 72 °C for 45 s — and a final extension step at 72 °C for 5 min. The PCR products were purified following the ExoSAP protocol and sequencing was conducted using the forward primer given above. Sequences were aligned using the Clustal algorithm implemented in BioEdit 7.0.9.0. (Hall, 1999), with final adjustments by eye.

All our new sequences were further analyzed together with those published by Kasapidis et al. (2005), Stamatis et al. (2009) and Sert et al. (2009), after download from GenBank. For each of the downloaded haplotypes exact numbers of individuals were taken from the original reference. Sequences published by Pierpaoli et al. (1999), Fickel et al. (2008), Fickel et al. (unpublished; direct submission to GenBank), Andersen et al. (2009) and Strzala et al. (unpublished; direct submission to GenBank) , were, however, excluded in order to avoid too large an alignment reduction or due to the fact that the exact number of individuals per haplotype could not be retrieved from the published data. Our final data set consisted of 456 sequences with full alignment length of 412 bp, and 394 bp without sites with gaps or missing data. Due to the presence of sites with ambiguous data and inconsistent single gap positions, all subsequent analyses were performed excluding the sites with gaps or missing data.

Overall molecular diversity indices (h-haplotype diversity, π-nucleotide diversity, k-mean number of pairwise differences) were calculated using DnaSP v5.10 (Librado and Rozas, 2009). For the analysis on phylogenetic relationships among the haplotypes, a median-joining (MJ) network (Bandelt et al., 1999) was constructed with the software Network 4.6.0.0 (available at http://www.fluxus-engineering.com/sharenet.htm). All positions were equally weighted and ε parameter was set to 0. The network was rooted using haplotypes of North African cape hares (GenBank accession numbers: DQ207740-7) as an out-group. After network calculations, the additional maximum parsimony post-processing option was used to remove superfluous median vectors. Network approaches are more suitable for determining the relationships among haplotypes in intraspecific studies as they allow inferring the presence of ancestral haplotypes and putative alternative evolutionary pathways (e.g., Posada and Crandall, 2001; Zachos et al., 2010). To complement the MJ network analysis, phylogenetic relationships among haplotypes were assessed by Bayesian inference using MrBayes v3.2.2 (Ronquist et al., 2012) via the CIPRES Science Gateway v3.3 Miller et al. (2010). Four Markov chains (one cold and three heated) were run simultaneously for 50 million generations, with trees sampling every 100 generations. The HKY model with stationary state frequencies fixed to be equal was used and among-site substitution rate heterogeneity was set using an invariable and five gamma-distributed substitution rate categories, adjusting the priors to the suggested best fit nucleotide substitution model (K2P+G+I) determined by MEGA6 (Tamura et al., 2013). The posterior distributions of all parameters were examined using Tracer v.1.5 and the first 20% of sampled trees were discarded as a burn-in, while the remaining trees were used to build a 50% majority-rule consensus tree. The trees were rooted using European rabbit (*Oryctolagus cuniculus*) sequences (GenBank accession numbers: NC_001913.1) as out-group, in order to avoid low substructuring resolution which may be caused by close evolutionary relatedness of North African cape hare.

Based on the results of the MJ and Bayesian inference analyses all sequences were split into three major haplogroups: Anatolia/Middle East (AMh), Balkans (BLh) and central Europe (cEUh). Molecular diversity indices for each defined group were calculated using DnaSP v5.10 and Arlequin v3.5.1.2 (Excoffier and Lischer, 2010). Genetic differentiation among those three major haplogroups was estimated by calculating F_{ST} values, based on pairwise differences with significance

tested for 10000 permutations, as implemented in Arlequin. An analysis of molecular variance (AMOVA) was performed to examine the total genetic variation between the three major haplogroups, as a result of geographical distribution between haplotypes and their pairwise distances. Arlequin was also used to perform mismatch distribution analyses. The validity of the models was analyzed by means of the sum of squared deviations (SSD) between observed and expected mismatches. Deviations of DNA sequence variability under the expectations of neutral theory of evolution, were assessed by performing Fs (Fu, 1997) and D (Tajima, 1989) tests (using Arlequin). The program DnaSP was used to run F* and D* tests (Fu and Li, 1993), based on the estimations of segregating sites.

Demographic changes in the effective population size (Ne) over time for each major haplogroup were assessed with the coalescent-based method Bayesian Skyline Plot (Drummond et al., 2005), using BEAST v1.8 (Drummond et al., 2012) via the CIPRES portal. Three independent runs were performed, each with a 100 million generations, with sampling every 10000 generations. The strict molecular clock and piecewise-linear Bayesian skyline tree prior were used, as well as a mutation rate of 12.4% per million years (Fickel et al., 2008). Appropriate models of nucleotide substitution for each group were determined using MEGA6. The results of each independent run were combined in LogCombiner v1.7.4. Bayesian Skyline Plots were analyzed and visualized in Tracer v1.5, using the combined files for each group, with the first 10% generations discarded as a burn-in.

To get better insight into the phylogeographic patterns and molecular diversity indices across the Balkans, this haplogroup was further divided into three subgroups: Southeastern Balkans (SEB), Southern Balkans (SB) and Greek islands that harbor exclusively B-lineages (GI-B), according to the results of our MJ network analysis. Molecular diversity indices, AMOVA, mismatch distributions and neutrality tests were computed for each defined group, using Arlequin and DnaSP, following the same procedure described above. Furthermore, Bayesian skyline Plot was constructed for each of the subgroups SEB and SB, using BEAST via CIPRES, as described above.

In order to infer possible ancient introgression of mountain hare mtDNA into the presently studied brown hares, all currently obtained sequences were phylogenetically compared with mountain hare sequences published by Zachos et al. (2010) (GenBank accession numbers HM032104-HM032123). This analysis was performed using a neighbour joining (NJ) approach based on pairwise TN93 distances, with the option of uniform evolutionary rate among sites (Saitou and Nei, 1987), as implemented in MEGA6. Statistical support of internal nodes was calculated with 1000 bootstrap repetitions.

Results

The alignment of 456 CR-1 mtDNA sequences of brown hares, with final length of 394 bp, was used for the MJ network construction (Fig. 2). A total of 253 CR-1 mtDNA haplotypes (55.48%) were revealed (Table S1). Three major haplogroups could be defined in the MJ network: Anatolia/Middle East (AMh), Balkans (BLh), and central Europe (cEUh). The AM haplogroup was connected by one branch and 13 mutational steps to the BL haplogroup, while the latter was connected by two branches, each with two mutational steps, to the cEU haplogroup. The undetected haplotype (median vector) within the AMh was connected to the outgroup branch leading to North African *L. capensis* haplotypes through 41 mutational steps (Fig. 2). Our outgroup routing of the MJ network suggested that the AMh was the most ancestral among all in our study. The obtained grouping was supported by a BI tree (Fig. S2), where clusters corresponding to BLh and cEUh haplogroups were separated with posterior probability values of 100% and 99%, respectively. The AMOVA (Tab. 1) and pairwise FST values (Tab. 2) indicated the significant variation among those three haplogroups.

The molecular diversity indices for each major haplogroup, the results of the mismatch analyses and neutrality tests are displayed in Tab. 3. Our total dataset revealed 117 parsimony informative sites and 33 singletons out of a total of 150 variable sites. The haplotype diversity (h) for the total dataset was 0.987, the nucleotide diversity (π) equal to 0.035, and the average number of nucleotide differences (k) equal to 13.844.

Table 1 – Molecular variance (AMOVA) for three major haplogroups (AMh, BLh and cEUh) in *L. europaeus*.

Source of variation	% of variation	Fixation index (F_{ST})
Among haplogroups	59.85	0.598*
Between haplogroups	40.15	

* p<0.0001

The mismatch distribution did not show a statistically significant deviation from expectations under a sudden expansion model in the total dataset or in any of the three major haplogroups (Fig. S3). Negative values were obtained for all applied neutrality tests, though not all significant. Only Fu's Fs test showed statistically significant negative values for the total dataset and for each major haplogroup, supporting the "recent expansion model". Fu and Li's D* revealed a statistically significant negative value only for the total dataset, whereas the Tajima's D test showed a statistically significant negative value in the BLh.

The molecular diversity indices, the numbers of polymorphic sites, and the Ti/Tv ratios decreased from Anatolia to the Balkans and further to central Europe. As that suggests a different evolutionary timing of demographic characteristics, the BSPs were performed separately from each major haplogroup to infer demographic changes in the effective population size over time (Fig. 3), which further supported gradual demographic expansion events in northward direction and starting in the south. The distribution of the three major haplogroups is depicted in Fig. 4 for each geographic region.

According to the MJ network, the BLh is substructured into three phylogeographically meaningful subgroups, namely the "Greek Islands that harbor exclusively B-lineages" (GI-B), the "Southern Balkans" (SB), and the "Southeastern Balkans" (SEB). Whereas the SEB subgroup holds a central position within the BLh, the GI-B and the SB subgroups appear to represent younger evolutionary offshoots of the SEB subgroup (Fig. 2). The observed subgrouping of the BLh was supported by our AMOVA results (Tab. 4) and pairwise FST- statistics (Tab. 5).

The subgroup-specific mismatch analyses and Fu's Fs tests statistically supported a model of sudden expansion for each subgroup, while other neutrality tests (except for Tajima's D value in the SB subgroup) showed negative but statistically insignificant values (Tab. 6). The molecular diversity indices suggest different demographic histories for each subgroup. Since the GI-B subgroup comprised only 21 sequences, we performed BSPs separately for the SEB and SB subgroups, respectively (Fig. 5). The geographical distribution of all AMh, BLh (separately for each subgroup), and cEUh haplogroups further supported the observed phylogeographic structuring patterns (Fig. 6).

The neighbour-joining tree (not shown) of all our newly obtained haplotypes from the Balkans and the 20 CR-1 mtDNA haplotypes of *Lepus timidus* published by Zachos et al. (2010) clearly split the haplotypes into two clades, each corresponding to the lineage of one species, *Lepus europaeus* or *L. timidus*, with the node bootstrap value of 100.

Discussion

Sequence variation of the CR-1 mtDNA is commonly used to develop phylogeographic models in mammals (e.g. Durka et al., 2005 for *Castor fiber*, Zachos and Hartl, 2011 for *Cervus elaphus*, Alexandri

Table 2 – Pairwise F_{ST} values among the three defined haplogroups of *L. europeus*.

	AM	BL
Anatolia/Middle East (AMh)		
Balkans (BLh)	0.609*	
Central Europe (cEUh)	0.706*	0.415*

* p<0.001

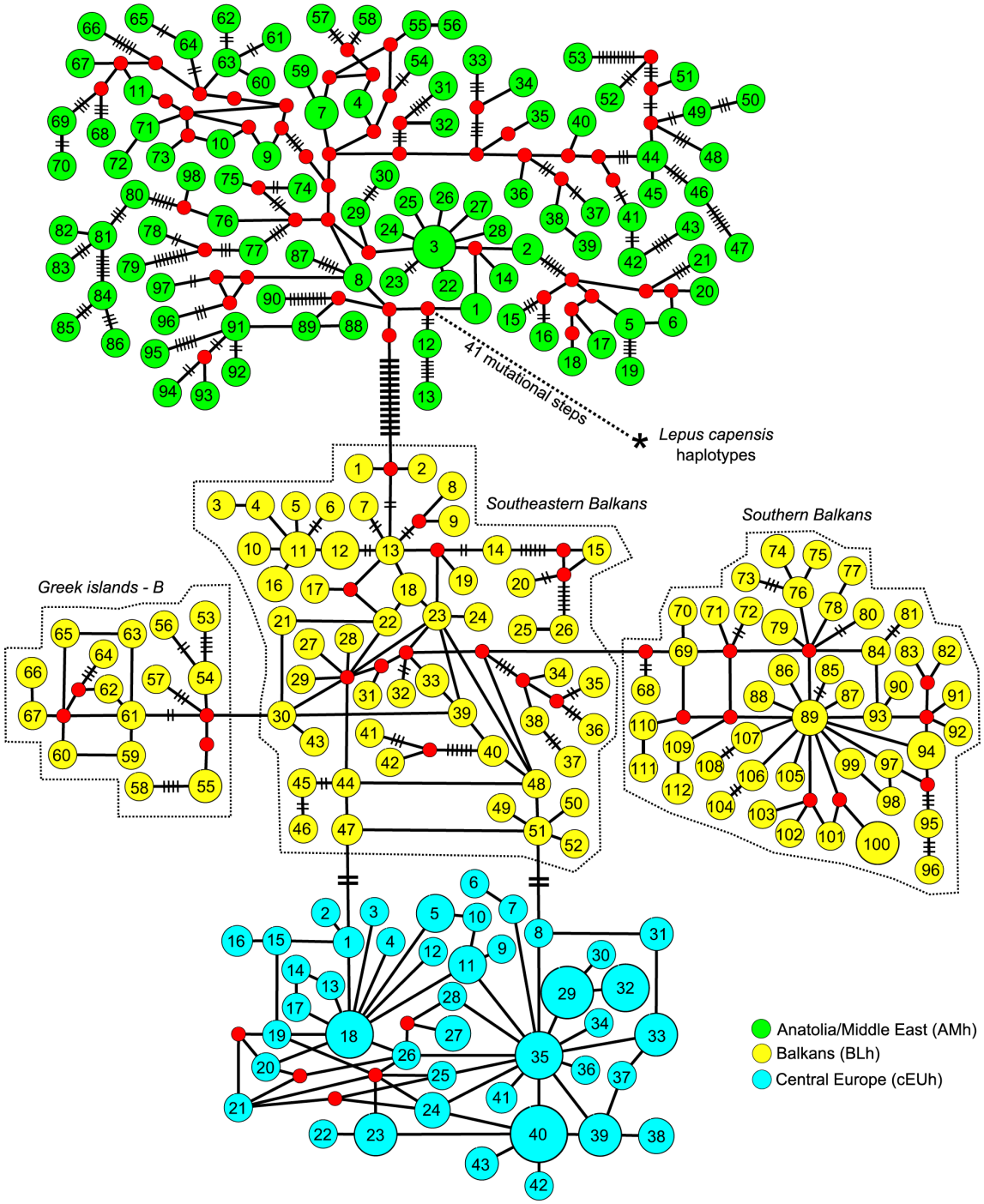

Figure 2 – Median-joining network of the mtDNA control region haplotypes of *Lepus europaeus*. Circle sizes are proportional to haplotype frequencies. Circles are coloured according to haplogroup membership as shown in the legend. Numbers of mutational steps between haplotypes are indicated by dashes, if more than one step is present. Red circles represent median vectors. Haplotype names correspond to those in Table S1.

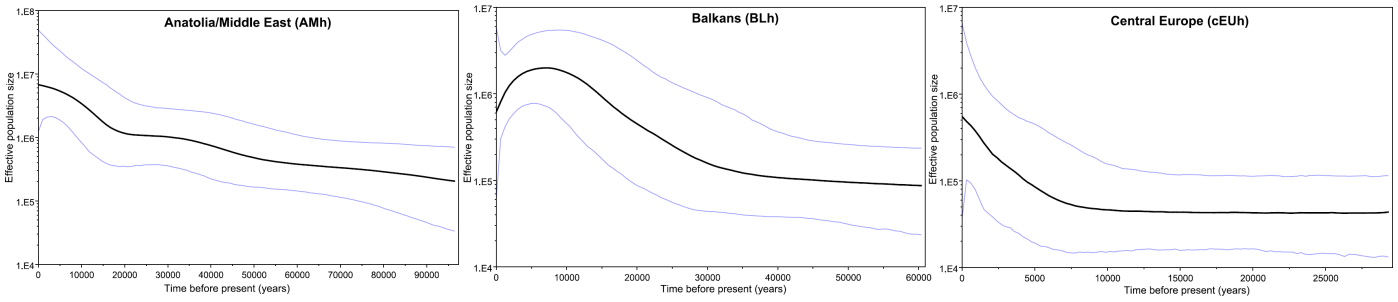

Figure 3 – Bayesian skyline plots showing the modeled demographic history of the three major haplogroups of *L. europaeus*. Black lines represent mean values of estimated population sizes, and blue lines represent the 95% highest probability density intervals.

et al., 2012 and Veličković et al., 2015 for *Sus scrofa*, Hirata et al., 2013 for *Ursus actros*; see also Avise, 2004 for general aspects). We used this marker to refine the proposed phylogeographic late Pleistocene and Holocene phylogeographic scenario for brown hares from the Balkans, by filling a critical sampling gap in the central Balkans (see Kasapidis et al., 2005; Stamatis et al., 2009). Our improved and extensive sampling led to a refined phylogeographic model of postglacial colonization of southeastern and central Europe by brown hares.

In the first phylogeographic model based on mitochondrial d-loop sequences, Kasapidis et al. (2005) found two distinct clades separating the Balkan and central European lineages from Anatolian/Middle Eastern lineages, with both of them coexisting in northeastern Greece and Bulgaria. Using a larger sampling and combining d-loop sequence and mtDNA RFLP data, Stamatis et al. (2009) concluded that postglacial colonization of Europe started from only one late glacial source region in the central or south-central Balkans. Five major RFLP-haplogroups were identified in their study: the "Anatolian/Middle East type haplogroup" (Turkey, Israel, north-eastern Greece, Bulgaria); the "south-eastern European type haplogroup" (Greece, Bulgaria, Croatia, Italy); the "European type haplogroup" — further divided into subgroup EU-A (central Europe, U.K., Spain, France, Netherlands, Germany, Bulgaria) and subgroup EU-B (Greece, Crete, Bulgaria) —; and the "intermediate haplogroup" (Greece, Bulgaria). Only in northeastern Greece and Bulgaria, haplotypes of all haplogroups were found. Thus, Stamatis et al. (2009) speculated that this region might represent the source region for the recolonization process of most other parts of Europe, but left it open to further meaningful geographical sampling.

Our MJ network supports in principle the phylogeographic scenarios as summarized above. However, our extensive sampling from the Balkans, specifically from central and northern parts, revealed additional three subgroups within the BL haplogroup: Southeastern Balkans (SEB), Southern Balkans (SB) and Greek islands excluding those harboring A-lineages (GI-B) off the Anatolian coast. These results led to the following phylogeographical interpretation: 1) ancient gene flow from Anatolia to the southeastern Balkans, 2) the southeastern central Balkans acted as source for populations both in the southern and northern Balkans, 3) populations from central and northwestern Europe are all originating from the northern Balkans, 4) those Greek islands (Crete, Kythera, Naxos) that harbour exclusively B-lineages were colonized either from the southeastern central Balkans (i.e., Bulgaria, northern and northeastern mainland Greece) or the Peloponnese recently or in historic times (by anthropogenic translocation).

Our more comprehensive sampling allowed us to identify the southeastern central Balkans (SEB) as the primary source region for most, if not all, other Balkan brown hare populations; delineated by northeastern Greece, south and northwestern as well as south-central Bulgaria, northeastern Macedonia (FYROM), southeastern and southwestern Serbia. This conclusion is supported by the central phylogenetic position of most of the haplotypes from this region in our network. Some of the haplotypes (22%) in this region represent typical Anatolian/Middle East lineages that were hypothesized as stemming from gene flow across the late Pleistocene and early Holocene land bridge across the area that is now the Sea of Marmara, the Bosphorous, and the Dardanelles (Kasapidis et al., 2005; Stamatis et al., 2009). Correspondingly, molecular diversity indices (Hd, π, k) in this region were the highest among all other studied regions, except for Anatolia and the Middle East. Moreover, our coalescence theory-based time estimates of population expansion clearly indicate the oldest expansion period (20000 BP) for this region in the Balkans, whereas brown hares from the "Southern Balkans"(Greece) started to expand slightly later (17000 BP), and brown hares from the northern Balkans, central, north-central, and western Europe expanded even much later (8000 BP). Actually, this

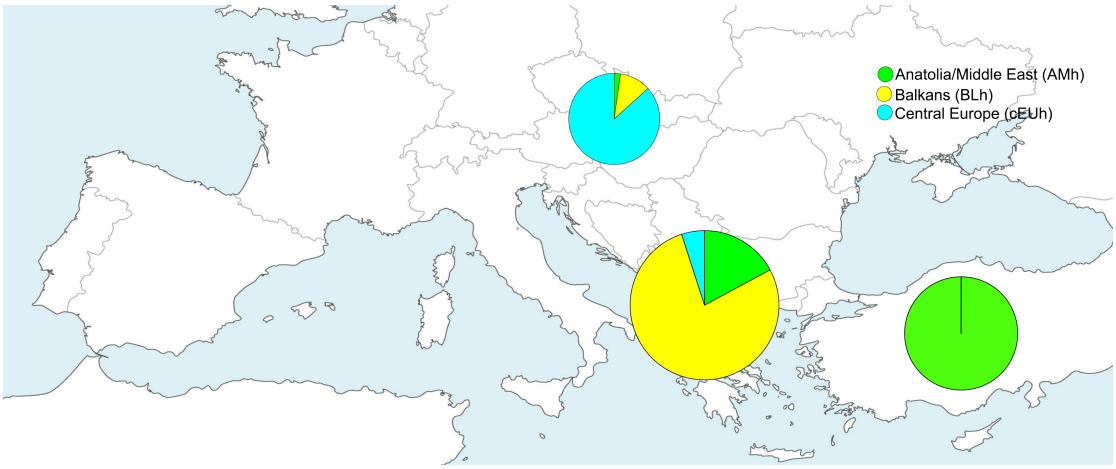

Figure 4 – Proportional distribution of brown hares assigned to one of the three haplogroups in central Europe, the Balkans, and Anatolia. Colours distinguish haplogroups within pie charts. Pie chart size is proportional to the total number of haplotypes within each region, while the size of chart slices represents the percentage of haplotypes belonging to each haplogroup.

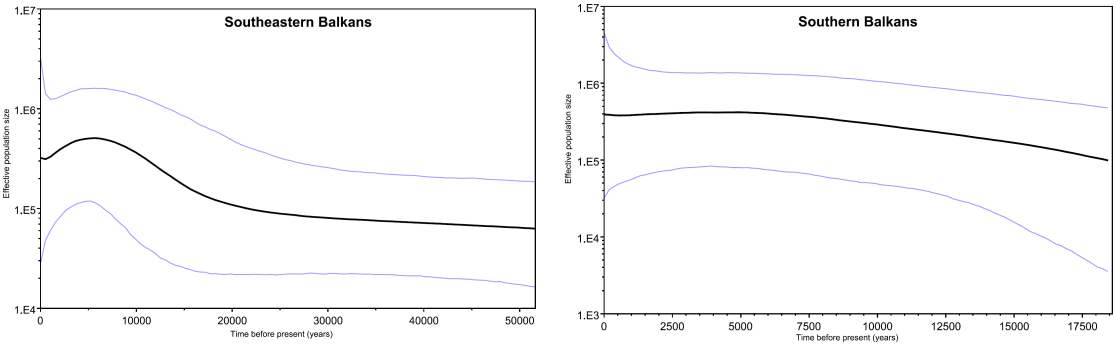

Figure 5 – Bayesian skyline plots showing the demographic history of the two groups Southeastern and Southern Balkans of *L. europaeus*. Dark lines represent medians of the population size, and blue lines represent the 95% probability intervals.

Table 3 – Molecular diversity indices, mismatch distribution analysis, and neutrality test for the three major haplogroups (AMh, BLh and cEUh) based on the CRl mtDNA sequences in *L. europaeus*.

	AMh	BLh	cEUh	All
Molecular diversity				
Number of sequences (n)	114	168	174	456
Number of haplotypes (h)	98	112	43	253
Number of polymorphic sites (S)	104	85	32	150
Transition/transversions ratio(Ti/Tv)	1.70	2.61	8.33	1.82
Haplotype diversity (h)	0.995	0.993	0.923	0.987
Nucleotide diversity (π)	0.033	0.019	0.009	0.035
Average number of pairwise differences (k)	12.672	7.517	2.772	13.844
Mismatch distribution and neutrality test				
Sum of squared deviations (SSD)	0.001	0.002	0.003	0.008
P	0.90	0.53	0.21	0.53
Tajima's D	-1.149	-1.547*	-1.229	-1.133
Fu's Fs	-24.188**	-24.620**	-26.504**	-23.610**
Fu and Li's F*	-1.424	-1.895	-1.914	-2.212
Fu and Li's D*	-1.015	-1.476	-1.955	-2.331*

AMh: Anatolia/Middle East; **BLh**: Balkans; **cEUh**: Central Europe.
* p <0.05 ** p <0.01

time schedule of geographical population expansion very nicely fits the climate and the concomitant vegetation changes within the last 20000 years from the late glacial maximum to the presence providing suitable hare habitats (Kasapidis et al., 2005). Finally, our network routed by cape hares sensu lato (*Lepus capensis*) from North Africa (Tunisia and Egypt, west of the Nile) identified the Anatolian haplotypes as ancestral to all those found in brown hares from Europe (i.e., BLh; cEUh).

Overall, our MJ network shows only one large evolutionary gap (13 substitutions) between Anatolia and all haplotypes from Europe. However, this evolutionary divergence is negligible given the maximum haplotype divergence (SEB: 16, SB: 12, GI-B: 8, cEUh: 10 substitutions) within the currently studied regions in Europe and the minimal evolutionary divergence between the most distantly related haplotypes within the BLh & cEUh network. Similarly, within the Anatolian phylogroup (Fig. 1) a minimum of 33 mutation steps were observed between the two most divergent haplotypes without major phylogenetic gaps (see also Sert et al., 2009). This currently observed pattern of intraspecific mtDNA evolution, namely relatively shallow reticulate differentiation across large geographic areas with meaningful geographical partitioning, conforms by and large to the patterns observed in cape hares, *L. capensis* s.l. from South and North Africa (Ben Slimen et al., 2008; Suchentrunk et al., 2009) and mountain hares, *L. timidus* (Hamill et al., 2006). Larger phylogenetic breaks have, however, been detected in American snowshoe hares, *L. americanus* (Melo-Ferreira et al., 2014); but no meaningful geographic differentiation of mtDNA lineages has been reported for Yarkand's hares, *L. Yarkandensis*, too (Li et al., 2006).

Table 4 – AMOVA results for the three subgroups (SEB, SB,GI-B) within the Balkans haplogroup in *L. europaeus*.

Source of variation	% of variation	Fixation index (F_{ST})
among subgroups	34.24	0.342*
within subgroups	65.76	

* p <0.0001

Table 5 – Pairwise F_{ST} values among the three defined subgroups (SEB, SB, GI-B) within the Balkans region in *L. europaeus*.

	SEB	GI-B
Southeastern Balkans (SEB)		
Greek Islands (GI-B)	0.284*	
Southern Balkans (SB)	0.295*	0.543*

* p <0.01

Our composite network architecture corresponds to an intermediate position of phylogeographic categories III ("Shallow Gene Tree – Linages Allopatric") and IV ("Shallow Gene Tree – Linages Sympatric") of Avise (2000). It translates into a continuous evolutionary dynamics across contiguous regions that have not been sundered for long periods of time and it does not particularly suggest a scenario of secondary contact. Furthermore, numerous alternative trajectories between European haplotypes within geographically meaningful haplogroups and the existence of several geographically dispersed star-like radiation centres support very recent (mitochondrial) evolutionary diversification in brown hares from Europe. Our demographic history simulations support this interpretation.

One haplotype from northern Serbia collapsed to the AM haplogroup, and two from Pleven (Bulgaria) grouped into the cEU haplogroup. For those cases we suggest human-mediated translocations, as widely known to have occurred in historic times and as still being carried out (e.g, Mamuris et al., 2001, 2002; Kasapidis et al., 2005; Suchentrunk et al., 1998, 2001, 2006). Mamuris et al. (2001) showed that mtDNA of hares released from a breeding station into a wild population has been passed over to the next generations. Assuming such translocation events for the above mentioned samples would not alter essentially our phylogeographic conclusions. Likewise, we did not find any mtDNA introgression from mountain hares, *L. timidus*, into the brown hares in our study, as reported in other brown hare populations (e.g. Alves et al., 2003; Suchentrunk et al., 2005; Melo-Ferreira et al., 2009, 2012; Thulin et al., 2006). Such introgression could have happened after the late glacial maximum or early Holocene when Mountain hares roamed the Balkans as far south as what is currently northern Greece (Vapa et al., 2007).

In conclusion, based on a geographically meaningful data set, all our results are congruent in supporting that brown hares from Anatolia have spread to southeastern and southcentral parts of the Balkans that

Table 6 – Molecular diversity indices and parameters of mismatch distribution analysis among defined groups of brown hares sampled across the Balkans based on the mtDNA control region sequences.

	SEB	SB	GI-B
Molecular diversity			
Number of sequences (n)	75	72	21
Number of haplotypes (h)	52	45	15
Number of polymorphic sites (S)	52	47	26
Transition/transversions ratio(Ti/Tv)	3.0	3.9	7.67
Haplotype diversity (h)	0.986	0.978	0.962
Nucleotide diversity (π)	0.019	0.011	0.016
Average number of pairwise differences (k)	7.357	4.103	5.371
Mismatch distribution and neutrality tests			
Sum of squared deviations (SSD)	0.002	0.004	0.001
P	0.69	0.10	0.98
Tajima's D	-1.101	-1.894*	-0.985
Fu's Fs	-24.942**	-25.878**	-5.583**
Fu and Li's F*	-1.032	-1.920	-0.783
Fu and Li's D*	-0.662	-1.373	-0.617

SEB: Southeastern Balkans; **SB**: Southern Balkans; **GI-B**: Greek Islands.
* p <0.05 ** p <0.01

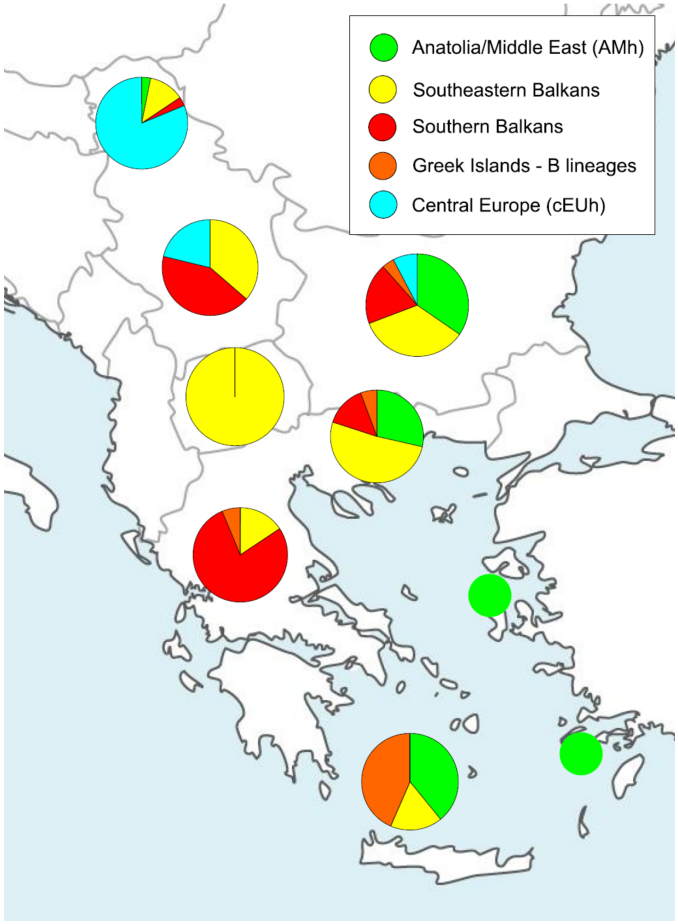

Figure 6 – Geographical distribution of mtDNA control region haplotypes of *L. europaeus* in the Balkan Peninsula among countries where samples originated from. Colours distinguish major haplogroups and groups within the Balkans haplogroup within pie charts as indicated in figure legend. Pie chart size is not proportional to the total number of haplotypes within each region, while size of chart slices represents percentage of haplotypes belonging to each group.

has further acted as a source region for gene flow to southern as well as central and northern parts of the Balkan Peninsula. Central and western European populations of brown hares have originated from the northern Balkans in a postglacial expansion, with no signal of old ancestry. However, according to Strzała et al. (2008), hares from southeastern Poland harbor the highest genetic diversity among all studied hares from central Europe, which suggests gene flow from further eastern populations into eastern central Europe. To clarify the phylogeographic patterns in brown hares from eastern Europe a more comprehensive study based on comprehensive geographical samples (e.g., Romania, Moldova, Ukraine, Russia) is necessary.

References

Andersen L.W., Fredsted T., Wincentz T., Pertoldi C., 2009. Brown hares on the edge: genetic population structure of the Danish brown hare. Acta Theriol. 54: 97–110.

Antoniou A., Magoulas A., Platis P., Kotoulas G., 2013. Assessing the genetic landscape of a contact zone: the case of European hare in northeastern Greece. Genetica 141(1–3): 23–40.

Alexandri P., Triantafyllidis A., Papakostas S., Chatzinikos E., Platis P., Papageorgiou N., Larson G., Abatzopoulos T.J., Triantaphyllidis C., 2012. The Balkans and the colonization of Europe: the post-glacial range expansion of the wild boar, *Sus scrofa*. J. Biogeogr. 39(4): 713–723.

Alves P.C., Ferrand N., Suchentrunk F., Harris D.J., 2003. Ancient introgression of *Lepus timidus* mtDNA into *L. granatensis* and *L. europaeus* in the Iberian peninsula. Mol. Phylogent. Evol. 27: 70–80.

Avise J.C., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.

Avise J.C., 2004. Molecular markers, natural history and evolution. Sinauer, Sunderland.

Bandelt H.J., Forster P., Röhl A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16: 37–48.

Ben Slimen H., Suchentrunk F., Memmi A., Ben Ammar Elgaaied A., 2005. Biochemical genetic relationships among Tunisian hares (*Lepus* sp.), South African cape hares (*L. capensis*), and European brown hares (*L. europaeus*). Biochem. Genet. 43: 577–596.

Ben Slimen H., Suchentrunk F., Elgaaied A.B.A., 2008. On shortcomings of using mtDNA sequence divergence for the systematics of hares (genus *Lepus*): an example from cape hares. Mamm. Biol. 73(1): 25–32.

De Beaufort F., 1991. La faune des mammiferes de Grece: caracteristiques, endemisme, particularismes. Biol. Gallo-Hell. 18: 99–106.

Djan M., Obreht D., Vapa L., 2006. Polymorphism of mtDNA regions in brown hare (*Lepus europaeus*) populations from Vojvodina (Serbia and Montenegro). Eur. J. Wildl. Res. 52: 288—291.

Drummond A.J., Rambaut A., Shapiro B., Pybus O.G., 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol. Biol. Evol. 22(5): 1185–1192.

Drummond A.J., Suchard M.A., Dong X., Rambaut A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29: 1969–1973.

Durka W., Babik W., Ducroz J.F., Heidecke D., Rosell F., Samjaa R., Saveljev A.P., Stubbe A., Ulevicius A., Stubbe M., 2005. Mitochondrial phylogeography of the Eurasian beaver *Castor fiber*. Mol. Ecol. 14(12): 3843–3856.

Excoffier L., Lischer H.E., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10: 564–567.

Fickel J., Hauffe H., Pecchioli E., Soriguer R., Vapa Lj., Pitra C., 2008. Cladogenesis of the European brown hare (*Lepus europaeus* Pallas, 1778). Eur. J. Wildl. Res. 54(3): 495–510.

Fickel J., Lieckfeld D., Pitra C., 1999. Analyse der genetischen Diversität und Struktur in benachbarten Populationen des Feldhasen (*Lepus europaeus*, Pallas 1778). Z. Jagdwiss. 45: 230–237.

Fickel J., Schmidt A., Putze M., Spittler H., Ludwig A., Streich W.J., Pitra C., 2005. Genetic structure of populations of European brown hare: implications for management. J. Wildl. Manage. 69: 760–770.

Fu Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915–925.

Fu Y.X., Li W.H., 1993. Statistical tests of neutrality of mutations. Genetics 133: 693–709.

Gökaşan E., Demirbağ E., Oktay F.Y., Ecevitoglu B.S., Şimşek M., Yüce H., 1996. On the origin of the Bosporus. Mar. Geol. 140: 183–199.

Hall T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95–98.

Hamill R.M., Doyle D., Duke E.J., 2006. Spatial patterns of genetic diversity across European subspecies of the mountain hare, *Lepus timidus* L. Heredity 97(5): 355–365.

Hartl G.B., Suchentrunk F., Nadlinger K., Willing R., 1993. An integrative analysis of genetic differentiation in the brown hare *Lepus europaeus* based on morphology, allozymes and mitochondrial DNA. Acta Theriol. 38(Suppl. 2): 33–57.

Hewitt G.M., 1999. Post-glacial re-colonization of European biota. Biol. J. Linnean Soc. 68: 87–112.

Kasapidis P., Suchentrunk F., Magoulas A., Kotoulas G., 2005. The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (*Lepus europaeus*) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations. Mol. Phylogenet. Evol. 34: 55–66.

Hirata D., Mano T., Abramov A.V., Baryshnikov G.F., Kosintsev P.A., Vorobiev A.A., Raichev E.G., Tsunoda H., Kaneko Y., Murata K., Fukui D., Masuda R., 2013. Molecular phylogeography of the Brown Bear (*Ursus arctos*) in northeastern Asia based on analyses of complete mitochondrial DNA sequences. Mol. Biol. Evol. 30(7): 1644–1645.

Kocher T.D., Thomas W.K., Meyer A., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sc. USA 6106–6200.

Li Z., Xia L., Li Y., Yang Q., Liang M., 2006. Mitochondrial DNA variation and population structure of the yarkand hare *Lepus yarkandensis*. Acta Theriol. 51(3): 243–253.

Librado P., Rozas J., 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

Lopez-Martinez N., 2008. The Lagomorph Fossil Record and the Origin of the European Rabbit. In: Alves P.C., Ferrand N., Hacklander K. (Eds). Lagomorph Biology: Evolution, Ecology and Conservation. Springer, Heidelberg and Berlin, pp. 27–46.

Mamuris Z., Sfougaris A.I., Stamatis C., 2001. Genetic structure of Greek brown hare (*Lepus europaeus*) populations as revealed by mtDNA RFLP-PCR analysis implications for conserving genetic diversity. Biol. Conserv. 101: 187–196.

Mamuris Z., Sfougaris A.I., Stamatis C., Suchentrunk F., 2002. Genetic structure of Greek brown hare (*Lepus europaeus*) populations based on the random amplified polymorphic DNA (RAPD) method. Biochem. Genet. 40: 323–338.

Melo-Ferreira J., Alves P.C., Freitas H., Ferrand N., Boursot P., 2009. The genomic legacy from the extinct *Lepus timidus* to the three hare species of Iberia: contrast between mtDNA, sex chromosomes and autosomes. Mol. Ecol. 18: 2643–2658.

Melo-Ferreira J., Boursot P., Carneiro M., Esteves P.J., Farelo L., Alves P.C., 2012. Recurrent introgression of mitochondrial DNA among hares (*Lepus* spp.) revealed by species-tree inference and coalescent simulations. Syst. Biol. 61(3): 367–381.

Melo-Ferreira J., Seixas F.A., Cheng E., Mills L.S., Alves P.C., 2014. The hidden history of the snowshoe hare, *Lepus americanus*: extensive mitochondrial DNA introgression inferred from multilocus genetic variation. Mol. Ecol. 23(18): 4617–4630.

Miller M.A., Pfeiffer W., Schwartz T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetics trees. In Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA, pp. 1–8.

Pierpaoli M., Riga F., Trocchi V., Randi E., 1999. Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. Mol. Ecol. 8: 1805–1817.

Posada D., Crandall K.A., 2001. Intraspecific phylogenetics: Trees grafting into networks. Trends Ecol. Evol. 16(1): 37–45.

Rogers A.R., Harpending H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9(3): 552–569.

Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 539–542.

Saitou N., Nei M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Sert H., Ben Slimen H., Erdogan A., Suchentrunk F., 2009. Mitochondrial HVI sequence variation in Anatolian hares (*Lepus europaeus* Pallas, 1778). Mamm. Biol. 74: 286–297.

Sert H., Suchentrunk F., Erdogan A., 2005. Genetic diversity in brown hares (*Lepus europaeus* Pallas, 1778) from Anatolia and differentiation among Anatolian and European populations. Mamm. Biol. 70: 171–186.

Smith S., Sandoval-Castellanos E., Lagerholm V.K., Napierala M., Sablin M., Von Seth J., Fladerer F.A., Germonpré M., Wojtal P., Miller R., Stewart J.R., Dalén L., 2017. Non-receding hare lines: genetic continuity since the Late Pleistocene in European mountain hares (*Lepus timidus*). Biol. J. Linnean Soc. 120: 891–908.

Stamatis C., Suchentrunk F., Moutou K.A., Giacometti M., Haerer G., Djan M.,Vapa L., Vukovic M., Tvrtkovic N., Sert H., Alves P.C., Mamuris Z., 2009. Phylogeography of the Brown hare (*Lepus europaeus* Pallas, 1778) in Europe: a legacy of south-eastern Mediterranean refugia? J. Biogeogr. 36: 515–528.

Strzała T., Stamatis C., Kosowska B., Moska M., Marszałek-Kruk B., Mamuris Z., 2008. Genetic variability of the Polish brown hare (*Lepus europaeus*) based on PCR-RFLP mtDNA analysis (preliminary results). EJPAU 11(1).

Suchentrunk F., Ben Slimen H., Kryger U., 2009. Molecular evidence of conspecificity of South African hares conventionally considered *Lepus capensis* L., 1758. Mamm. Biol. 74(5): 325–343.

Suchentrunk F., Ben Slimen H., Sert H., 2008. Phylogenetic aspects of nuclear and mitochondrial gene-pool characteristics of South and North African cape hares (*Lepus capensis*) and European brown hares (*L. europaeus*). In: Alves P.C., Ferrand N., Hacklander K. (Eds). Lagomorph Biology: Evolution, Ecology and Conservation. Springer, Heidelberg and Berlin, pp.65–85.

Suchentrunk F., Ben Slimen H., Stamatis C., Sert H., Scandura M., Apollonio M., Mamuris Z., 2006. Molecular approaches revealing prehistoric, historic, or recent translocations and introductions of hares (Genus *Lepus*) by humans. Hum. Evol. 21: 151–165.

Suchentrunk F., Hartl G.B., Flux J.E.C., Parkes J., Haiden A., Tapper S., 1998. Allozyme heterozygosity and fluctuating asymmetry in brown hares *Lepus europaeus* introduced to New Zealand: Developmental homeostasis in populations with a bottleneck history. Acta Theriol. Suppl. 5: 35–52.

Suchentrunk F., Jaschke C., Haiden A., 2001. Little allozyme and mtDNA variability in brown hares (*Lepus europaeus*) from New Zealand and Britain - A legacy of bottlenecks? Mamm. Biol. 66: 48–59.

Suchentrunk F., Mamuris Z., Sfougaris A.I., Stamatis C., 2003. Biochemical genetic variability in brown hares (*Lepus europaeus*) from Greece. Biochem. Genet. 41: 127–140.

Suchentrunk F., Mamuris Z., Stamatis C., Ben Slimen H., Hacklander K., Haerer G., Giacometti M., 2005. Introgressive hybridization in wild living mountain hares (*L. timidus varronis*) and brown hares (*L. europaeus*) and morphological consequences. Mamm. Biol. 7: 39–40.

Suchentrunk F., Michailov C., Markov G., Haiden A., 2000. Population genetics of Bulgarian brown hares *Lepus europaeus*: allozymic diversity at zoogeographical crossroads. Acta Theriol. 45: 1–12.

Tajima F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Thulin C.G., Fang M., Averianov A.O., 2006. Introgression from *Lepus europaeus* to *L. timidus* in Russia revealed by mitochondrial single nucleotide polymorphisms and nuclear microsatellites. Hereditas 143: 68–76.

Vapa Lj., Djan M., Obreht D., Hammer S., Suchentrunk F., 2007. Allozyme variability of brown hares (*Lepus europaeus*) from the Vojvodina (Serbia and Montenegro), compared to central and southeastern European populations. Acta Zool. Acad. Sci. Hung. 53(1): 75–87.

Veličković N., Djan M., Ferreira E., Stergar M., Obreht D., Maletić V., Fonseca C., 2015. From north to south and back: the role of the Balkans and other southern peninsulas in the recolonization of Europe by wild boar. J. Biogeogr. 42(4): 716–728.

Yaltirak C., Alpar B., Sakinc M., Yuce H., 2000. Origin of the strait of Canakkale (Dardanelles): regional tectonics and the Mediterranean-Marmara incursion. Mar. Geol. 164: 139–156.

Zachos F.E., Hartl G.B., 2011. Phylogeography, population genetics and conservation of the European red deer *Cervus elaphus*. Mamm. Rev. 41(2): 138–150.

Zachos F.E., Ben Slimen H., Hackländer K., Giacometti M., Suchentrunk F., 2010. Regional genetic in situ differentiation despite phylogenetic heterogeneity in Alpine mountain hares. J Zool 282: 47–53.

Zhelev C., 2015. Status and influence of some ecological factors on the stocks of brown hare (*Lepus capensis* Linnaeus, 1758) in lowland habitats in Bulgaria. PhD thesis, Faculty of Forestry, Sofia, Bulgaria.

Associate Editor: R. Caniglia

Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 List of mtDNA control region haplotypes detected in 456 sequences of *L. europaeus*, with names (H), frequency (f) and sampling locality for each haplotype.

Figure S2 Bayesian phylogenetic tree showing relationships among 253 haplotypes of *L. europaeus*. The trees were rooted using the *Oryctolagus cuniculus* sequence (AN: NC_001913.1).

Figure S3 Observed and expected mismatch distributions in (a) Anatolia/Middle East (AMh); (b) Balkans (BLh) and (c) central Europe (cEUh) brown hare major haplogroups.