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

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

Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

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

Materials and methods

Sample collection

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

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

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

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

Different regions of the mitochondrial DNA were amplified. PCR protocols and primers (Le.H-Dloop_F, Le.L-Dloop_R [15] for the control region (CR) and LepCyb2L_F, LepD2H_R [4] for cytochrome b (cyt b) + tRNA-Thr + tRNA-Pro + control region) were used to the amplification. PCRs were carried out in a total volume of 25 µl, using the following sequence of steps: denaturation at 94 °C for 5 minutes, followed by 35 cycles of amplification 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, and a final step at 72 °C for 5 minutes. The forward sequencing reaction was performed by Macrogen Europe (The Netherlands).

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

Ethics statement

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

Sequence analysis

Two datasets were created from the sequences. The first dataset comprised 137 CR mtDNA sequences with a total length of 358 bp. The second dataset comprised 105 concatenated sequences cyt b + tRNA-Thr + tRNA-Pro + CR, with a total length of 916 bp after alignment. Alignment was performed using Seqscape 2.6 (Applied Biosystems) and ClustalW in MEGA 6 [27], respectively. The aligned sequences were deposited in GenBank with the Accession numbers: MG865671-MG865724 for CR and MG841060- MG841112 for the cyt b + tRNA-Thr + tRNA-Pro + CR region (S1 and S2 Tables). The European Rabbit (Oryctolagus cuniculus) (GenBank: AJ001588) [28] was used as an outgroup for the phylogenetic analyses. DAMBE 6 [29] was used to analyze substitution saturation.

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

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

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

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

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

Results

MtDNA control region sequences (358 bp)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 1. Comparison of genetic statistics for the brown hares sequenced in this study, originating from Central-Eastern Europe, based on the 916-bp mtDNA sequences (cyt b + 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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

that different species of hares have been introduced in the region up to this day [53]. Overall, the presence of both major lineages (and the European subclades) of brown hare in Corsica could be the result of several human-mediated introduction events from different origins [54]. Likewise, a contact zone between mountain hares (L. timidus) and brown hares can be observed in Lithuania, as recorded in different populations of brown hares [9, 48,55-57].

The network result, in accordance with Stamatis et al. [6], showed that there are relatively close relationships between some haplotypes belonging to CE and several haplotypes from SEE (Fig 3). This finding indicates that one haplotype of the first subclade is only connected by one, so far undetected, haplotype, to another haplotype from the second subclade. However, the network analysis based on the longer sequence (916 bp) (Fig 5) does not provide strong support for this hypothesis. Overall, the close phylogenetic relationships between the two subclades SEE and CE in large geographic ranges of Europe support the idea that the brown hare colonized the current spatial ranges, when ecological conditions in these areas became suitable for the species after the Last Glacial Maximum [6, 58]. Also, the presence of a large number of unique haplotypes in South-Eastern Europe (the Balkans) and Anatolia is evidence for maintenance of a high proportion of the pre-glacial brown hare diversity in these areas during at least the last glacial period. Other studies have demonstrated the high intraspecies diversity of brown hare in these areas [6, 15, 18].

We discovered large contact zones for brown hares in several countries of Central-Eastern Europe. These findings support the existence of probable glacial refugia during the LGM in some of these areas (especially in Southern Europe), where the refugial populations probably underwent genetic differentiation [8], resulting in a number of genetic clusters. Following the retreat of the glaciers, the genetically isolated populations colonized Europe and formed secondary contact zones [59]. Our findings are in accordance with others [6-8, 15] who suggest the post-glacial population expansion scenario from southern refugia (such as Iberia,

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

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

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

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

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

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

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

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744

Supporting information

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

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

Figure 1

Click here to download Figure Figure 1_Rev.jpg

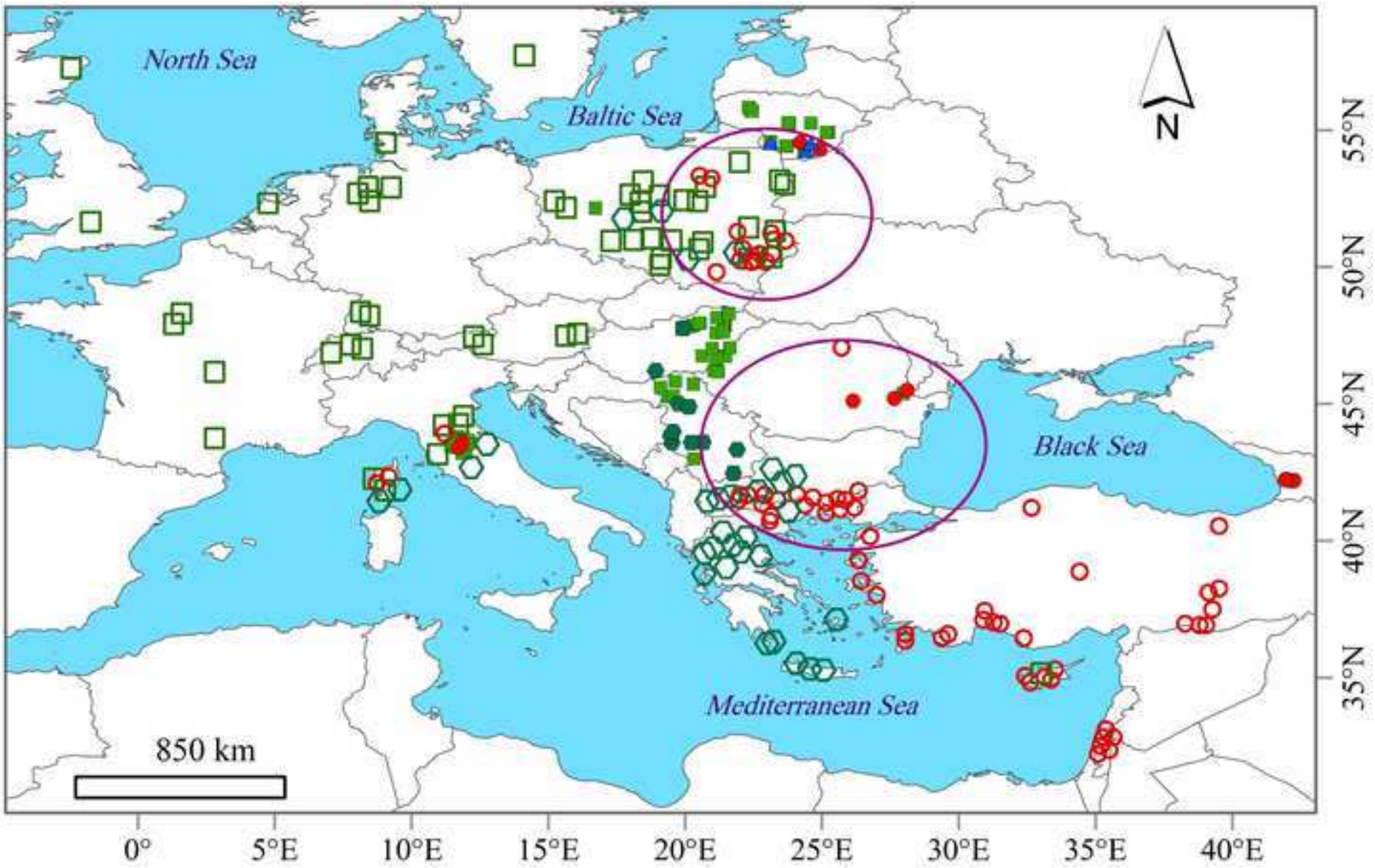

Figure 2

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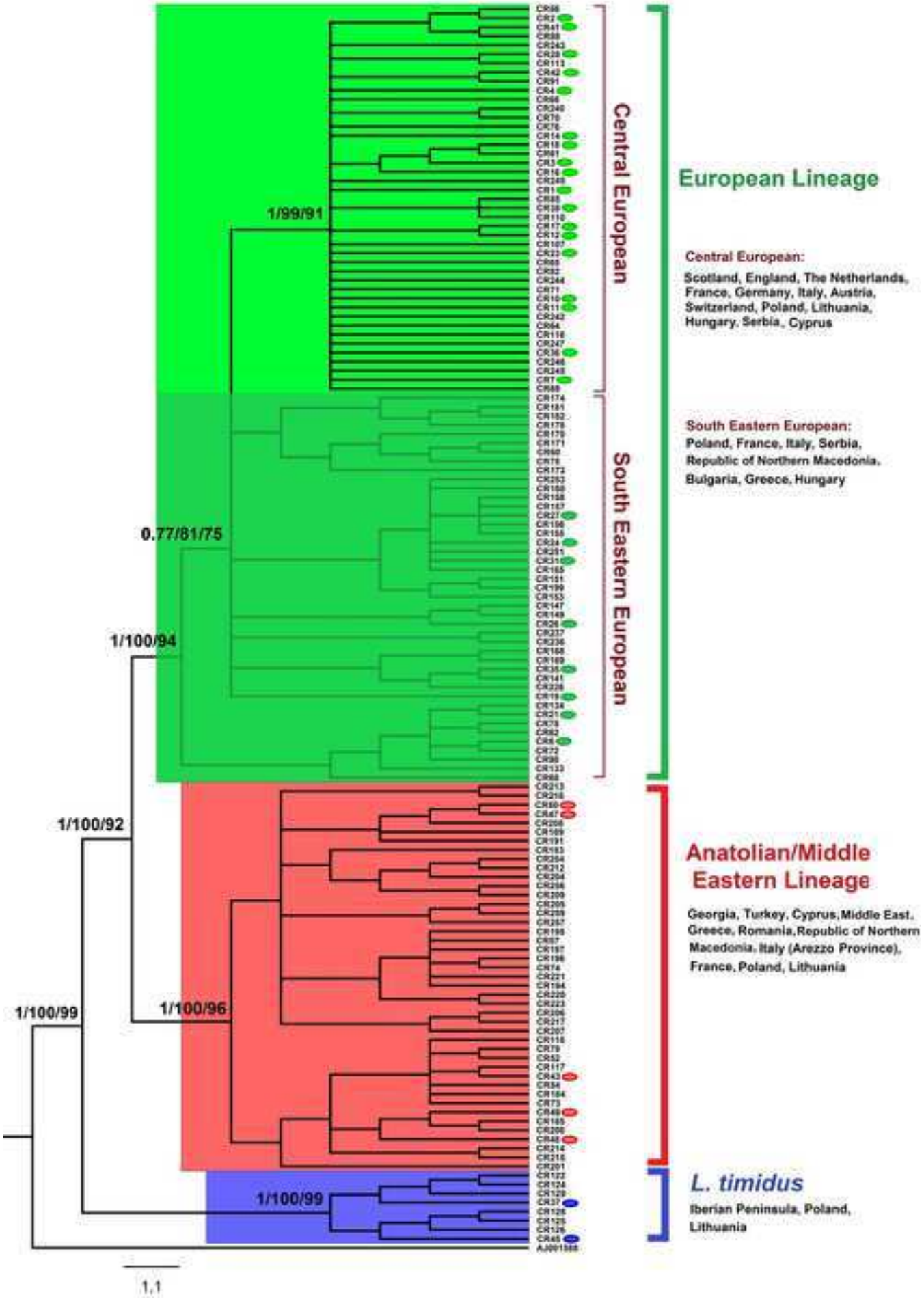

Figure 3

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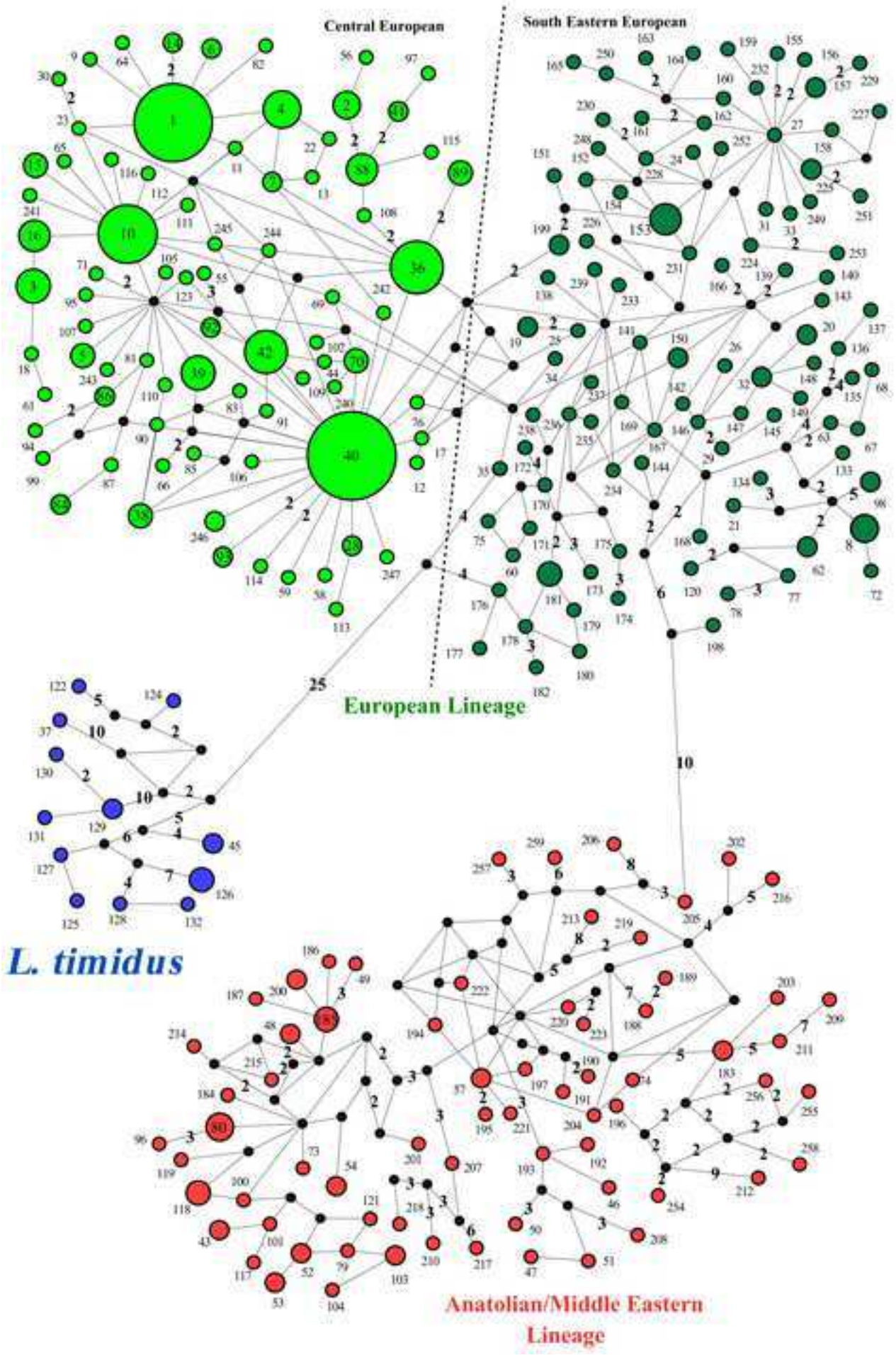

Figure 4

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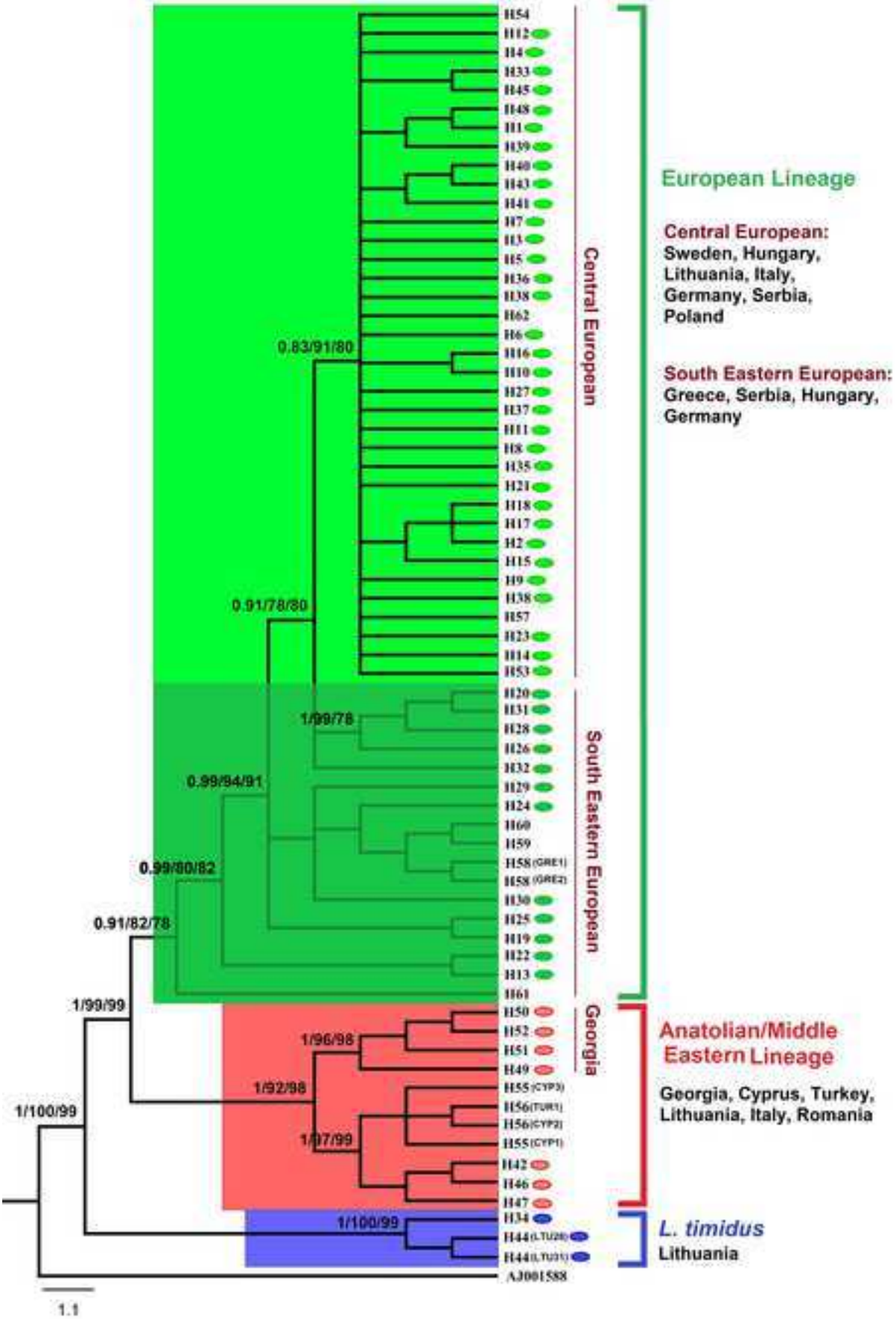

Figure 5

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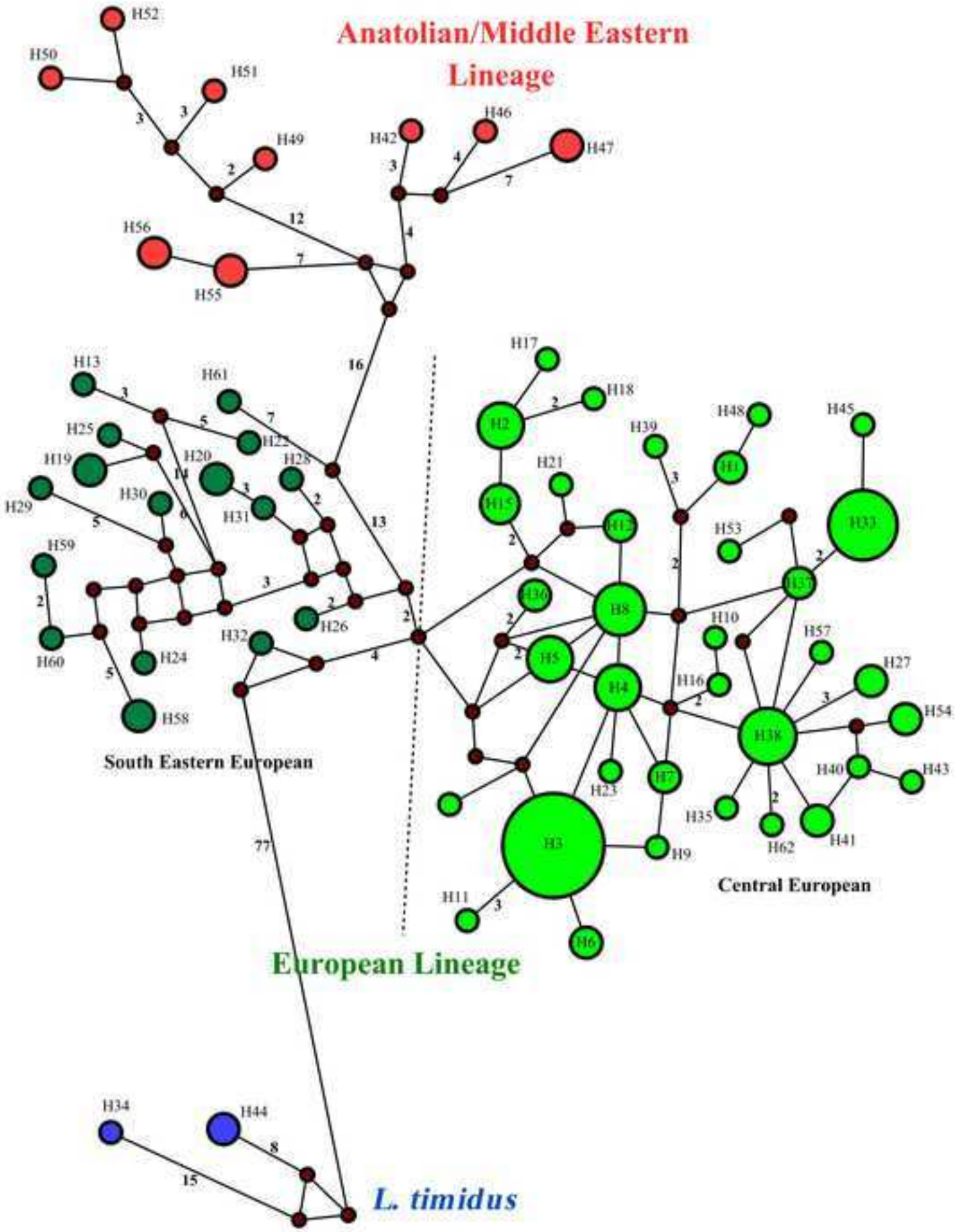

Figure 6

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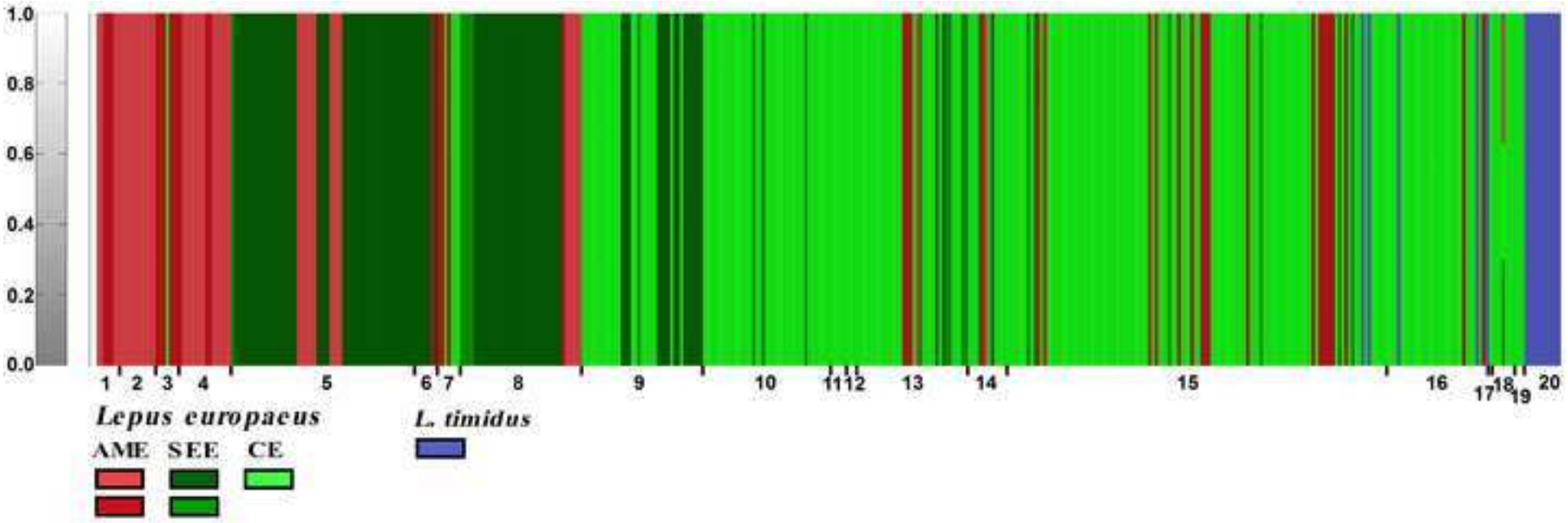

Large-scale mitochondrial DNA analysis reveals new light on the phylogeography of Central and Eastern-European Brown hare (Lepus europaeus Pallas, 1778)

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

Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

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

Materials and methods

Sample collection

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

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

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

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

Different regions of the mitochondrial DNA were amplified. PCR protocols and primers (Le.H-Dloop_F, ~~and~~ Le.L-Dloop_R [15] for the control region (CR) and LepCyb2L_F, ~~and~~ LepD2H_R [4] for cytochrome b (cyt b) + tRNA-Thr + tRNA-Pro + control region) were used ~~for~~ to the amplification. PCRs were carried out in a total volume of 25 µl, using the following sequence of steps: denaturation at 94 °C for 5 minutes, followed by 35 cycles of amplification 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, and a final step at 72 °C for 5 minutes. The forward sequencing reaction was performed by Macrogen Europe (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

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

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

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

Results

MtDNA control region sequences (358 bp)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 1. Comparison of genetic statistics for the brown hares sequenced in this study, originating from Central-Eastern Europe, based on the 916-bp mtDNA sequences (cyt b + 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	-15.340**	-1.455*
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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

also probably due to human-mediated translocations, as reported in other studies (e.g., [51]. Furthermore, the occurrence of L. europaeus in Corsica is recent and artificial, as it is known that different species of hares have been introduced in the region up to this day [53]. Overall, the presence of both major lineages (and the European subclades) of brown hare in Corsica could be the result of several human-mediated introduction events from different origins [54]. Likewise, a contact zone between mountain hares (L. timidus) and brown hares can be observed in Lithuania, as recorded in different populations of brown hares [9, 48,55-57].

The network result, in accordance with Stamatis et al. [6], showed that there are relatively close relationships between some haplotypes belonging to CE and several haplotypes from SEE (Fig 3). This finding indicates that one haplotype of the first subclade is only connected by one, so far undetected, haplotype, to another haplotype from the second subclade. However, the network analysis based on the longer sequence (916 bp) (Fig 5) does not provide strong support for this hypothesis. Overall, the close phylogenetic relationships between the two subclades SEE and CE in large geographic ranges of Europe support the idea that the brown hare colonized the current spatial ranges, when ecological conditions in these areas became suitable for the species after the Last Glacial Maximum [6, 58]. Also, the presence of a large number of unique haplotypes in South-Eastern Europe (the Balkans) and Anatolia is evidence for maintenance of a high proportion of the pre-glacial brown hare diversity in these areas during at least the last glacial period. Other studies have demonstrated the high intraspecies diversity of brown hare in these areas [6, 15, 18].

We discovered large contact zones for brown hares in several countries of Central-Eastern Europe. These findings support the existence of probable glacial refugia during the LGM in some of these areas (especially in Southern Europe), where the refugial populations probably underwent genetic differentiation [8], resulting in a number of genetic clusters. Following the retreat of the glaciers, the genetically isolated populations colonized Europe and formed

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

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

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

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

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

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

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

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Supporting information

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

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