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Mitochondrial Phylogeography and Population History of the Balkan Short-Tailed Mouse (*Mus macedonicus* Petrov and Ružić, 1983) in Turkey and Surrounding Areas

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Abstract

As a contribution to our understanding of postglacial colonisation history of Anatolia, the Caucasus and the Middle East, we increased the existing phylogeographic coverage of the widespread Balkan short-tailed mouse *Mus macedonicus*. This added 92 new mitochondrial D-loop sequences (73 new haplotypes) from Anatolia and Thrace to generate a total dataset for the species of 221 sequences (174 haplotypes). We confirmed the previously described existence of a northern lineage (Anatolia, the southern Balkans, the Caucasus, Iran and Syria) and southern lineage (Israel and Lebanon) and generated Bayesian Skyline Plots to show demographic expansion after the Last Glacial Maximum (LGM) in the northern lineage but not the southern. We used haplotype networks to reveal haplotypes close to the ancestral condition of the northern lineage and to infer spread through its range, including colonisation of the southern Balkans. Our various phylogenetic reconstructions also show finer-scale geographic structuring. *M. macedonicus* likely occupied two separate glacial refugia in the vicinities of Israel and Lebanon (southern lineage) and Anatolia, Georgia and Iran (northern lineage) although further work is needed for precise localisation. *M. macedonicus* has become a well-worked model system for the phylogeography of a region deserving more attention.

Keywords: Bayesian Skyline Plot; colonisation history; D-loop; glacial refugia; haplotype network; Last Glacial Maximum; mitochondrial DNA

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1. Introduction

Our knowledge of colonisation history of animals and plants has been greatly enhanced by studies involving a phylogeographic approach, i.e., using genetic markers (particularly DNA sequences) to identify potential source areas and routes of colonisation, employing phylogenetics to identify lineages that can then be tracked geographically [1]. This approach has been applied particularly to colonisation of Europe by temperate species after the Last Glacial Maximum (LGM), including identifying glacial refugia, areas where the temperate species would have been able to survive the cold, arid conditions associated with the LGM [2]. The southern European glacial refugia of Iberia, Italy and the Balkans have been viewed as particularly important [2,3]. More neglected has been the possibility

of southern refugia further to the east in Anatolia (Asian Turkey) and the Middle East, not only as sources of colonisation for Europe, but also in relation to colonisation within that region. The relevance of Anatolia and the Middle East to the colonisation of Europe is illustrated by phylogeographic studies of the bent-winged bat, *Miniopterus schreibersii* (Kuhl, 1817), which apparently survived the LGM along the coast of Syria, Lebanon, Israel and Egypt and, post-LGM, colonised first Anatolia and then Europe from there [4]. Anatolia itself can also be a site of refugia and there have been several syntheses localising these [5–7]. Of particular interest, there have been phylogeographic studies of various small and small–medium-sized terrestrial mammals, localising LGM refugia in Anatolia and inferring colonisation both within Anatolia and into Europe (and Asia): on the white-breasted hedgehog, *Erinaceus concolor* Martin, 1838 [8], the yellow-necked fieldmouse, *Apodemus flavicollis* (Melchior, 1834) [9], the Anatolian ground squirrel, *Spermophilus xanthoprymnus* (Bennett, 1835) [10], the bicolored shrew, *Crocidura leucodon* (Hermann, 1780) [11], the brown hare *Lepus europaeus* Pallas, 1778 [12], the stone/beech marten *Martes foina* (Erxleben, 1777) [13] and the long-eared hedgehog *Hemiechinus auritus* (Gmelin, 1770) [14].

Another species that has been studied from these respects is the Balkan short-tailed mouse, *Mus macedonicus*, and this forms the subject of this paper. Here we have been able to considerably expand the geographic coverage of DNA sequences for this species over previous studies, enhancing it as a model system for the phylogeography of Anatolia and the Middle East. *M. macedonicus* can be found throughout Anatolia and down into Iran and Israel, but is also present in the Caucasus and the southern Balkans—the latter region inspiring its scientific and vernacular names. Although closely related to the house mouse (*Mus musculus* Linnaeus, 1758), *M. macedonicus* is not so tied to human and livestock habitations, although it can survive well in agricultural fields as well as in natural habitats [15]. Therefore, the genetic structure of this species has probably not been influenced to the same extent by humans as in *M. musculus* and is instead likely to reflect the particular refugia the species occupied at the LGM, and range expansion from these. Phylogeographic studies on *M. macedonicus* have largely been based on the analysis of mitochondrial D-loop sequences. Initial studies by Prager et al. [16,17] and Gündüz et al. [18] were not geographically wide-ranging enough to show genetic subdivision within the species. However, subsequent studies by Orth et al. [19], Macholán et al. [20] and Rajabi-Maham and Azizi [21] demonstrated the existence of two major lineages, one southern lineage restricted to Israel and Lebanon, and another, more wide-ranging northern lineage found in Syria, Iran, Anatolia, the Caucasus and the southern Balkans. Orth et al. [19] named these lineages as subspecies, *M. macedonicus macedonicus* (northern) and *M. macedonicus spretoides* (southern), and suggested that they occupied two distinct LGM refugia to the north (Caucasus) and south (within the Middle East).

The aim of our study was to document mitochondrial D-loop sequence variation in Turkey in *M. macedonicus*, a key, central geographic area in the distribution of the species. In this way, we could carry out a more detailed phylogeographic analysis of the D-loop variation in *M. macedonicus* over a defined geographic area than conducted hitherto. At the same time, we aimed to fill a substantial gap in the geographic coverage of D-loop variation in *M. macedonicus* as a whole, and thereby substantially enhance our understanding of the refugial, demographic and colonisation history of the species.

2. Materials and Methods

2.1. Sampling and Sequencing

The new *M. macedonicus* sampled in the present study comprises 92 individuals from 56 previously unsampled localities in Turkey (localities 1–17, 19–33 and 35–58 in Supplementary Information, Table S1) and were collected between 2001 and 2009. Tissue from these

individuals was preserved in absolute ethanol and maintained at 4 °C in the sub-collection İG/PÖ (tissue collections of first and second authors, İ.G. and P.Ö.) at the Department of Biology, Faculty of Sciences, Ondokuz Mayıs University, Samsun, Turkey (OMÜ). DNA was extracted from small pieces of tail tissue using phenol/chloroform [22] or with the Qiagen Dneasy^{TM} Tissue Kit (Hilden, Germany) following manufacturers' instructions. The whole D-loop and flanking regions were amplified and both strands sequenced following [18]. Each sequence comprised 1016 base pairs (bp) of tRNA-Thr (67 bp), tRNA-Pro (68 bp) and the whole D-loop (881 bp), between positions 15,290 and 16,305, including indels relative to reference mtDNA sequence of *M. macedonicus* [23] (GenBank accession: NC_085424). For simplicity, these complete sequences are called "D-loop" and are numbered to incorporate the Turkish haplotypes *mac*Turkey.4–*mac*Turkey.6 already described [18]. To generate a continuous series, our new sequences are denoted Tr.1–Tr.3 and Tr.5–Tr.76 (we obtained new specimens with the same sequence as *mac*Turkey.5 and *mac*Turkey.6, which we now label Tr.5 and Tr.6) (see Supplementary Information, Table S1). All sequences are deposited in the DDBJ database (accession numbers: LC896954–LC897028.

2.2. Genetic Diversity Estimates

Genetic diversity estimates were obtained for the Turkish dataset. Nucleotide diversity (π) and haplotype diversity (*H*d) were calculated using ARLEQUIN v3.5 [24]. The number of segregating sites (*S*) and average number of nucleotide differences (*k*) were computed using DNASP v6 [25].

2.3. Phylogenetic Analysis

For the Turkish sequences, nucleotide composition was analysed and frequency of each haplotype was estimated using DNASP. The phylogenetic relationships among haplotypes were reconstructed using three different approaches: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP and ML analyses were performed in PAUP, v4.10b [26] and BI analyses in MRBAYES, v3.1.2 [27]. The most appropriate DNA substitution models for our dataset were established using the Akaike information criterion (AIC) [28] implemented in the software jModeltest v2.0 [29] and the suggested model was subsequently employed in the ML and BI analyses. MP analyses were performed using a heuristic search algorithm, 100 random addition replicates and tree bisection reconnection (TBR) swapping. The relative stability of each tree generated was assessed with bootstrap analysis using the same resampling options as described above for MP (1000 bootstrap replicates, except 10 random addition replicates). ML analysis was performed using starting trees obtained by neighbour joining and TBR branch swapping with model parameters. BI analysis was performed with 4 chains of 15,000,000 generations or until the average standard deviation of the split frequencies fell below 0.01, trees were sampled every 100 generations and the burn-in value was set to 25% of the sampled trees. The software tool TRACER v1.7.1 [30] was used to observe the parameters and to determine the number of trees needed to reach stationarity (burn-in). After discarding burn-in trees and evaluating convergence, the remaining samples were retained to generate a 50% majority rule consensus tree and to calculate posterior probabilities.

To describe the fine-scale relationships among Turkish haplotypes of *M. macedonicus*, we also used a network-based approach, appropriate for closely related sequences [31]. Haplotype networks were computed with a median-joining algorithm (MJ) implemented in the software NETWORK v10.1.0.0 [32].

For an analysis of *M. macedonicus* throughout its range, we used the same 1033 base pair length of D-loop as available in previously published sequences available from GenBank (between positions 15,290 and 16,322), including indels relative to the mitogenome

reference sequence of *M. macedonicus* [23] (GenBank accession: NC_085424) (Supplementary Information, Table S1). The full dataset contained 221 *M. macedonicus* sequences, comprising 174 haplotypes. These were used to construct an MJ network, following the same approach as for the Turkish haplotypes. Additionally, we conducted preliminary phylogenetic analysis using rooted trees that showed low branch support except for the northern and southern lineages already described by Orth et al. [19] and Macholán et al. [20]. Due to the greater information content of the network (mutation steps between haplotypes, alternative relationships not visible in a bifurcating tree, identification of haplotypes that are not observed but likely important), we focus here on the network that we obtained.

2.4. Demographic Analysis

To estimate historical demographic parameters and to test for departures from neutrality and possible population expansion for the Turkish dataset, Tajima's D [33], Fu's F_s [34], Strobeck's S [35] and Ramos-Onsins and Rozas's R_2 [36] statistics were calculated with DNASP. We also performed a Mismatch Distribution Analysis [37,38] for the Turkish dataset to examine historical changes in population size, using ARLEQUIN. A smooth, unimodal distribution indicates population growth [37]. To compare the observed data with the expected data under the sudden expansion model, we conducted goodness-of-fit tests based on the sum of squared deviations (SSD) and Harpending's raggedness index (r_g) [39,40], using 10,000 parametric bootstrap replicates.

Bayesian coalescent genealogy sampling, using the Monte Carlo Markov chain (MCMC) method implemented in BEAST v.2.7.7 [41], was used to estimate the pattern of demographic change for each of the two main lineages that were identified in phylogenetic analyses of *M. macedonicus* throughout its range. The whole 1033 bp alignment of sequences was used and the substitution model was HKY [42] with gamma correction. A strict molecular clock was used, given that rate variation is not expected among closely related sequences, with the rate fixed at 4×10^{-7} substitutions site^{-1} year^{-1}. This rate is appropriate for intraspecific sequence data; it has previously been used in a study of mitochondrial D-loop variation in the closely related *M. musculus* [43] and was originally inferred from the timing of colonisation of that species during the Neolithic period [44]. Posterior parameter distributions were obtained from four MCMC chains which were each run for 20 million generations, with the first 2 million generations discarded as burn-in. Log files were examined using TRACER v.1.7.2 [30], to check for convergence. The log and tree files were combined using LOGCOMBINER in the BEAST v.2.7.7 package. Bayesian Skyline Plots (BSP), showing effective female population size over time [45], were obtained using TRACER and graphics prepared using R v.4.5.1.

3. Results

3.1. Haplotype Distribution Within Turkey

Details of new specimens, including their collection location and D-loop haplotypes, are provided in Supplementary Information, Table S1 and mapped in Figure 1. A total of 73 new haplotypes (Tr.1–Tr.3 and Tr.7–Tr.76) and 2 previously described (*mac*Turkey.5 and *mac*Turkey.6) by Gündüz et al. [18] were found in Turkey (i.e., Tr.5 and Tr.6, respectively, in this study), based on the concatenated sequence of mtDNA, comprising 1016 bp of tRNA-Thr, tRNA and the whole D-loop. One haplotype, *mac*Turkey.4, described before by Gündüz et al. [18] was not recorded in the present study, but it was included in the molecular analysis. Thus, considering this 1016 bp sequence, the full dataset from Turkey comprises 96 specimens from 58 localities that display a total of 76 haplotypes.

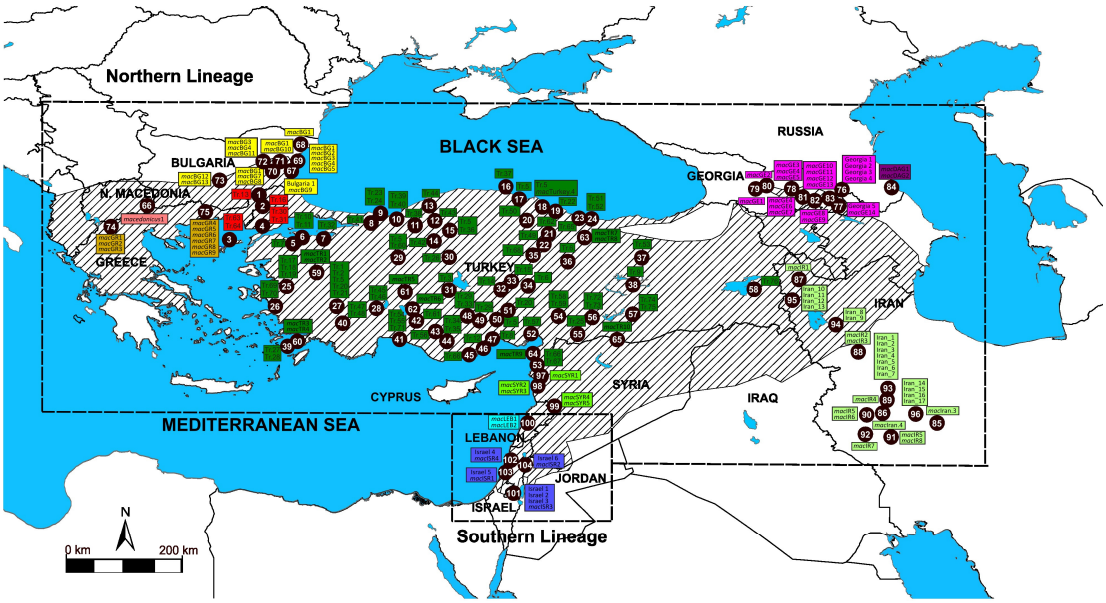


Figure 1. Geographic distribution of sampling localities and D-loop haplotypes of *Mus macedonicus* analysed here, from Turkey and nearby countries. Numbers in circles indicate locality codes where specimens were collected, while D-loop haplotype IDs for each locality are shown in boxes (see also Supplementary Information, Table S1). Colouring is by country (with Anatolia and Thrace also distinguished). The hatched area represents the distribution range of *Mus macedonicus* according to the IUCN Red List (https://www.iucnredlist.org/fr/species/13966/115117069 (accessed on 21 June 2025)). Some samples from Iran were obtained from locations south-west of the species range shown in this map.

Each locality harboured between one and five haplotypes and most individuals sequenced had a unique D-loop haplotype, although there were numerous exceptions (Figure 1 and Supplementary Information, Table S1). These include three individuals with the same haplotype from locality 43 (Tr.56) and two individuals with the same haplotype in the following localities: 7 (Tr.32), 12 (Tr.43), 16 (Tr.37), 18 (*mac*Turkey.4: [18]), 36 (Tr.6), 37 (Tr.53), 42 (Tr.61) and 48 and 49 (Tr.29). Haplotype Tr.5 was widespread, being observed in geographically distant populations (localities 15, 17, 18, 27, 29, 50). Additionally, single individuals from localities 27 and 51 had the Tr.20 haplotype, likewise for haplotype Tr.66 in localities 45 and 53 and, as already indicated, pairs of individuals in localities 48 and 49 had the Tr.29 haplotype.

3.2. Phylogenetic Analysis of the Turkish Data

MP analysis resulted in three most parsimonious trees (length = 214) with only minor variation in positioning of terminal branches. The best-fit model selected under AIC was General Time Reversible (GTR) with gamma correction (G) of 0.2100 and proportion of invariable sites (I) of 0.6920, and this was used in subsequent ML and BI analyses. The trees generated from MP, ML and BI analyses were highly congruent and all showed a lack of subdivision into major lineages; instead, there is partitioning into multiple small clusters of related haplotypes, with some clusters statistically well-supported (Figure 2). The lack of major subdivision but clustering of related haplotypes is also evident in the MJ network (Supplementary Information, Figure S1). The most abundant and widespread haplotype (Tr.5) is close to the centre of the network. There is a star-like pattern centred on a hypothetical haplotype one mutation step from Tr.3, Tr.5 and Tr.51. Such a star-like configuration is indicative of a relatively recent demographic expansion with the central haplotype likely ancestral, and the surrounding haplotypes having evolved from it through a series of mutational events during range expansion, as considered in more detail below.

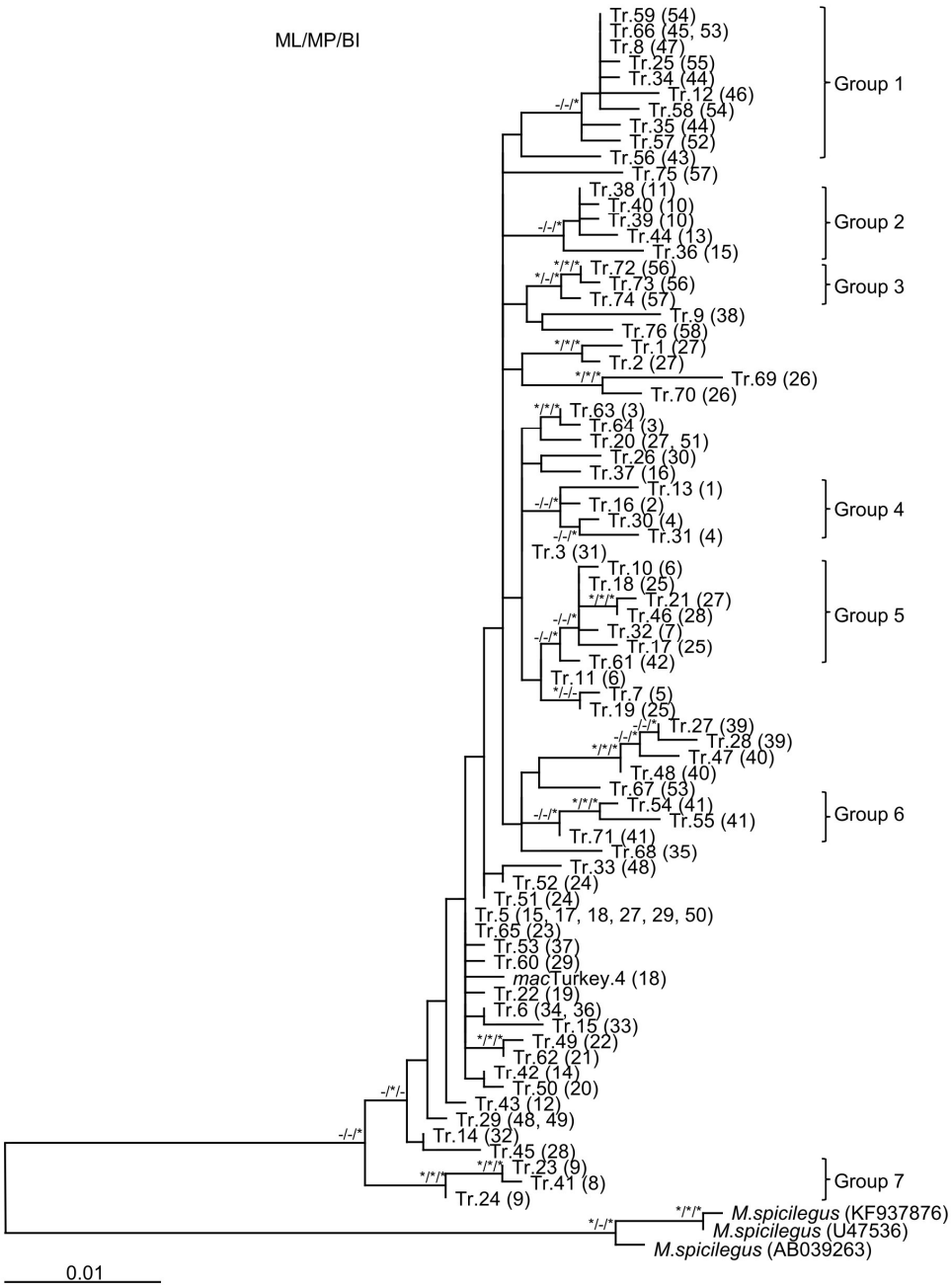


Figure 2. Results of ML, MP and BI analyses combined on a ML tree based on D-loop sequences of *Mus macedonicus* from Turkey. Asterisks at nodes indicate bootstrap values ≥70% (ML and MP analyses) and posterior probabilities ≥0.80 (BI analysis) and dashes are given for lower values. Map codes are given in parentheses after haplotype IDs (see Figure 1 and Supplementary Information, Table S1). Groups 1–7 are related haplotypes which also have geographic affinity (see text).

Considering further the clusters of three or more related haplotypes with high statistical support in at least one out of the ML, MP and BI phylogenies (as indicated in Figure 2), those haplotype clusters show clear geographical localisation. Going down the tree from top to bottom in Figure 2, and relating to the geographic distribution of haplotypes in Figure 1: the cluster of haplotypes Tr.8, Tr.12, Tr.25, Tr.34, Tr.35, Tr.57, Tr.58, Tr.59 and Tr.66 (Group 1) are all found in southern Anatolia, the cluster of haplotypes Tr.36, Tr.38, Tr.39, Tr.40 and Tr.44 (Group 2) are all found in north-western Anatolia, the cluster of haplotypes Tr.72, Tr.73 and Tr.74 (Group 3) are all found in south-eastern Anatolia, the cluster of haplotypes Tr.13, Tr.16, Tr.30 and Tr.31 (Group 4) are all found in Thrace, the cluster of haplotypes

Tr.10, Tr.17, Tr.18, Tr.21, Tr.32, Tr.46 and Tr.61 (Group 5) are all found in western Anatolia, the cluster of haplotypes Tr.54, Tr.55 and Tr.71 (Group 6) are all found in south-western Anatolia and the cluster of haplotypes Tr.23, Tr.24 and Tr.41 (Group 7) are all found in north-western Anatolia. The same clusters of related haplotypes in the MP, ML and BI trees are also evident in the MJ network (Supplementary Information, Figure S1).

3.3. Demographic History: Genetic Diversity Within Turkey and Tests of Neutrality

A total of 96 individuals analysed had 76 haplotypes, a haplotype diversity (H_d) of 0.992 (SD = 0.004) and a nucleotide diversity (π) of 0.008% (SD = 0.004). There were 86 segregating sites (S) and the average number of nucleotide differences (k) was 8.163. Tests indicated deviations from neutrality, with Tajima's D significantly negative (−1.68384, $p = 0.0180$), suggesting an excess of low-frequency polymorphisms, indicative of a recent population expansion or purifying selection. Similarly, Fu's F_S was highly negative (−24.59230, $p = 0.0001$), further indicating population expansion. Additional evidence of recent population expansion comes from the significant positive values of Ramos-Onsins' R_2 statistic (0.0438) and the high values of Strobeck's S index (1.00). In addition, the mismatch distribution for the whole dataset appeared to support the hypothesis of population growth, as it had the expected unimodal distribution curve (Supplementary Information, Figure S2). Both the SSD value (0.00034, $p = 0.9726$) and raggedness value, r_g (0.00367, $p = 0.9204$) were low and non-significant, supporting the idea that the species has undergone recent expansion.

3.4. Phylogenetic Analysis of D-Loop Across the Species Range

Another MJ network was generated to show the relationships among individuals sampled from the whole range of *M. macedonicus*. These were inferred by combining our new mitochondrial sequences with 129 D-loop sequences available from GenBank generally from countries surrounding Turkey including Bulgaria, Georgia, Greece, Iran, Israel, Lebanon and Syria (Figure 1 and Supplementary Information, Table S1). The final dataset analysed included 221 D-loop sequences.

As previously found by Orth et al. [19] and Macholán et al. [20] we found two well-separated groups in *M. macedonicus*, one widespread northern lineage and a southern lineage incorporating all sequences from Israel and Lebanon (Figure 3; see Figure 1 and Supplementary Information, Table S1). All the Turkish sequences from our previous analysis were in the northern lineage.

A network is a useful means to describe the relationships among closely related individuals, as in the present dataset where the sequences are all from a single species. When the haplotypes are coloured according to geographic location (Figure 3), the network does suggest that, as with the Turkish data alone (see above), haplotypes cluster to some degree by geography, which we describe below. Also echoing Supplementary Information, Figure S1, the same hypothetical haplotype is central to the network, one mutation step from Tr.3, Tr.5 and Tr.51. The central haplotype is also one mutation step from a Georgian haplotype *mac*GE1 and an Iranian haplotype *mac*IR6. Thus, these most central haplotypes of those so far described are scattered over a large area of Anatolia, Georgia and Iran: locations 15, 17, 18, 24, 27, 29, 31, 50, 79 and 90 on Figure 1 (see also Supplementary Information, Table S1). Based on our network this large area is the most likely area of origin of the current *M. macedonicus* populations of the northern lineage.

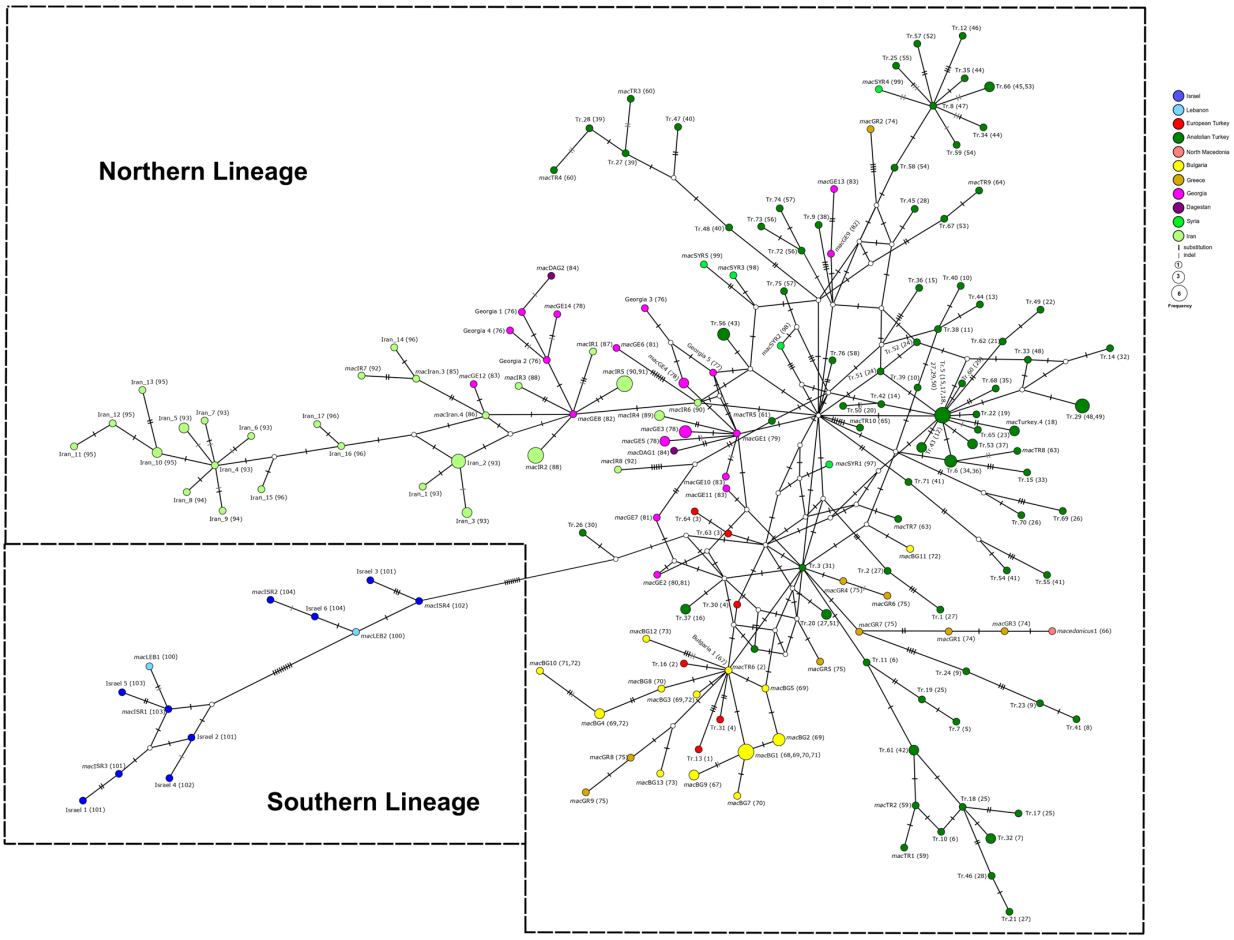


Figure 3. Median-joining network for D-loop sequences, using our new 92 sequences and the 129 sequences (101 haplotypes) available from GenBank (for the geographical origins of the sequences, see Figure 1 and Supplementary Information, Table S1). The size of each circle is proportional to the frequency of the particular haplotype in the sample. Median vectors, which represent either extant unsampled sequences or extinct ancestral sequences, are indicated by blank circles. Hash marks represent mutation steps between haplotypes.

The Georgian and Iran haplotypes (light green and purple in Figure 3) occur mostly together (along with the two Dagestan haplotypes) in the same part of the network, although showing plenty of diversity (the Iranian haplotype *mac*IR8 is nineteen mutations different from Iran_11). The Bulgarian haplotypes (yellow in Figure 3) form a tighter cluster elsewhere in the network. The central haplotype within the Bulgarian cluster (Bulgaria 1) is only one mutation step removed from Tr.3, suggesting an Anatolian origin for the Bulgarian *M. macedonicus* populations. Considering Bulgaria 1 as likely ancestral to the cluster of Bulgarian haplotypes is interesting because haplotypes in Thrace (European Turkey) and Greece (red and brown, respectively, in Figure 3), which are geographically close, appear to derive from the same haplotype. Other Greek haplotypes are scattered elsewhere in the network. There is another intriguing cluster consisting of *mac*GR1, *mac*GR3 and *mac*GR7 and *macedonicus*1 found in the two localities sampled in Greece and the single sampling locality in North Macedonia (Supplementary Information, Table S1). One of the clusters of haplotypes located in north-western Anatolia previously identified from Figure 2 (Tr.23, Tr.24 and Tr.41; see above) appears to derive from this cluster of Greek haplotypes, although with four mutation steps from *mac*GR7 (Figure 3). Finally, the Syrian haplotypes (medium green in Figure 3) are scattered throughout the network, but always most closely related to haplotypes from Turkey, Syria's geographic neighbour to the north,

rather than being closely related to haplotypes from Lebanon and Israel (light and dark blue in Figure 3), Syria's geographic neighbours to the south. As stated earlier, the Lebanese and Israeli haplotypes are highly divergent from all other *M. macedonicus* haplotypes and form a distinct southern lineage nine mutation steps from the nearest northern lineage haplotype (Figure 3).

3.5. Demography of Mus macedonicus

The Bayesian Skyline Plot (BSP) for the northern lineage of *M. macedonicus* shows clear population expansion around 15–20,000 years ago and also at about 5–7000 years ago (Figure 4a). However, the BSP for the southern lineage (Figure 4b) only shows demographic expansion at the more recent of these times.

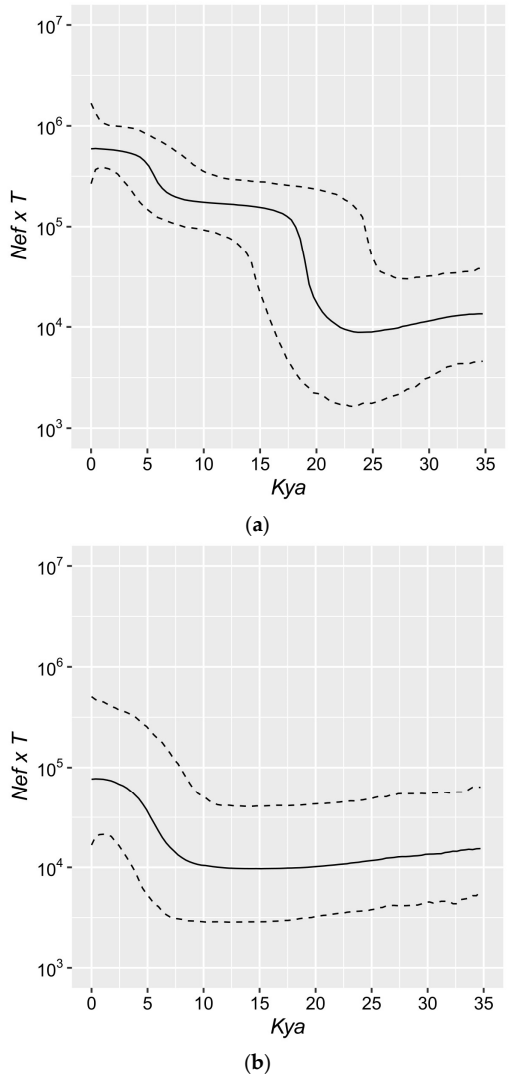


Figure 4. Bayesian Skyline Plots showing effective female population size (*Nef* × T) with time (kya) for (**a**) the northern lineage and (**b**) the southern lineage of *Mus macedonicus*. The solid line is the median and the dashed lines represent 95% highest posterior density (HDP) limits.

4. Discussion

The northern and southern lineages that were recovered in our trees and networks have previously been identified and have been named as separate subspecies *M. macedonicus macedonicus* and *M. macedonicus spretoides*, respectively, by Orth et al. [19]. There are size differences in the skull, but these differences can be ascribed to Bergmann's Rule, with

the southern lineage living in warmer conditions (and associated smaller skull). Thus, the distinction between the proposed subspecies is, at present based solely on genetics, both mitochondrial DNA and allozyme markers [19]. Therefore, the northern and southern lineages may be considered 'cryptic subspecies' in an analogous way that the term 'cryptic species' is used to define forms that are considered sufficiently genetically distinct to warrant a designation as separate species [46].

From the demographic analysis (see Figure 4), the estimated dates for coalescence of the northern lineage samples (median 27.159 kya; 95% HPD limit 18.671–39.098 kya) and southern lineage samples (median 24.201 kya; 95% HPD limit 15.250–34.714 kya) suggest that these two lineages originated by the time of the LGM and this fits well with the contention of Orth et al. [19] that the two lineages occupied different refugia at the LGM.

Orth et al. [19] suggested quite a wide possible region for the LGM refugium for the southern lineage including the area occupied by Israel, Lebanon, Jordan, Syria and Iraq. However, already based on the samples collected by Macholán et al. [20], it is only their new specimens from Lebanon that were of the southern lineage. Their new specimens from various locations in Syria (as well as their new specimens from Iran and Turkey) were of the northern lineage. Likewise, all our new specimens from Turkey were of the northern lineage, similar to those specimens from Iran collected by Rajabi-Maham and Azizi [21]. Therefore, the most likely refugial area for the southern lineage is best considered the current range of that lineage in Israel and Lebanon, until further evidence is available. While our BSP for the northern lineage shows population increase 15–20,000 years ago, as would be expected on population expansion from a limited LGM refugial area to a wider post-LGM distribution, there was no population expansion detected at that time in the southern lineage. This finding and the high mtDNA diversity seen in the southern lineage [20] further support the contention that the southern lineage is a stable population that has long occupied the area currently defined by Israel and Lebanon. That stability may reflect adequate temperatures (and other aspects of climate, e.g., humidity) for *M. macedonicus* during the LGM, late Pleistocene and Holocene both in terms of its physiology and habitat and resources. There is a long archaeological record of *M. macedonicus* in Israel [20,47] and that area has been defined as one of the Mediterranean glacial refugial areas for herbaceous plants and trees as well as a hotspot for plant diversity [5].

The location of the LGM refugium of the northern lineage is more difficult to ascertain. As we have already described, the BSP suggests a post-LGM expansion from a relatively small refugial area to the current very large distribution of that lineage. Orth et al. [19] suggest that the refugium was just south of the Caucasus, based on likely suitable climatic areas rather than an inference from the genetics of *M. macedonicus*. From inspection of the network in Figure 3, while the diversity of haplotypes in Georgia is large, it is not substantially larger than other geographic regions. The network is dominated by haplotypes from the northern lineage and has a star-like configuration. Considering those haplotypes nearest the centre of the star as most ancestral, those haplotypes one mutation removed from the putative ancestral sequence are found in Georgia, Iran and Anatolia suggesting a rapid and extensive expansion from the refugium. Interestingly, one of those haplotypes, Tr.5, is particularly widespread—found in six distantly spaced localities around Anatolia—suggesting that it was one of the haplotypes that did participate in the expansion. Although the LGM refugium of the northern lineage of *M. macedonicus* is likely to be somewhere within that large region of Georgia, Iran and Anatolia, where precisely it was located is unclear from the genetics. Therefore, we turn again to a consideration of climatic and ecological conditions. Demirtaş et al. [48] have used ecological niche modelling to infer suitable areas during the LGM for *M. macedonicus* and, considering the eastern part of the range of the species, the potential sites for a refugium include the eastern Black Sea coast

and adjacent upland area, the lowlands south of the Greater Caucasus Mountains and in the vicinity of the Zagros Mountains in Iran close to the southern limit of the Iranian specimens that have been included in this study. For comparison, there is an ecological niche modelling study of the tortoise, *Testudo graeca*, including a subspecies *T. g. ibera* with a similar current distribution to the northern lineage of *M. macedonicus* [49]. Once again, the eastern Black Sea coast is identified as possible glacial refugium along with the south-east of the Caucasus (on the coast of the Caspian Sea) and further east along the south of Caspian Sea. Thus, the suggestion of Orth et al. [19] that the refugium was in the Caucasus lowlands is reasonable. However, the Caucasus refugium is usually considered as a source of northwards expansion into the extreme east of Europe after the LGM [2] and the same logic may favour the vicinity of the Zagros Mountains in Iran as the refugial area for the northern lineage of *M. macedonicus*. This would mean that there was northwards population expansion on climate warming after the LGM to northern Iran, Anatolia and Georgia. Another consideration for LGM refugia for *M. macedonicus* is that it is a species that lives in open areas, avoiding forests [20]. Thus, where it has been possible to use pollen records to study vegetation to the south of the Caucasus and along the Black Sea coast, it is notable that those areas that would have been climatically suitable for temperate species like *M. macedonicus* at the LGM would have been dominated by forest [50]. Therefore, we may expect that *M. macedonicus* may not have existed centrally in such a glacial refugium, but in adjacent areas with temperature conditions that it could tolerate but somewhat drier conditions favouring the shrubby habitat that it likes [15].

In addition to the subdivision of *M. macedonicus* into a northern and southern lineage, there is evidence of further geographic subdivision in the northern lineage, albeit with low statistical support, as already noted by Macholán et al. [20]. On the network that they generated they noted a phylogenetic separation between Asian and European haplotypes. With our large addition of haplotypes from Turkey, that separation no longer holds, but there is plenty of interesting geographic signal and overall it is possible to investigate the demography and subdivision of the northern lineage in a more nuanced way than possible hitherto.

First, we wish to reiterate that the genetic data show evidence of recent population expansion in various ways. The tests of neutrality and the mismatch distribution for the Turkish data (Supplementary Information, Figure S2) indicate this. Also, the BSP for all the northern lineage data shows a population increase not only soon after the LGM, but also 5–7000 years ago. The population increase after the LGM is expected: the refugium of the northern lineage is likely to have been considerably smaller than the distribution the lineage would have attained once the climate ameliorated. The later population increase is less expected, but could represent human activities affecting the landscape and thereby habitat availability and access to resources for *M. macedonicus*. Interestingly, a population increase at about 5–7000 years ago is also seen in the BSP for the southern lineage. This range of times matches the Chalcolithic period in the Middle East when there was continued agricultural exploitation of the environment, with use of many crops including cereals and olives [51,52]. *M. macedonicus* does well in such agricultural habitat [15] and so it is not difficult to imagine that any increase in such resources and habitat would have led to an increase in population.

In that demographic context, we can now consider the geographic patterning within the northern lineage of *M. macedonicus*. There is undoubtedly a clustering of related haplotypes within defined geographic areas. This can be seen within small regions of Anatolia and also at a broader scale of individual countries over the species range (e.g., Iran and Bulgaria in the network: Figure 3). This is to be expected in a small mammal with limited dispersal, and which has expanded its range through natural movements.

Within local geographic areas it is not surprising that individuals should have a recent common ancestor and therefore related haplotypes, and that if a country is colonised from one source area, the founders may be related and the expansion through the country will also carry their genetic signature.

The genetic clustering with geography in Anatolia evident in our tree and network is coupled with low divergence between clusters, as also seen in other species, e.g., the brown hare *Lepus europaeus* [12]. This is to be expected with expansion from a single LGM refugium within or close to Anatolia as we have been asserting for the northern lineage of *M. macedonicus* (and Orth et al. [19] before us). Other taxa, such as the Anatolian ground squirrel, *Spermophilus xanthoprymnus* [10] apparently had multiple LGM refugia within Anatolia, and show much clearer phylogeographic structuring across the landmass (see also [6]).

Particularly intriguing is the genetic comparison of European (Balkan) and Asian populations of *M. macedonicus*. Based on our assumption of a single LGM refugium for the northern lineage, the Balkans would have been colonised from Anatolia after the lineage expanded throughout Anatolia. The land connection between Anatolia and Thrace (the part of Turkey within the Balkans) existed until about 8000 years ago and species such as *M. macedonicus* and *L. europaeus* would have had to cross by then [12]. Considering the network of haplotypes of *M. macedonicus* (Figure 3) within the Balkans, there is a particularly clear star-like pattern centred on the Bulgarian haplotypes, but also including haplotypes from Greece, North Macedonia and Thrace. This suggests that starting with the central haplotype of that star-like pattern in the Balkans, Bulgaria 1, there was an accumulation of derivative haplotypes each distinguished by new mutations. That central haplotype is one mutation removed from the Tr.3 sequence which is itself one of the haplotypes very closely related to (one mutation off) the central haplotype of the northern lineage expansion. This close relationship of a Balkan haplotype (Bulgaria 1) to the central haplotype of the northern lineage expansion makes sense given that the Balkans were colonised when there was a land bridge before 8000 years ago, and can likely be considered as part of the early post-LGM expansion from the northern lineage refugium. Likewise, *L. europaeus* in the Balkans is very similar to *L. europaeus* in Anatolia, but it lost some of the Anatolian diversity on colonisation [12].

Another cluster of haplotypes worthy of mention are those *M. macedonicus* collected from near the Bosporus on the Anatolian side (Tr.23, Tr.24 and Tr.41). These are the best differentiated cluster of haplotypes in the phylogeny of Turkish sequences (Figure 2). In the network (Figure 3) the cluster is affiliated with a cluster of haplotypes from Greece and North Macedonia and it indeed appears that a Greek haplotype may be ancestral. Therefore, it may be that this rather distinctive set of Anatolian sequences derives from a colonisation event in the reverse direction from expected, i.e., from the Balkans to Anatolia. Unusual geographic relationships can be ascribed to particular scenarios of natural population expansions, contractions and replacements. This is the case for unexpected distributions of mitochondrial lineages in another small mammal, the bank vole, *Clethrionomys glareolus* in both Britain and southern Scandinavia [53]. As in the *M. macedonicus* case, these unusual distributions relate to land bridge colonisations.

5. Conclusions

M. macedonicus is a particularly valuable model system for investigating the colonisation history of species in Anatolia, the Caucasus, the Middle East and into the Balkans, being widely distributed in that area and having a solid basis of previous work on which to build. Before our current study, there was relatively little known about the genetic variation of *M. macedonicus* in Turkey (Anatolia and Thrace), and yet this area encompasses a large,

central part of the distribution of the species. Our substantially enhanced sampling of Turkey has enabled us to construct a detailed MJ network of the whole species which shows clear geographic structuring and allows us to infer D-loop haplotypes close to the ancestral state for the northern of the two main mitochondrial lineages in *M. macedonicus.* This lineage occurs in Anatolia, the Balkans, the Caucasus, Iran and Syria. From the distribution of inferred ancestral haplotypes, the LGM refugium for the northern lineage could have been in the Caucasus, as suggested by Orth et al. [19]. This fits well with the Caucasus as being a known refugial area of temperate species [2], but that relates to postglacial colonisation of eastern Europe which is south-to-north postglacial colonisation, while in the case of *M. macedonicus* it would be colonisation from the Caucasus southwards. From the distribution of ancestral haplotypes, the vicinity of the Zagros Mountains in Iran is also a possible refugial area for the northern lineage, which would imply a more 'normal' postglacial colonisation (for the northern hemisphere) of south-to-north. While the exact site of the northern lineage LGM refugium is uncertain, the BSP for this lineage indicates population increase after the LGM, which is supported by neutrality tests for Turkish *M. macedonicus*. The northern lineage spread from its easterly refugium (wherever exactly that was) throughout the Caucasus, Anatolia, Iran, Syria and southern Balkans. The data for Turkey, in particular, show fine geographic structure, with related haplotypes in close geographical proximity, as expected for a small mammal with limited birth-to-breeding dispersal. There are also signs of local population expansions, presumably part of the overall postglacial expansion, as seen particularly clearly in the southern Balkans. In contrast to what we have inferred for the northern lineage, the southern lineage of *M. macedonicus*, currently located in Israel and Lebanon, in the BSP does not show evidence of population expansion after the LGM. Until further evidence is available it seems most reasonable to suggest that this lineage had an LGM refugium in the vicinity of Israel and Lebanon and it has not expanded its distribution substantially from that area. Overall, our study has provided many new insights into the phylogeography of *M. macedonicus.* Clearly, to understand the colonisation history of Anatolia, the Caucasus, the Middle East and into the Balkans requires more than the study of one species, but *M. macedonicus* together with what is already known of other study systems such as *Spermophilus xanthoprymnus*, *Lepus europaeus* and *Miniopterus schreibersii* illustrates the interest and richness of the area, both focusing on the region itself, but also considering impacts elsewhere, including Europe and Asia. Further detailed studies on a range of taxa would be very worthwhile to provide a more complete picture.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d17110740/s1. Table S1. The list and geographic origin of D-loop sequences of *Mus macedonicus* used in this study. Figure S1. Median-joining network for 96 *Mus macedonicus* D-loop sequences, comprising 76 haplotypes, from Turkey. Figure S2. Mismatch distribution for D-loop sequences of *Mus macedonicus* from Turkey, compared with the sudden expansion model of Rogers [38].

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Turkey. *Mus macedonicus* was considered a pest rodent and not subject to ethical guidelines. All individuals were collected and euthanized according to international standards of humane animal care and handling.

Data Availability Statement: The sequence data presented in the study are openly available at DDBJ (https://www.ddbj.nig.ac.jp/index-e.html, accessed 16 October 2025).

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Abbreviations

The following abbreviations are used in this manuscript:

BI	Bayesian inference
bp	base pairs
BSP	Bayesian Skyline Plot
DDBJ	DNA Data Bank of Japan
GTR	general time reversible
IUCN	International Union for the Conservation of Nature
kya	kilo-years ago
LGM	Last Glacial Maximum
MCMC	Markov chain Monte Carlo
MJ	Median-Joining
ML	Maximum Likelihood
MP	Maximum Parsimony
OMÜ	Ondokuz Mayis University
TBR	tree bisection and reconnection

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