

Phylogeography and genetic structure of the long-fingered bat (*Myotis capaccinii*) across Europe: insights from mitochondrial DNA and ddRAD-seq Data

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Abstract:

We aimed to investigate the phylogeography and genetic structure of the long-fingered bat (*Myotis capaccinii*) across Europe using mitochondrial DNA and ddRAD-seq data, and to investigate, using high-resolution SNP data, whether the previously recorded mito-nuclear discordance in the species reflects a biological reality or was due to the low resolution of the nuclear microsatellites used in earlier studies. A total of 265 wing tissue samples were collected from 40 sites across 10 countries. Mitochondrial cytochrome-b sequences were analyzed for haplotype diversity and phylogenetic relationships. ddRAD-seq was performed on 144 samples to obtain nuclear SNPs. Phylogenetic trees, haplotype networks, Bayesian skyline plots, mismatch distributions, AMOVA, and STRUCTURE analyses were used to infer genetic structure and historical demography. Three distinct mitochondrial clades (B, C1, C2) were identified, with divergence times estimated at ~350K and ~120K years BP. Clade B showed a star-like haplotype network and highest diversity, suggesting refugial origin in Anatolia. ddRAD-seq data corroborated mtDNA results and revealed longitudinal patterns of postglacial expansion and admixture. STRUCTURE analysis supported clustering into two or three genetic groups with admixture zones. Bayesian skyline plots indicated recent population expansions during the Holocene. The genetic structure of *Myotis capaccinii* reflects historical isolation in glacial refugia followed by postglacial expansions. Integrative use of mtDNA and ddRAD-seq data effectively resolved phylogeographic patterns and mito-nuclear discordance. Mountain ranges acted as barriers to female gene flow, while nuclear data revealed ongoing interbreeding. This study highlights the importance of combining genomic approaches to understand evolutionary history and inform conservation strategies.

Keywords: mitochondrial DNA, phylogeography, mitochondrial clades, *Myotis capaccinii*, ddRAD-seq, mito-nuclear discordance.

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ABSTRACT

Aim:

To investigate the phylogeography and genetic structure of the long-fingered bat (*Myotis capaccinii*) across Europe using mitochondrial DNA and ddRAD-seq data, and to assess, using high-resolution SNP data, whether the previously recorded mito-nuclear discordance in the species reflects a biological reality or was due to the low resolution of the nuclear microsatellites used in earlier studies.

Location:

Europe, including Northern Africa, Iberian Peninsula, Italy, Balkans, Anatolia, and the Near East.

Taxon:

Myotis capaccinii (long-fingered bat), a widely distributed bat species in the Mediterranean region.

Methods:

A total of 265 wing tissue samples were collected from 40 sites across 10 countries. Mitochondrial cytochrome-b sequences were analyzed for haplotype diversity and phylogenetic relationships. ddRAD-seq was performed on 144 samples to obtain nuclear SNPs. Phylogenetic trees, haplotype networks, Bayesian skyline plots, mismatch distributions, AMOVA, and STRUCTURE analyses were used to infer genetic structure and historical demography.

Results:

Three distinct mitochondrial clades (B, C1, C2) were identified, with divergence times estimated at ~350K and ~120K years BP. Clade B showed a star-like haplotype network and highest diversity, suggesting refugial origin in Anatolia. ddRAD-seq data corroborated mtDNA results and revealed longitudinal patterns of postglacial expansion and admixture. STRUCTURE analysis supported clustering into two or three genetic groups with admixture zones. Bayesian skyline plots indicated recent population expansions during the Holocene.

Main conclusions:

The genetic structure of *Myotis capaccinii* reflects historical isolation in glacial refugia followed by postglacial expansions. Integrative use of mtDNA and ddRAD-seq data effectively resolved phylogeographic patterns and mito-nuclear discordance. Mountain ranges acted as barriers to female gene flow, while nuclear data revealed ongoing interbreeding. This study highlights the importance of combining genomic approaches to understand evolutionary history and inform conservation strategies.

INTRODUCTION

The evolution and distribution of species was significantly influenced by the Pleistocene cycles of isolation and recolonization. During the glacial maxima of the ice ages, many species survived in isolated refugia, which acted as genetic reservoirs that formed and preserved unique evolutionary lineages (Hewitt, 1999; Schmitt, 2007; Habel *et al.*, 2010; Husemann *et al.*, 2014). As ice sheets retreated, populations expanded from these refuges, leading to contact between previously isolated groups in "suture zones," areas rich in biodiversity where different evolutionary lineages meet and potentially interbreed (Hewitt, 1999; Swenson & Howard, 2005; Bilton *et al.*, 2021). These zones and refugia provide valuable insights into historical biogeographical processes and are essential for understanding how species are distributed geographically and have dispersed over time, with implications for their taxonomy and conservation (Dufresnes *et al.*, 2023; Gippner *et al.*, 2024). Examining these patterns also helps predict how species might respond to future climate change, offering a guide for biodiversity management (Razgour *et al.*, 2019; Carroll *et al.*, 2024).

The long-fingered bat, *Myotis capaccinii* Bonaparte, 1837 (terra typica: Sicily, Italy), represents an interesting case of a widely distributed species (spanning Northern Africa in the south, the eastern parts of the Iberian Peninsula in the west, and the Persian Mesopotamia in the east (Hutson *et al.*, 2008; Koopman, 1994)) and whose distribution of intraspecific diversity might have been shaped by isolation in ice age refugia. The species has two defined subspecies, specifically *M. c. capaccinii* from northwestern Africa to Yugoslavia and *M. c. bureschi* (Heinrich, 1936) (terra typica: Karamlek, Stiranca Mountains: Bulgaria) from Bulgaria to Iran (Kumerloeve, 1975; Deblase 1980; Harrison & Bates, 1991). A study by Bilgin *et al.* (2008) showed the presence of two main mitochondrial clades distributed along an eastern-western axis with a contact zone in southeastern Europe; however, this study only comprised samples from Turkey, Greece, and Bulgaria, a small portion of the species' entire distribution. When samples from the whole distribution range of *M. capaccinii* were analyzed in the PhD dissertation of Viglino (2012), a second shallower mitochondrial break was detected north of the Appenine peninsula, increasing the number of mtDNA clades to three. This broader sampling confirmed the deep mitochondrial subdivisions identified in earlier studies. However, nuclear DNA analyses revealed a different pattern. Microsatellite data (Bilgin *et al.* 2008) failed to detect the mitochondrial subdivision structure, and where genetic breaks were identified, they did not align geographically with the mitochondrial boundaries (Viglino 2012). Instead, nuclear microsatellite analyses indicated a single genetic break separating western populations (spanning from Spain to Tunisia) from eastern populations (ranging from Italy to Iran), rather than the mitochondrial break observed between Italy and southeastern Europe.

Such discordance between mitochondrial and nuclear markers are not uncommon in bats (e.g., Centeno-Cuadros *et al.* 2019, Çoraman *et al.* 2019, Furman *et al.* 2014), and the causes can either be biological (e.g., introgression, incomplete lineage sorting, hybridization) or be induced by methods of analyses. Specifically, the microsatellite markers used in Bilgin *et al.* (2008) and Viglino (2012) were initially developed for other species and may have been affected by homoplasmy for allele size or the presence of null alleles (Putman & Carbone, 2014; Zhang & Hewitt, 2003).

To determine if the detected mito-nuclear discordance is due to biological or methodological issues, single nucleotide polymorphisms (SNPs) obtained by double-digested restriction-site-associated DNA sequencing (ddRAD-seq) provides an alternative, for species or populations having limited apparent variation (Lemopoulos *et al.*, 2019) or when not using species-specific markers. Providing information from thousands of loci, the potential advantage of SNPs generated through ddRAD-seq over microsatellites includes the added power and precision for detecting structure in weakly and/or clinally divergent populations, and improved resolution for understanding historical patterns of genetic structuring compared to microsatellites (Andrews *et al.*, 2016). Furthermore, the ddRAD-seq approach can provide more reliable inferences on population structure (Bruneaux *et al.*, 2013) and improved resolution for data sets with fewer individuals compared to the microsatellite approach (Jeffries *et al.*, 2016). In addition, issues like homoplasmy, null alleles, and scoring inconsistencies that might significantly affect microsatellite analyses are not as problematic for SNP datasets (Zimmermann *et al.* 2020), and hence the latter provide more robust basis for evaluating mito-nuclear discordance.

In this study, our main goal is to analyze samples from the whole range of *M. capaccinii* with ddRAD-seq approach and see if SNP data also show the discordance in microsatellites described above. If the mito-nuclear discordance was due to low resolution of microsatellites, we would expect to observe mtDNA differences reflected in nDNA when using the SNP data. On the other hand, if the SNP data also show mito-nuclear discordance, there would be greater support for a biological rather than methodological basis of the observation. In addition, in this study, we expanded the mtDNA dataset by adding localities from the Near East and the Caucasus and by intensifying sampling across both contact zones (northern Italy/Alps and the Balkans), thereby enabling a more precise delimitation of the three mitochondrial clades and a clearer reconstruction of their postglacial history.

MATERIALS AND METHODS

Sampling

A total of 273 tissue samples from 10 countries and 40 populations (including two unknown populations from Turkey and Greece) were analyzed. However, a subset of 265 samples (excluding eight samples from the two unknown populations) was used for most analyses (Figure 1 & Supplementary Table 1). For the purposes of this study, each sampling site is considered a population, provided that at least one sample was available. Sample sizes per site ranged from 1 to 27 (mean = 6.8, median = 4), with 18 sites having fewer than five samples. Per country, sample sizes ranged from 1 (Albania, Lebanon, Syria) to 113 (Turkey). Detailed sample sizes are provided in Supplementary Table 1. This heterogeneity in sampling effort was considered when interpreting population-level analyses.

Laboratory Procedures

For the DNA extractions, one biopsy punch from each bat was used, and a PureLink® Genomic DNA Mini Kit (Invitrogen) was utilized, following the protocols of the manufacturer. For mitochondrial DNA, about 700 base pairs (bp) of the cytochrome-b gene (cyt-b) was amplified using the primers Molcit-F (Ibanez *et al.*, 2006) and MVZ-16 (Smith and Patton, 1993). The PCR products were purified and sequenced by Macrogen Inc. (South Korea) using the same primers. Sequencer v.4.7 (Gene Codes Corp.) was used to align and edit the sequences and ensure that they were coding for protein. DnaSP v.5 (Rozas *et al.*, 2017) was used to prune identical haplotypes.

To obtain genome-wide nuclear SNPs for a subset of 144 samples, DNA extractions were submitted to double-digest RAD-seq (ddRAD-seq) protocol (Peterson *et al.*, 2012; DaCosta and Sorenson, 2014), (12x12 P3 and P7 indices were used for multiplexing). These 144 samples were selected to have at least one ddRAD-seq library to be prepared for each country that samples were collected from, with the aim to have good geographic coverage of the entire study area, and the three mitochondrial clades mentioned above. Genomic DNA was fragmented using restriction enzymes, and barcoded sequencing adapters with compatible overhangs were ligated to the resulting fragments. Following size selection, PCR amplification, and quantification, the prepared libraries were sequenced on a single lane of an Illumina HiSeq 2500 platform. The ddRAD-seq libraries for which adequate data were obtained are given in Supplementary Table 2.

Statistical Analyses

For mitochondrial DNA analyses, DnaSP was used to assess descriptive statistics, such as the number of haplotypes, haplotype (h) and nucleotide (π) diversities, and the number of polymorphic sites (Nei and Tajima, 1981; Tajima, 1983; Graur and Li, 2000). The software was also used to determine demographic parameters, including Tajima's D (Tajima, 1989), Fu's FS (Fu, 1997), D (Fu & Li, 1993), and Harpending's raggedness index (r) (Harpending *et al.*, 1993). The significance of these statistics was assessed with 1000 coalescent simulations. DnaSP was then used to determine mismatch distributions for hypotheses of constant or expanding population sizes. Mismatch distributions were also investigated using Arlequin v.3.5, which, in the case of an expanding population, is able to distinguish between spatial and demographic expansions (Excoffier & Lischer, 2010). Pairwise differences were used for molecular distance, and one hundred bootstrap replicates were analyzed.

PopArt v.1.7 (Leigh & Bryant, 2015) was used to construct a haplotype network of unique haplotypes identified from the 265 analyzed samples utilizing the TCS network option (Clement *et al.*, 2000). The relationship between haplotypes was also investigated by constructing the main kinds of phylogenetic trees with MEGA X (Kumar *et al.*, 2018). The substitution model for the maximum likelihood analysis, which was carried out in MEGA X, was determined as the Hasegawa-Kishino-Yano (HKY) model (no Gamma distributed rate or invariant sites) with jModeltest v.0.1.1 (Darriba *et al.*, 2012; Guindon & Gascuel, 2003). On the other hand, for the analysis of neighbor-joining tree, a maximum composite likelihood model was used. For all three methods, nodal support was evaluated by 1000 bootstrap replicates. For the neighbor-joining and maximum likelihood analyses, trees were rooted based on the topology of the haplotype network, which clearly identified the most ancestral haplotypes (Clement *et al.*, 2000). Another phylogenetic tree, which also estimated the divergence times, was constructed with Bayesian inference using BEAST v.1.8.4 (Suchard *et al.*, 2018). For this analysis, the HKY substitution model, Yule process, and a strict molecular clock with rates of 4.8% divergence per million years (as suggested for the cyt-b gene; Bilgin *et al.*, 2008; Ruedi & Mayer, 2001) were used. The analysis was run for 50,000,000 generations, logging every 5000 states and discarding the first 10% states as burn-in, resulting in effective sample sizes (ESS) greater than 1000 for all traces. We have also used a second approach where we used an uncorrelated relaxed clock with a lognormal distribution and an outgroup species (i.e., *Myotis ricketti*, which is estimated to have diverged from *M. capaccinii* circa 8 million years ago according to Stadelmann *et al.*, 2007). This analysis was run for 250 million generations, logging every 25,000 generations and discarding the first 1000 states as burn-in. The results were visualized using Tracer 1.6 (Rambaut *et al.*, 2018).

To ensure comparability with prior studies and address the structural consistency of our designated clades, we performed a combined analysis integrating our newly generated dataset with previously published sequences. We aligned the eight cytochrome-b sequences from Bilgin *et al.* (2008) (GenBank accessions EU475878.1–EU475885.1) with the 265 sequences generated in this study using the MUSCLE algorithm in MEGA X. Because the amplified regions targeted in the two studies differed, the alignment was trimmed to the maximum common overlapping region, resulting in a shortened sequence of 205 bp.

Haplotype determination for this truncated dataset was performed using DnaSP v.6. To visualize the relationships between the combined haplotypes without forcing artificial bifurcations on a short sequence, we constructed a statistical parsimony network using the TCS algorithm (Clement *et al.*, 2000) implemented in PopART v.1.7 (Leigh & Bryant, 2015). Phylogenetic tree reconstruction was also attempted in MEGA X; however, due to the short sequence length and the resulting inability to reliably calculate pairwise distances, Neighbor-Joining (NJ) could not be computed, and a Maximum Likelihood (ML) tree was generated instead to assess basic topology.

To detect possible recent changes in effective population sizes (Drummond *et al.*, 2012), BEAST was also used to compute Bayesian skyline plots (BSPs) separately for the three clades B, C1, and C2 which were determined during the haplotype network construction (see results). For this analysis, all available sequences for the three clades were used (N = 64, 108, and 93 for clades B, C1, and C2, respectively), and the same parameters that were used in the Bayesian phylogenetic construction were applied, only substituting the Bayesian skyline coalescence with the Yule process. The final analysis was run for 250 million generations for all three clades, logging every 25,000 generations. Again, the first 10% of the states were discarded as burn-in.

Differentiation in mtDNA among clades was explored using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992), and the significance was evaluated using 1000 replications. The software Arlequin v.3.5 was used to carry out AMOVA (Excoffier & Lischer, 2010). Arlequin was also used to determine F_{ST} values among populations and carry out an exact test of population differentiation, using 100,000 steps in Markov chain and 10,000 dememorization steps. In addition, Mantel tests were carried out with Arlequin to check for possible isolation-by-distance (IBD), using three sample sets consisting of samples belonging to clades B, C1, and C2, see below. The presence of an IBD effect was checked within clades, as well as within regions harboring clades C1-C2, B-C1, and B-C1-C2.

In the ddRAD-seq data set, for genotyping and SNP discovery, the raw data were processed for aligning ddRAD DNA regions by using ‘STACKS’ software pipeline (Stacks Manual on Stacks’ Web Site) to build loci from short-read sequences. Before executing the pipeline, the optimal parameters were determined using a subset of the libraries. The de novo pipeline was run multiple times, altering only one parameter per run, as described by Paris *et al.* (2017). The optimization process used the r80 method, as recommended by Paris *et al.* (2017), to identify loci present in at least 80% of the samples within each group. The decision framework from Paris *et al.* (2017) was employed to test values of m ranging from 2 to 7 and $M=n$ from 0 to 9. After parameter selection, the stages of STACKS Pipeline were followed as: process_radtags to clean and demultiplex the raw data, ustacks to build loci de novo, cstacks for assembling a catalogue of loci, and sstacks for matching against the catalogue. Subsequently, the data was filtered so that only samples with less than 10% missing data were used for subsequent analyses. With the data processed on the Stack pipeline, F_{ST} and nucleotide diversity (HWE) were estimated for each sampling location. The final data was exported in structure format for further use in the determination of clusters in STRUCTURE version 2.3 (Catchen *et al.*, 2013).

Data Statement

The double-digest RAD sequencing (ddRAD-seq) data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1492173. The 144 *Myotis capaccinii* individuals are registered as BioSamples SAMN61478237–SAMN61478380, and the corresponding raw sequence reads are available as SRA runs SRR39526783–SRR39526926.

RESULTS

mtDNA Phylogeography

The final trimmed sequence alignment of cyt-b was 671 bp long. All unique haplotypes are available with Genbank Accession Numbers: MZ695449-MZ695721. Descriptive statistics for the clades, populations and geographical regions are given in Supplementary Table 3. A total of 17 distinct haplotypes were detected in the 273 analyzed sequences for a total of 40 polymorphic sites, 36 of which were parsimony informative. The haplotype diversity of all samples was 0.802 (± 0.015 = 1 SD), and nucleotide diversity was 0.01716 (± 0.00072). Tajima’s D statistic was 2.0976 but not significant, as well as Fu’s F_S and D and Harpending’s raggedness index (r).

The haplotype network clearly revealed the existence of three distinct mitochondrial clades, which are referred to as B, C1 and C2, following Bilgin *et al.* (2008) (see also Viglino (2012)) (Figure S8). Among the 265 samples that were used to construct this network, the most common haplotype was H6, which was one of the four haplotypes (H5-H8) detected in clade C1 and was found in 93 samples. Clade B, on the other hand, having nine haplotypes (H9-H17), showed the highest number of distinct haplotypes, of which H10 was the most common one found in 34 samples. The other seven haplotypes differed from H10 by one to three bp and clustered around it, forming a clear star-like pattern. There were five haplotypes in clade C2 (H1-H4), the most common of which were H1 and H2, found in 55 and 34 samples, respectively. No clear star-like pattern was observed in clades C1 and C2. Clade B was the most diverged one, differing from haplotypes of clade C1 at least by 26 bp and from haplotypes of clade C2 by at least 23 bp. C1 and C2 differed from each other by seven bp at least. The geographical transition zone between clades B and C1 was detected in Thrace, close to the Turkish border with Greece. In fact, haplotypes from both clades were found at the same site in Koufovouno, Greece (Figure 3a). The transition zone between clades C1 and C2, on the other hand, was localized around the Alps (Figure 3b).

Haplotype diversities of clades B, C1 and C2 were $0.718 (\pm 0.049)$, $0.242 (\pm 0.050)$, $0.521 (\pm 0.031)$ and the nucleotide diversities were $0.00155 (\pm 0.0002)$, $0.00099 (\pm 0.00021)$, $0.00095 (\pm 0.00012)$, respectively. None of the other descriptive statistics were significant for any clade or site, except for Fu and Li's D in the Koufovouno population in Greece, as all 25 polymorphic sites of this site were parsimony informative with no singleton variable sites (see Discussion). Tajima's D was negative for all clades, indicating population expansion, however none of the values were significant.

When the 17 haplotypes found in *M. capaccinii* were analyzed together, all three phylogenetic tree reconstruction methods, namely Bayesian inference, neighbor-joining, and maximum likelihood, showed the presence of the same three clades, with high bootstrap support, as well as the same subclades within clades, such as (H1, H2, H3) in clade C2, (H6, H8) in clade C1, and (H13, H16) in clade B (Figure 4, Supplementary Figures 1-2). The F_{ST} values and their significance, as well as the average number of pairwise differences and haplotype distances, well reflect the clear separation among the three clades (Supplementary Figures 3-5).

The divergence times between clades B and C, and between clades C1 and C2, were estimated by analyzing the 17 haplotypes together using a strict molecular clock with a rate of 4.8% divergence per million years, and a phylogenetic tree was constructed via Bayesian inference (Figure 4, Supplementary Figure 13). The divergence time between clades B and C was calculated as 340,150 years (95% HPD Interval = 224,290 – 464,020 years). The divergence between C1 and C2 was

determined as 124,990 years (95% HPD Interval = 64,480 – 190,480 years). When an uncorrelated relaxed clock was selected and *M. ricketti* was used as an outgroup, the divergence time of *M. capaccinii* and *M. ricketti* was adjusted by the program as 7,743,300 years (95% HPD Interval = 5,613,400 – 9,633,600 years). In this case, the divergence time of clades B and C was calculated as 370,400 years (95% HPD Interval = 87,450 – 877,900 years). The divergence of C1 and C2 was determined as 129,000 years (95% HPD Interval = 21,464 – 333,400 years) (Supplementary Figure 14).

When the mismatch distributions were plotted for a constant-size model and for a model of demographic expansion using DnaSP, the expansion model fitted better than the constant-size population model for clade B and slightly better for clade C2, whereas the mismatch distributions and raggedness index values showed no signs of a demographic expansion for clade C1 (Supplementary Figure 6, Supplementary Table 3). To address whether combining clades C1 and C2 would produce a clearer demographic signal, we performed a combined mismatch analysis under a constant-size model in DnaSP. The combined dataset ($n = 173$) yielded a bimodal mismatch distribution with peaks at 0 and 7–8 pairwise differences, a raggedness index of 0.1292, and a high mean absolute error (0.9560), indicating poor fit to a unimodal expansion model (Supplementary Figure 6). This bimodal pattern is consistent with population structure or admixture rather than a single expansion event, further supporting the treatment of C1 and C2 as distinct evolutionary lineages. When the mismatch distributions were calculated using Arlequin, for clades C1 and C2, the mismatch distributions were inconclusive, the least-square procedure did not converge after 2000 steps, and the raggedness index values showed no clear signal of demographic expansion (Supplementary Figure 6). Yet for clade B, plots were produced, indicating a spatial, rather than a demographic expansion (Supplementary Figure 7). Bayesian skyline plots, on the other hand, indicated onset of population expansions to around 2,000 years, 1,700 years, and 1,000 years before present (BP) for clades B, C1, and C2, respectively (Figure 5).

When AMOVA was used to compare clades B and C, the global F_{ST} was 0.86 and was significantly greater than 0 ($P < 0.001$). When clades B and C were grouped together (i.e., comparing B vs. C1+C2), the percentage of variation attributed to among-group differences was 86.11%, whereas within-group variation was only 13.89%, consistent with the deeper divergence time estimated between clade B and the C clade (~350,000 years BP) relative to the shallower split between C1 and C2 (~125,000 years BP). When clade C was divided into C1 and C2, and the three clades were analyzed together, the F_{ST} was 0.96 ($P < 0.001$). This time, among-group differences constituted 95.57% of all variation, whereas within-group variations constituted only 4.43%. Additionally, the Mantel test for the populations carrying clade C1 haplotypes detected a significant IBD effect in the

corresponding geographical area (Mantel test $R^2 = 0.52$, $P < 0.01$, when Koufovouno in Greece was excluded; $R^2 = 0.59$, $P < 0.01$, when Koufovouno and the Italian populations were excluded), whereas no significant IBD effect was detected for clade B and clade C2 populations in the east and west areas, respectively. Significant IBD was detected for regions harboring clades C1-C2, B-C1, and B-C1-C2 ($P < 0.01$ for all, $R^2 = 0.36$, $R^2 = 0.05$, and $R^2 = 0.1$, respectively).

When we aligned the eight cytochrome-b sequences from Bilgin *et al.* (2008) with our sequences, trimming the combined dataset to the 205 bp overlapping region yielded 11 variable sites and collapsed the 273 sequences into 9 distinct haplotypes (Supplementary Table 5, Supplementary Figure 15). Despite the limited phylogenetic signal inherent to this shortened fragment, the TCS haplotype network successfully recovered the primary structural divergence between Clades B and C.

Crucially, the single sequence defined as Clade C in Bilgin *et al.* (2008) (EU475885.1) collapsed perfectly into Hap_1, grouping seamlessly with 184 of our newly generated Clade C sequences. Similarly, the remaining seven GenBank sequences from the 2008 study, previously identified as Clade B, clustered strictly with our Clade B samples, collapsing into Hap_5 and Hap_9. (Note that the original six Clade B haplotypes from the 2008 study collapsed into just two haplotypes here due to the reduced number of informative sites within the 205 bp window). Because of this reduced sequence length, the finer distinction between our sub-clades C1 and C2 was not resolved; however, this does not impact the broader Clade B versus Clade C validation.

Maximum Likelihood tree reconstruction further demonstrated the statistical limitations of the 205 bp fragment. While the dominant Hap_1 (containing the Bilgin *et al.* (2008) Clade C sequence) was correctly isolated, the lack of sufficient mutational steps caused the algorithm to artificially group minor European C haplotypes (Hap_2, Hap_3, Hap_4) into an unresolved polytomy alongside Clade B haplotypes (Supplementary Figure 16). This is a recognized artifact of forcing bifurcating tree algorithms on short, low-variation intraspecific data, reinforcing that the TCS network provides the most accurate visualization for this combined, truncated dataset.”

ddRAD-seq

The ddRAD-seq output of the 144 samples showed that 23 samples resulted in fewer than 5635 reads of 150 bp long and were therefore discarded. The remaining 121 samples used for further analyses had an average read size of 327,234 (95% CI: 295,537-358,930), with a range of 47,709 to 1,545,968 reads per sample. Highest coverage and data retention were observed when m, representing

the minimum number of raw reads required to form a stack, was 3 and M, representing the number of mismatches allowed between stacks to merge them into a putative locus, was 6 and when $n = M$ (Supplementary Figures 9-12; Supplementary Table 4). The Stacks pipeline was run under these optimized parameters.

For variant sites, the maximum observed heterozygosity value observed among the populations is 0.35329 for the population Tsoutouro (Greece, clade C1), while the maximum expected heterozygosity and nucleotide diversity values are respectively 0.31516 and 0.34671 for the population Kıyıköy (Turkey, clade B). The minimum value for observed and expected heterozygosity is respectively 0.14758 and 0.11955 for the population Alcampell (Spain, clade C2) for variant sites, while the minimum nucleotide diversity value, 0.14609, belongs to the population of Ichkeul Cave (Tunisia, clade C2). For all sites, the maximum observed heterozygosity and maximum nucleotide diversity values observed among the populations are respectively 0.00075 and 0.00073 for the population Yenisu (Turkey, clade B), while the maximum expected heterozygosity value is 0.00066 for the Kıyıköy (Turkey, clade B) population. The minimum value for observed heterozygosity is 0.00034 for Alcampell (Spain, clade C2), Ichkeul Cave (Tunisia, clade C2), and Slovenia (Slovenia, clade C1) populations; the minimum value for expected heterozygosity is 0.00027 for only the Alcampell (Spain, clade C2) population; and the minimum nucleotide diversity value is 0.00031 for the population of Ichkeul Cave (Tunisia, clade C2) (Tables 1 & 2).

The visual representation of the matrix of pairwise F_{st} based on ddRAD-seq data (Figure 6) shows consistency with the differentiation of the mtDNA clades B, C1, and C2, with a color gradient observed when transitioning from the comparisons of the mitochondrial clades B to C2. The highest F_{st} values can be observed as expected between B and C2 populations as the darkest shades of red. The level of differentiation between B and C1, or C1 and C2 is expected to be lower than that of between B and C2 populations, and corresponding zones shows a result in the expected direction as lighter shades of red. Lowest F_{st} values is expected to be between populations within the same clade represented as lightest shades of red and the results are in line with this expectation. However, the pattern is not absolutely clear-cut, as there are some exceptions to this gradient (e.g. Lombardia-ITA or Slovenia-SLV), potentially indicative of nuclear introgression between different mitochondrial clades.

The results of the Structure analysis indicated that based on Evanno *et al.* (2005), $K=2$ had the highest support (Figure 7). The samples sorted by longitude broadly show an assignment into two clusters (corresponding to clades B and C1+C2), with an area of admixed genetic ancestry in between. A plot of mean assignment probability values to either of these two putative clusters (B or C1+C2) aligned with longitude (Figure 8) also shows the decreasing probabilities from west to east to belong

to the first cluster (and inversely so for the second cluster). Finally, for Structure results set with K=3 groups (Figure 9), populations/individuals assigned to the three mitochondrial clades B, C1 and C2 further show admixed genotypes. A similar plot of average assignment probabilities by longitude shows a similar decrease in C1 and B in the east-west direction as in K=2, but in this plot a decrease from the highest assignment probabilities around the longitude of 10-15E as one moves east and west is also observed.

DISCUSSION

Phylogeography of *M. capaccinii* part 1: The separation of clades

In this study, two distinct classes of genetic markers (mtDNA and nuclear ddRAD-seq) were used to illuminate the phylogeographic history of *M. capaccinii*. The mtDNA analyses supported a major subdivision of haplotypes into three main clades, namely clades B, C1, and C2. Bilgin *et al.* (2008) previously showed a major break between clades B and C1 in the Balkans, with clade B mainly restricted to Anatolia and clade C1 located in Turkish Thrace, Greece and Bulgaria. With an expanded geographic sampling, we show here that bats carrying clade C1 haplotypes occur west to northern Italy, and evidence of a new clade (C2) found in southern Italy, France, Iberia and north-western Africa. The separation of clades C1 and C2 in Italy coincides with the Apennine Mountains and runs along the south-western Alps. This suggests that these mountains represent potential major geographical barriers for female lineages in *M. capaccinii*, restricting their movements across these mountain chains. This is confirmed by AMOVA analyses of mtDNA structure which all support a strong effect of these barriers on the segregation of haplotypes (up to 95% of variance explained).

The second major mtDNA contact zone detected in this expanded mtDNA analysis is found in eastern Europe, where clade C1 and B meet near the geographic border of Turkish and Greek Thrace. This second break, however, does not coincide with a mountain range, and bats from one locality (Kouvofouno; Fig. 3) carry haplotypes of both major lineages. This area of mtDNA admixture therefore suggests that the two major clades meet in a restricted area, as they did not expand much beyond this contact zone. The reason for a strong restriction of female gene flow across short geographic areas might reflect a strong female philopatry to nursery colonies evidenced in other *Myotis* bats (e.g., Kerth *et al.* 2000; Nagy *et al.* 2013). Interestingly, the Thrace contact zone between two major mtDNA clades is also found in another congener, *M. myotis* showing strong female philopatry (Castella *et al.* 2001; Furman *et al.* 2020), despite high potential ability for dispersal movements.

Neighbor-joining and maximum likelihood reconstructions strongly supported the existence of the three clades with an earlier divergence of clade B, and a further split between clades C1 and C2. Using Bayesian inference, these divergence times were estimated to be about 350,000 years BP for the divergence of B and C1+C2, and 130,000 years BP for the divergence of C1 and C2 using a rate of 4.8% divergence per million years. Both of these divergence times seem to coincide with the Pleistocene (Habbe, 2007; Cheng *et al.*, 2009; Litt *et al.*, 2007).

The divergence of clades C1 and C2, as well as the genetic similarity of individuals from Iberia, France, southern Italy, and North Africa, can be explained by the geographical changes that took place at the estimated time of divergence of these clades, as well as the existence of geographical barriers (i.e., the Alps and the Apennines). During that time (circa 130,000 BP), the Tyrrhenian Sea was much narrower than it is today, and the level of the Mediterranean Sea was significantly lower, so that there could be a substantial genetic exchange between populations of Spain, the Balearic Islands, North Africa, Sardinia, and the Apennine Peninsula (Shackleton *et al.*, 1984). The sharing of haplotype H2 among populations of Tunisia, Mallorca, Sardinia, and Latium is an example of this exchange (Supplementary Figure 8). It is also possible that Tunisia was colonized relatively recently, directly through the Strait of Sicily (rather than through the Gibraltar Strait), as haplotype H1 is encountered both in Sicily and Tunisia (Supplementary Figure 8), suggesting that *M. cappacinii* dispersal is more hindered by mountain chains than by sea channels. Even though Sicily and Tunisia are separated by a distance of about 140 km, there is evidence of high levels of genetic similarity among Sicilian and Tunisian populations in other taxa (Husemann *et al.*, 2014; Stöck *et al.*, 2008; Cosson *et al.*, 2005; Habel *et al.*, 2009, 2010, 2011), although there are some variations to this pattern as well. For instance, there is evidence that the butterfly *Melanargia galathea* colonized Italy through the Strait of Sicily, and then recolonized Tunisia back from Sicily (Habel *et al.*, 2011).

The overall nucleotide diversity ($\pi = 0.01716$) observed in this study is slightly lower than the ~ 0.02 reported by Bilgin *et al.* (2008). This difference likely reflects the expanded geographic sampling in the present study, which includes western populations (Iberia, France, Italy, North Africa) where diversity is lower, whereas the earlier study focused primarily on eastern populations (Turkey, Greece, Bulgaria) where clade B—the most diverse clade—predominates. Within clade B alone, nucleotide diversity ($\pi = 0.00155$) is consistent with values reported for other *Myotis* species with similar phylogeographic structure (Ruedi & Castella, 2003; Salicini *et al.*, 2013).

The ddRAD individuals are a subset of those characterized for mtDNA, so the two datasets are not sampled independently of one another. They were not, however, selected by mtDNA clade attribution: the criterion applied was geographic coverage. Because the mitochondrial lineages of *M.*

capaccinii are almost entirely partitioned by geography, a range-wide design was known in advance to deliver substantial samples of all three, and lineage coverage was secured without lineage serving as a selection criterion. Results of population structure based on nuclear ddRAD-seq with $K=2$ and $K=3$ groups largely corroborate the major mitochondrial structure in *M. capaccinii*. As this class of markers reflects both the contribution of males and females (not only female's contribution as in mtDNA), these markers suggest that interbreeding is ongoing between different clades, especially between clades C and B. At $K=3$, animals representing clades C1 and C2 have admixed nuclear genotypes, suggesting high interbreeding between these two groups. The highest assignment probabilities for the clade that corresponds to C1 were detected in northern Italy and Slovenia, suggesting a refugial origin for those populations. This observation is in line with fossil records and genetic evidence indicating that temperate mammal species, such as red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and wild boar (*Sus scrofa*), survived in these specific refugia (Sommer and Nadachowski, 2006), however a denser sampling within Italy and/or along the Adriatic coast is needed to evaluate this hypothesis more robustly.

Phylogeography of *M. capaccinii* part 2: The expansion

The mitochondrial break between clades B and the C, and the gradient of longitudinal differentiation, with a contact zone in between in the nuclear DNA data suggests refugial survival and differentiation in the Iberian and Anatolian peninsulas for clades C and B respectively, with subsequent range expansions. The Bayesian Skyline plots based on mtDNA suggest a relatively recent, post-glacial expansion for all three clades during the Holocene. The final range of the population expansions for the clades was dated to start around 2,000 years BP, 1,700 years BP, and 1,000 years BP for clades B, C1, and C2, respectively. Thus, the final expansions started long after the end of the Pleistocene, when the climate was warmer and the deglaciation of continental ice sheets had long been completed (Walker, 1995). The mismatch distributions, however, showed that a spatial expansion model better fitted the data for clade B, rather than a demographic expansion or a constant size model, whereas for clades C1 and C2, the results were inconclusive. To assess whether combining C1 and C2 would clarify these signals, we performed a combined mismatch analysis, which yielded a bimodal distribution characteristic of population structure rather than a single expansion event (Supplementary Figure 6). For clade C1, this may stem from the fact that there were an excess of three pairwise differences in the observed data, which stems from the three nucleotide differences of the most common haplotypes of this clade, namely haplotypes 5 and 6, of which haplotype 5 was only encountered in northern Italy and haplotype 6 only in the Thrace & Balkans region. This in turn, was possibly caused by the presence of a large area between these two locations that was not sampled in this study. Although the mismatch distribution tests were inconclusive and

unable to generate a signal for population expansion or distinguish between spatial and demographic expansion possibilities for clade C1, the significant IBD effect supports the possibility of a post-glacial spatial expansion. The combined C1+C2 mismatch results further support that these clades represent distinct evolutionary units with a history of secondary contact rather than a simple expansion. Fu's FS and Tajima's D statistics were negative for all clades, again indicating population expansion, yet none of these values were significant at the $P < 0.05$ level (Tajima, 1989). The only significant statistic among all detected values was Fu's and Li's D value for the Koufovouno colony, which was significantly positive, indicating an excess of parsimony informative sites, which was probably due to the fact that this population showed haplotypes from different clades. This in turn, may stem from the fact that colonies from Greece and Turkey mix in autumn (and probably in spring) when they are in transience to their winter quarters in Bulgaria (Papadatou *et al.* 2008, 2009). However, the presence of clade B in Thrace and the absence of clade C1 in Anatolia indicate that the range expansion of the older clade B, rather than just seasonal migrations, may be the real reason for their syntopical occurrence in the same caves.

The star-like haplotype network of clade B also indicated a recent population expansion. The most common haplotype of this clade, haplotype 10, was also the haplotype with the largest number of connections or descendant nodes among all 16 haplotypes, indicating it may be the most ancestral haplotype. Additionally, clade B had the highest haplotype and nucleotide diversities among the three clades, and Anatolia and the Middle East, hosting individuals carrying clade B haplotypes, were the regions with the highest haplotype and nucleotide diversities, suggesting ancestral/refugial populations within. Pockets of glacial refugia, where animal and plant species have been isolated and differentiated during the LGM, have been proposed for the Iberian, Italian, and Balkan peninsulas and south-western and eastern Anatolia (Hewitt, 1999; Veith *et al.*, 2003; Schmitt, 2007). The "refugia within refugia" hypothesis suggests that such pockets, or parts of major refugial peninsulas, are suitable for the persistence of species and populations (Bilgin, 2011; Bilgin *et al.*, 2016; Gürün *et al.*, 2019; Canestrelli *et al.*, 2007; Gómez & Lunt, 2007; Medail & Diadema, 2009). The phylogeography of several bat species, including *Nyctalus noctula* (Petit *et al.*, 1999), *Myotis myotis* (Ruedi & Castella, 2003), *Rhinolophus ferrumequinum* (Rossiter *et al.*, 2007), *Rhinolophus hipposideros* (Dool *et al.*, 2013), *Plecotus austriacus* (Razgour *et al.*, 2013), *Pipistrellus pipistrellus* (Boston *et al.*, 2014), *Myotis escalerai*, and *Myotis crypticus* (Puechmaile *et al.*, 2012; Salicini *et al.*, 2013; Razgour *et al.*, 2019), also supports this hypothesis. Glacial refugia most likely played a crucial role in promoting genetic diversity and differentiation for *M. capaccinii* as well, as shown by the mitochondrial splits which were dated back to the Pleistocene.

The gradual expansion of the mitochondrial clades can be observed in ddRAD-seq analysis of assignment probabilities longitudinally from both to west to east and east to west. Classical examples of inferring refugial isolation in Europe are generally based on observing genetic breaks geographically (Hewitt 1999, Schmidt 2007) and matching them with the Iberian, Italian or Balkan/Anatolian peninsulas in terms of origin. The post-isolation expansion scenarios are then based on the genetic similarity of non-peninsular populations with the peninsular ones. In this study, we used the mtDNA data to map the refugial areas, and our ddRAD-seq analyses provided extra line of evidence on the expansion out of the refugia, beyond the classical examples of distribution of genetic diversity mentioned above, showing the strength of combined use of mtDNA and ddRAD-seq in outlining glacial and post-glacial phylogeography of species. This integrative approach adds to the recent line of studies across Europe, where the combination of mitochondrial and genome-wide nuclear data consistently reveal complex patterns of glacial refugia and postglacial expansion of the continent (Dufresnes *et al.*, 2023; Gippner *et al.*, 2024).

The sharp mtDNA breaks we observe across major orographic barriers, contrasted with broad nuclear admixture and longitudinal clines in SNP assignment, are best explained by female philopatry and male-mediated gene flow in bats, a pattern widely documented and consistent with genetic methods for inferring sex-biased dispersal (Kerth, 2008; Prugnolle & de Meeus, 2002). In *Myotis*, classic cases include Bechstein's bat, where extreme matrilineal structure co-exists with extensive nuclear mixing due to dispersing males (Kerth *et al.*, 2002), and greater mouse-eared bat, where strong mtDNA differentiation but weak nuclear structure likewise points to male-biased dispersal (Castella *et al.*, 2001). Additional evidence comes from Natterer's bat, in which relatedness and parentage reveal female philopatry with males as the dispersive sex (Scott *et al.*, 2018), and from the northern long-eared bat, where high nuclear connectivity across scales is consistent with male movements during the mating season (Johnson *et al.*, 2014). A similar mito–nuclear contrast outside *Myotis* (e.g., pallid bat) shows deep mtDNA splits despite interbreeding at nuclear loci, underscoring that strong mtDNA discontinuities can co-occur with contemporary gene flow when dispersal is sex-biased (Arnold & Wilkinson, 2015). Taken together with our ddRAD-SNP results (admixture between C1 and C2; low–moderate nuclear F_{ST} across contact zones), these lines of evidence favor a biological explanation (sex-biased dispersal, secondary contact, postglacial dynamics) over a methodological explanation.

CONCLUSION

With this study, we combined ddRAD-seq-generated SNPs with mtDNA data throughout the range of *M. capaccinii* for a continent-scale evaluation of the mito-nuclear discordance observed in this

species. The three previously defined mtDNA clades (B, C1, C2) were delineated at a finer and larger scale. We show that the main mountain chains of Europe (the Alps and the Apennines) clearly separate the mitochondrial lineages, whereas sea straits such as Gibraltar appear to be relatively permeable in terms of historical gene flow, as expected for bats. Additionally, Thrace and the Alps appear to comprise contact zones that resulted from post-glacial expansions. In contrast, the SNP data show evidence for clinal variation, indicative of hybridization between mtDNA lineages. Therefore, the SNP data support the mito-nuclear discordance previously observed in microsatellites and suggest it to be of biological origin, likely due to sex-biased dispersal and secondary contact after post-glacial expansion.

TABLES

Table 1 – Mean observed heterozygosity, expected heterozygosity and nucleotide diversity values of variant positions for each population for ddRAD-seq.

Population ID	Observed Heterozygosity	Expected Heterozygosity	Nucleotide Diversity
Tadavan-IRN	0.24286	0.17768	0.2369
Halfeti-TUR	0.27713	0.25321	0.28938
Sarihidir-TUR	0.29258	0.25471	0.26922
Karan-TUR	0.27226	0.24426	0.25929
Kulu-TUR	0.27797	0.22886	0.26156
Aksu-TUR	0.27782	0.22388	0.26865
Beyyayla-TUR	0.2627	0.24023	0.28827
Nusret dudeni-TUR	0.29857	0.22378	0.29837
Yenisu-TUR	0.3464	0.2824	0.33888
Kiyikoy-TUR	0.35225	0.31516	0.34671
Koufovouno-GRE	0.29414	0.25985	0.28494
Maroneia-GRE	0.29714	0.25346	0.28621
Tsoutouro-GRE	0.35329	0.27418	0.32902
DBLUSF-GRE	0.28696	0.2655	0.30343

Slovenia-SLV	0.15914	0.14581	0.15269
Ichkeul-TUN	0.16176	0.13639	0.14609
Lombardia-ITA	0.15556	0.12299	0.16398
Provence-FRA	0.26474	0.18371	0.22046
SantMarti-SPA	0.23322	0.22028	0.2937
Alcampell-SPA	0.14758	0.11955	0.1594

Table 2 – Distribution of the 121 individuals in the final ddRAD dataset across countries and mitochondrial lineages.

Country	B	C1	C2	Total
Turkey	55	0	0	55
Greece	10	10	0	20
Slovenia	0	12	0	12
Tunisia	0	0	8	8
Spain	0	0	5	5
Algeria	0	0	4	4
Italy	0	1	3	4
Lebanon	3	0	0	3
France	0	0	3	3
Iran	3	0	0	3
Albania	0	1	0	1
Bulgaria	1	0	0	1
Romania	1	0	0	1
Syria	1	0	0	1
Total	74	24	23	121

FIGURES

Figure 1 – Map of sampling locations. The gray area is the current geographic range of *Myotis capaccinii* (spatial data source: International Union for Conservation of Nature, IUCN). The colors correspond to the mtDNA clades discussed below, B (red), C1 (green), and C2 (blue).

Figure 2 – The haplotype network for 265 cyt-b sequences (671 bp) and their distribution based on geographical regions. The sizes of the circles are proportional to the frequency of the haplotypes they represent. The small dots stand for missing haplotypes. Another version of this network, which is based on 38 populations instead of geographical regions, is given in Supplementary Figure 8.

Figure 3 – The sizes of the circles are proportional to the population sizes. a) The split zone between clades B and C1. b) The split zone between clades C1 and C2. The mountains extending along the France-Italy border, as well as the northern borders of Italy are the Alps, whereas the Apennine Mountains stretch from the northwest to the southeast of Italy. The Apennines, along with the Alps, act as a geographic barrier separating clades C1 and C2. The mountains separating France from Iberia are the Pyrenees.

Figure 4 – The Bayesian phylogenetic tree of 17 cyt-b haplotypes of *M. capaccinii*. The posterior probabilities are given as percentages on the nodes. The units on the reverse scale axis are years.

Figure 5 – Bayesian skyline plots for *M. capaccinii* clades (a) B, (b) C1 and (c) C2.

Figure 6. Matrix of Pairwise F_{ST} for 20 localities with their corresponding geographical mtDNA clade zone information.

Figure 7. The Structure plot of $K=2$ with individuals sorted by increasing longitude on the x-axis.

Figure 8. The Structure assignment probabilities to either of the populations (at $K=2$), sorted by longitude categories.

Figure 9. The Structure plot of $K=3$ with individuals sorted by increasing longitude on the x-axis.

Figure 10. The Structure assignment probabilities to either of the populations (at $K=3$), sorted by longitude categories.

SUPPLEMENTARY TABLES

Supplementary Table 1 – Sampling sites for *M. capaccinii*. N stands for the number of samples for mitochondrial DNA. The color codes representing different clades correspond to those in Supplementary Table 3.

Region	Population	Country	N	Map Code	Latitude	Longitude
Iberia	Almeria	Spain	10	1	37.39	-1.76
	Huesca	Spain	8	2	41.9	0.43
	Valencia	Spain	8	3	39.66	-0.43
	Mallorca	Spain	10	7	39.83	3.1
France	France	France	10	4	44.25	4.45
N. Africa	Tunisia	Tunisia	27	5	37.14	9.67
Italy	Sicily	Italy	5	6	37.48	13.55
	Lazio	Italy	5	8	41.31	13.49
	Sardegna	Italy	10	9	40.82	8.81
Italy	Lombardia	Italy	10	10	45.96	9.3
	Piemonte	Italy	2	11	45.89	8.53
Thrace - Balkans	Albania	Albania	10	12	40.67	20.99
	General Todorov	Bulgaria	1	13	41.47	23.27
	Krasen	Bulgaria	2	14	43.74	25.98
	Katratzides Mine	Greece	3	15	41.13	26.16
	V. Lefkimis	Greece	1	16	41.07	26.19
	Maronia Cave	Greece	18	17	40.93	25.5
	Tsoutouro Mine	Greece	31	18	41.15	26.19
	Dramia	Greece	1	20	35.28	24.32
Thrace - Balkans	Argyroupoli	Greece	1	21	35.35	24.25
	Koufovouno	Greece	31	19	41.35	26.45
Thrace - Balkans	Kav	Turkey	1	22	41.71	27.91
	Yenasu	Turkey	3	23	41.62	27.97
	Kuru	Turkey	1	24	41.84	27.56
Anatolia	Aksu	Turkey	5	25	40.76	30.96
	Boğazpınar	Turkey	7	26	40.02	28.02
	Nusret Düdeni	Turkey	3	27	39.62	28.16
	Beyyayla	Turkey	7	28	40.13	30.7
	Karan	Turkey	14	29	39.56	27.1
Thrace - Balkans	Kırıköy	Turkey	1	30	41.64	28.09
Middle East	Haql el Azim	Lebanon	1	31	34.4	36.02
	Bisotun	Iran	1	32	34.38	47.43
	Si Mili	Iran	1	33	31.7	49.4
	Eslamabad	Iran	3	34	34.27	46.79
Anatolia	Kulu	Turkey	1	35	39.09	33.08
	Sarıhıdır Nevşehir	Turkey	10	36	38.74	34.93
Middle East	Beaufort Castle	Lebanon	1	37	33.32	35.53
	Syria	Syria	1	38	36.33	39.08
Total number of samples analyzed:			265			

Supplementary Table 2 – Coordinate information of sampling sites for *M. capaccinii* with number of samples for which ddRAD-seq data was collected from each location.

	Population	Country	No of samples	Latitude	Longitude
1	Almeira	Spain	1	37.39	-1.76
2	Alcampell, Huesca	Spain	2	41.9	0.43
3	Sant Marti, Mallorca	Spain	2	39.83	3.1
4	Provence	France	2	44.25	4.45
5	Piemonte	Italy	1	45.89	8.53
6	Lombardia	Italy	2	45.96	9.3
7	Ichkeul Cave	Tunisia	8	37.14	9.67
8	Sicilia	Italy	1	37.48	13.55
9	Kosnica	Slovenia	12	46.22	14.97
10	Devoll	Albania	1	40.67	20.99
11	Tzanes	Greece	1	37.04	25.22
12	Tsoutouro Mines	Greece	3	41.15	26.19
13	General Todorov	Bulgaria	1	41.47	23.27
14	Maroneia	Greece	5	40.93	25.5
15	Koufovouno	Greece	6	41.35	26.45
16	Crete	Greece	1	35.28	24.32
17	Dupnisa	Turkey	1	41.84	27.55
18	Sivriiler	Turkey	1	41.81	27.92
19	Yenisu	Turkey	3	41.62	27.97
20	Kıyıköy	Turkey	6	41.64	28.09
21	Nusret Düdeni	Turkey	2	39.62	28.16
22	Kocaeli	Turkey	1	40.83	29.51
23	Beyyayla	Turkey	3	40.13	30.7
24	Aksu	Turkey	3	40.76	30.96
25	Kulu	Turkey	4	39.09	33.08
26	Karan	Turkey	9	39.56	27.1
27	Atakum	Turkey	4	41.40	36.18
28	Sarıhıdır	Turkey	10	38.74	34.93
29	Bitlis	Turkey	1	38.40	42.11
30	Halfeti	Turkey	4	37.25	37.85
31	Mrouj	Lebanon	1	33.35	35.45
32	Aamchite	Lebanon	1	34.15	35.67
33	Al Ghazli	Syria	1	36.33	39.08

Supplementary Table 3 – Descriptive statistics for the *M. capaccinii* samples. **h** is the haplotype diversity, π is the nucleotide diversity, **SV** is the number singleton variable sites, **PI** is the number of parsimony informative sites and **r** is Harpending's raggedness index, **Map Code** is the number associated with a given sample on **Figure 1** Koufovouno is not included in Thrace B and Thrace C1 geographical regions. The significant values are in bold, where the asterisk signifies $P < 0.02$. The color codes representing different clades correspond to those in Table 1. NA means the corresponding software was not able to calculate the values, usually because of a lack of polymorphisms or small sample size at the site.

Clade	Haplotypes	Size	Site	Country	Map Code	h	π	SV	PI	Tajima's D	Fu's F _s	Fu's D	r
C2	H1	10	Almeria	Spain	1	0	0	0	0	NA	NA	NA	NA
	H1	8	Huesca	Spain	2	0	0	0	0	NA	NA	NA	NA
	H1	8	Valencia	Spain	3	0	0	0	0	NA	NA	NA	NA
	H2	10	Mallorca	Spain	4	0	0	0	0	NA	NA	NA	NA
	H1	10	Cèze	France	5	0	0	0	0	NA	NA	NA	NA
	H1, H2	27	Ichkeul	Tunisia	6	0,462	0,00069	0	1	1,258	0,902	0,607	0,2189
	H1, H3, H4	5	Sicily	Italy	7	0,7	0,00289	2	2	0,273	0,278	0,273	0,7100
	H2	5	Lazio	Italy	8	0,000	0,00000	0	0	0,000	0,000	0,000	0,0000
C1	H2	10	Sardegna	Italy	9	0,000	0,00000	0	0	NA	NA	NA	NA
	H5	10	Lombardia	Italy	10	0,000	0,00000	0	0	NA	NA	NA	NA
	H5	2	Piemonte	Italy	11	0,000	0,00000	0	0	NA	NA	NA	NA
	H6	10	Albania	Albania	12	0,000	0,00000	0	0	NA	NA	NA	NA
	H6	1	G. Todorov	Bulgaria	13	NA	NA	0	0	NA	NA	NA	NA
	H6	2	Krasen	Bulgaria	14	0,000	0,00000	0	0	NA	NA	NA	NA
	H6	3	Katratzides	Greece	15	0	0	0	0	NA	NA	NA	NA
	H6	1	Lefkimis	Greece	16	NA	NA	0	0	NA	NA	NA	NA
	H6	18	Maronia	Greece	17	0	0	0	0	NA	NA	NA	NA
	H6, H8	31	Tsoutouro	Greece	18	0,065	0,00010	1	0	-1,145	-1,774	-1,693	0,7627
C1&B	H7	1	Dramia	Greece	19	NA	NA	0	0	NA	NA	NA	NA
	H7	1	Argyroupoli	Greece	20	NA	NA	0	0	NA	NA	NA	NA
B	H6, H9, H10	31	Koufovouno	Greece	21	0,185	0,00665	0	25	-1,017	0,961	1,693*	0,6791
	H9	1	Kav	Turkey	22	NA	NA	0	0	NA	NA	NA	NA
	H9	3	Yenasu	Turkey	23	0	0	0	0	NA	NA	NA	NA
	H9	1	Kuru	Turkey	24	NA	NA	0	0	NA	NA	NA	NA
	H10, H11	5	Aksu	Turkey	25	0,4	0,00060	1	0	-0,817	-0,772	-0,817	0,2000
	H10, H12	7	Boğazpınar	Turkey	26	0,286	0,00043	1	0	-1,006	-1,101	-1,049	0,2653
	H10	3	N. Düdeni	Turkey	27	0,000	0,00000	0	0	NA	NA	NA	NA
	H10, H13	7	Beyyayla	Turkey	28	0,571	0,00085	0	1	1,342	1,101	0,953	0,3469
	H10	14	Karan	Turkey	29	0,000	0,00000	0	0	NA	NA	NA	NA
	H10	1	Kıyıköy	Turkey	30	NA	NA	0	0	NA	NA	NA	NA
	H10	1	H. el Azim	Lebanon	31	NA	NA	0	0	NA	NA	NA	NA
	H10	1	Bisotun	Iran	32	NA	NA	0	0	NA	NA	NA	NA
	H10	1	Si Mili	Iran	33	NA	NA	0	0	NA	NA	NA	NA
	H10	3	Eslamabad	Iran	34	0,000	0,00000	0	0	NA	NA	NA	NA
	H14	1	Kulu	Turkey	35	NA	NA	0	0	NA	NA	NA	NA
	H15	10	Sarıhıdır	Turkey	36	0,000	0,00000	0	0	NA	NA	NA	NA
	H15	1	B. Castle	Lebanon	37	NA	NA	0	0	NA	NA	NA	NA
	H16	1	Syria	Syria	38	NA	NA	0	0	NA	NA	NA	NA
Clade	Haplotypes	Size	Geographical Region	Country	Code	h	π	SV	PI	Tajima's D	Fu's F _s	Fu's D	r
C2	H1, H2	36	Iberia	NA	NA	0,413	0,00062	0	1	1,047	0,815	0,574	0,2008
	H1	10	France	France	NA	0	0	0	0	NA	NA	NA	NA
	H1, H2	27	North Africa	Tunisia	NA	0,462	0,00069	0	1	1,258	0,902	0,607	0,2189
	H1, H2, H3, H4	20	Italy C2	Italy	NA	0,432	0,0011	2	2	-1,003	-0,952	-0,759	0,2209
C1	H5	12	Italy C1	Italy	NA	0,000	0,00000	0	0	NA	NA	NA	NA
	H6, H7, H8	68	Thrace C1	NA	NA	0,086	0,00022	1	2	-1,484	-0,933	-0,494	0,7872
B	H9, H10	6	Thrace B	NA	NA	0,333	0,0005	1	0	-0,933	-0,965	-0,950	0,2222
	H10, H15, H16	8	Middle East	NA	NA	0,464	0,00149	4	0	-1,535	-1,797	-1,665	0,2283
	H10-H15, H17	47	Anatolia	Turkey	NA	0,702	0,00140	2	4	-0,802	-0,713	-0,520	0,1548
C1	H5 - H8	111	All C1	NA	NA	0,242	0,00099	1	4	-0,622	-0,280	-0,048	0,6136
C2	H1 - H4	93	All C2	NA	NA	0,521	0,00095	1	3	-0,378	-0,341	-0,248	0,1695
B	H9 - H17	69	All B	NA	NA	0,718	0,00155	3	7	-1,353	-0,999	-0,584	0,1288
All	H1 - H17	273	All	NA	NA	0,802	0,01716	4	36	2,098	1,714	0,891	0,0626

Supplementary Table 4 – The number of assembled loci, the number of polymorphic loci and the number of SNPs information for each a) m and b) M, and c) the number of new polymorphic loci added for each iteration of M.

a) Parameter	Number of Assembled Loci	Number of Polymorphic Loci	Number of SNPs
m1	453	248	408
m2	5162	3561	7575
m3	5182	3564	7543
m4	4892	3339	7053
m5	4539	3043	6489
m6	4217	2794	5950

b)Parameter	Number of Assembled Loci	Number of Polymorphic Loci	Number of SNPs
M1	5205	3532	7257
M2	5265	3846	10438
M3	5273	4049	13586
M4	5281	4216	16654
M5	5298	4354	19350
M6	5300	4423	21081
M7	5288	4448	22432
M8	5255	4430	23065

c)Parameter	Number of New Loci Added
M1/M2	314
M2/M3	203
M3/M4	164
M4/M5	138
M5/M6	69
M6/M7	25
M7/M8	-18

Supplementary Table 5 – A complete list of samples and their corresponding haplotype assignments.

Number of variable sites: 11
Number of haplotypes, h: 9
Haplotype diversity, Hd: 0.4852
Hap_1: 185 [1-89 92 106-118 121-201 273]
Hap_2: 3 [90-91 93]
Hap_3: 12 [94-105]
Hap_4: 2 [119-120]
Hap_5: 64 [202-205 210-245 247-257 259-268 270-272]
Hap_6: 4 [206-209]
Hap_7: 1 [246]
Hap_8: 1 [258]
Hap_9: 1 [269]
Hap_1: 185 [MC001 Almeria MC002 Almeria MC003 Almeria MC004 Almeria MC005 Almeria MC006 Almeria MC007 Almeria MC008 Almeria MC009 Almeria MC010 Almeria MC021 Huesca MC022 Huesca MC023 Huesca MC024 Huesca MC025 Huesca MC026 Huesca MC027 Huesca MC028 Huesca MC011 Mallorca MC012 Mallorca MC013 Mallorca MC014 Mallorca MC015 Mallorca MC016 Mallorca MC017 Mallorca MC018 Mallorca MC019 Mallorca MC020 Mallorca MC035 Valencia MC036 Valencia MC029 Valencia MC030 Valencia MC031 Valencia MC032 Valencia MC033 Valencia MC034 Valencia MC059 Provenza MC060 Provenza MC061 Provenza MC062 Provenza

MC063|Provenza MC064|Provenza MC065|Provenza MC066|Provenza MC067|Provenza
 MC068|Provenza MC079|Tunisia MC080|Tunisia MC081|Tunisia MC082|Tunisia MC084|Tunisia
 MC085|Tunisia MC086|Tunisia MC087|Tunisia MC088|Tunisia MC337|Tunisia MC338|Tunisia
 MC340|Tunisia MC341|Tunisia MC342|Tunisia MC343|Tunisia MC344|Tunisia MC345|Tunisia
 MC346|Tunisia MC347|Tunisia MC348|Tunisia MC349|Tunisia MC350|Tunisia MC351|Tunisia
 MC352|Tunisia MC353|Tunisia MC354|Tunisia MC355|Tunisia MC115|Lazio MC116|Lazio MC117|Lazio
 MC118|Lazio MC119|Lazio MC120|Sardegna MC121|Sardegna MC122|Sardegna MC123|Sardegna
 MC124|Sardegna MC125|Sardegna MC126|Sardegna MC127|Sardegna MC128|Sardegna
 MC129|Sardegna MC089|Sicilia MC092|Sicilia MC069|Albania MC070|Albania MC071|Albania
 MC072|Albania MC073|Albania MC074|Albania MC075|Albania MC076|Albania MC077|Albania
 MC078|Albania MC235|G.Todorov MC057|Krasen MC058|Krasen MC197|Katratzides
 MC206|Katratzides MC285|Katratzides MC182|Lefkimis MC140|Maronia MC141|Maronia
 MC142|Maronia MC143|Maronia MC144|Maronia MC145|Maronia MC146|Maronia MC147|Maronia
 MC148|Maronia MC149|Maronia MC419|Maronia MC420|Maronia MC421|Maronia MC422|Maronia
 MC423|Maronia MC424|Maronia MC426|Maronia MC427|Maronia MC154|Tsoutouro
 MC156|Tsoutouro MC157|Tsoutouro MC158|Tsoutouro MC160|Tsoutouro MC163|Tsoutouro
 MC164|Tsoutouro MC168|Tsoutouro MC169|Tsoutouro MC170|Tsoutouro MC171|Tsoutouro
 MC173|Tsoutouro MC178|Tsoutouro MC183|Tsoutouro MC185|Tsoutouro MC210|Tsoutouro
 MC212|Tsoutouro MC213|Tsoutouro MC240|Tsoutouro MC242|Tsoutouro MC244|Tsoutouro
 MC250|Tsoutouro MC253|Tsoutouro MC254|Tsoutouro MC259|Tsoutouro MC263|Tsoutouro
 MC270|Tsoutouro MC271|Tsoutouro MC273|Tsoutouro MC287|Tsoutouro MC314|Tsoutouro
 MC132|Koufovouno MC134|Koufovouno MC136|Koufovouno MC137|Koufovouno MC139|Koufovouno
 MC179|Koufovouno MC184|Koufovouno MC186|Koufovouno MC187|Koufovouno MC189|Koufovouno
 MC191|Koufovouno MC192|Koufovouno MC193|Koufovouno MC194|Koufovouno MC195|Koufovouno
 MC207|Koufovouno MC214|Koufovouno MC252|Koufovouno MC260|Koufovouno MC261|Koufovouno
 MC268|Koufovouno MC276|Koufovouno MC277|Koufovouno MC284|Koufovouno MC302|Koufovouno
 MC308|Koufovouno MC316|Koufovouno MC425|Koufovouno EU475885.1]

Hap_2: 3 [MC090|Sicilia MC091|Sicilia MC094|Sicilia]

Hap_3: 12 [MC095|Lombardia MC096|Lombardia MC097|Lombardia MC098|Lombardia
 MC099|Lombardia MC100|Lombardia MC101|Lombardia MC102|Lombardia MC103|Lombardia
 MC104|Lombardia MC111|Piemonte MC112|Piemonte]

Hap_4: 2 [MC238|Argyroupoli MC236|Dramia]

Hap_5: 64 [MC135|Koufovouno MC190|Koufovouno MC218|Koufovouno MC376|Aksu
 MC037|Bogazpinar MC038|Bogazpinar MC039|Bogazpinar MC040|Bogazpinar MC041|Bogazpinar
 MC042|Bogazpinar MC043|Bogazpinar MC044|NusretD. MC045|NusretD. MC046|NusretD.
 MC381|Beyyayla MC382|Beyyayla MC383|Beyyayla MC384|Beyyayla MC385|Beyyayla
 MC386|Beyyayla MC388|Beyyayla MC399|Karan MC400|Karan MC401|Karan MC402|Karan
 MC403|Karan MC404|Karan MC405|Karan MC406|Karan MC408|Karan MC409|Karan MC410|Karan
 MC411|Karan MC412|Karan MC413|Karan MC430|Kav MC391|Kiyikoy MC393|Yenasu MC394|Yenasu
 MC395|Yenasu MC389|Kuru MC047|Sarihidir MC048|Sarihidir MC049|Sarihidir MC050|Sarihidir
 MC051|Sarihidir MC052|Sarihidir MC053|Sarihidir MC054|Sarihidir MC055|Sarihidir MC056|Sarihidir
 MC228|BeaufortC. MC225|H.el.Azim MC239|Bisotun MC233|SiMili MC150|iran MC151|iran
 MC152|iran EU475878.1 EU475879.1 EU475880.1 EU475882.1 EU475883.1 EU475884.1]

Hap_6: 4 [MC377|Aksu MC378|Aksu MC379|Aksu MC380|Aksu]

SUPPLEMENTARY FIGURES

Supplementary Figure 1 – The neighbor-joining tree for *M. capaccinii*. The bootstrap support values are given on the nodes.

Supplementary Figure 2 – The maximum likelihood tree for *M. capaccinii*. The bootstrap support values are given on the nodes.

Supplementary Figure 3 – a) F_{ST} values for 38 populations and three clades of *M. capaccinii*. The color codes of the clades correspond to those on Figure 1. b) The significance of the F_{ST} values. The color codes of the clades correspond to those on Figure 1.

Supplementary Figure 4 – Nei's distance and average number of pairwise differences within and between 38 populations of *M. capaccinii*.

Supplementary Figure 5 – The haplotype distance matrix of 265 *M. capaccinii* samples.

Supplementary Figure 6 – The observed and expected mismatch distributions in constant size and expanding population models for *M. capaccinii* clades B, C1, C2 and C (combined C1 and C2). The x-axis shows the number of pairwise differences between cyt-b haplotypes, and the y-axis represents the frequency of these differences. The raggedness index values are not significant ($P > 0.05$).

Supplementary Figure 7 – Observed and expected mismatch distributions under models of demographic and spatial expansions for clade B of *Myotis capaccinii*. A model of constant population size was rejected (99% confidence intervals of the mutation parameters θ_0 and θ_1 , which are related to the effective size of the population before and after expansion, respectively, were not overlapping). For both the demographic expansion and spatial expansion models, the raggedness index (r) was 0.1447 ($P < 0.001$).

Supplementary Figure 8 – The haplotype network for 265 cyt-b sequences (671 bp) and their distribution based on 38 populations of *Myotis capaccinii*. The sizes of the circles are proportional to the frequency of the haplotypes they represent. The small dots stand for missing haplotypes.

Supplementary Figure 9 – For visualization of optimization of *de novo* assembly of loci using STACKS, plot of mean coverage for the samples where each *Myotis capaccinii* individuals is represented as a box plot as mean coverage.

Supplementary Figure 10 – Plots of iterating values of minimum number of raw reads required to form a stack (m) for metrics: the number of Assembled loci; the number of Polymorphic Loci and the number of SNPs.

Supplementary Figure 11 – Plots of iterating values of minimum number of raw reads required to form a stack (M) for metrics: the number of Assembled loci; the number of Polymorphic Loci and the number of SNPs in *Myotis capaccinii* samples.

Supplementary Figure 12 – Plot of the number of new polymorphic loci added for each iteration of M (the distance between stacks) for the dataset.

Supplementary Figure 13 – The divergence tree of the most common haplotypes of three haplogroups of *M. capaccinii* obtained using a strict molecular-clock with a rate of 4.8% divergence per million years. The units on the reverse scale axis are years.

Supplementary Figure 14 – The divergence tree of the most common haplotypes of three haplogroups of *M. capaccinii* obtained using an uncorrelated relaxed clock and *M. ricketti* as an outgroup. The units on the reverse scale axis are million years.

Supplementary Figure 15 – Statistical parsimony haplotype network based on a 205 bp overlapping segment of the cytochrome-b (cyt-b) gene, combining the 265 sequences generated in this study with eight sequences from Bilgin *et al.* (2008) (GenBank accessions EU475878.1–EU475885.1). Haplotypes belonging to clade B (including accessions EU475878.1–EU475884.1) are colored orange, whereas haplotypes corresponding to clades C1 and C2 (including accession EU475885.1) are colored blue. A complete list of samples and their corresponding haplotype assignments is provided in Supplementary Table 5. Due to the limited phylogenetic signal within this truncated overlapping region, the finer differentiation between clades C1 and C2 could not be resolved.

Supplementary Figure 16 – Maximum likelihood phylogenetic tree of *Myotis capaccinii* based on the 205 bp overlapping cytochrome-b (cyt-b) region, combining the 265 sequences generated in this study with the eight sequences from Bilgin *et al.* (2008). The tree was constructed using the 9 unique haplotypes (Hap_1–Hap_9) identified across the 273 analyzed samples (see Supplementary Table 5). The tree is drawn to scale, with branch lengths indicating the number of substitutions per site. A total of 11 variable sites were identified within the 205 bp alignment. As noted in the text, due to the short

sequence length and limited phylogenetic signal, minor European C haplotypes (Hap_2–Hap_4) form an unresolved polytomy alongside clade B haplotypes rather than resolving into a distinct bifurcating clade.

REFERENCES

- Andrews, K. R., Good, J. M., Miller, M. R., Luikart, G., Hohenlohe, P. A., 2016. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nature Reviews Genetics*, 17, 81–92.
- Bilgin, R., Karataş, A., Çoraman, E., Morales, J. C., 2008. The mitochondrial and nuclear genetic structure of *Myotis capaccinii* (Chiroptera: Vespertilionidae) in the Eurasian transition, and its taxonomic implications. *Zoologica Scripta*, 37, 253–262.
- Bilgin, R., 2011. Back to the Suture: The Distribution of Intraspecific Genetic Diversity in and Around Anatolia. *International Journal of Molecular Sciences*, 12, 4080–4103.
- Bilgin, R., Gürün, K., Rebelo, H., Puechmaille, S. J., Maracı, Ö., Presetnik, P., Benda, P., Hulva, P., Ibanez, C., Hamidovic, D., Fressel, N., Horaček, I., Karataş, A., Karataş, A., Allegrini, B., Georgiakakis, P., Gazaryan, S., Nagy, Z., Abi-Said, M., Lučan, R. K., Bartonička, T., Nicolaou, H., Scaravelli, D., Karapandža, B., Uhrin, M., Paunović, M., Juste, J., 2016. Circum-Mediterranean phylogeography of a bat coupled with past environmental niche modelling: a new paradigm for the recolonization of Europe? *Molecular Phylogenetics and Evolution*, 99, 323–336.
- Bilton, D. T., McDevitt, A. D., Razgour, O., 2021. Biogeography and postglacial colonisation of Europe. In *The Handbook of the Mammals of Europe*, Springer, Cham.
- Boston, E. S. M., Puechmaille, S. J., Clissmann, F., Teeling, E. C., 2014. Further evidence for cryptic north-western refugia in Europe? Mitochondrial phylogeography of the sibling species *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*. *Acta Chiropterologica*, 16, 263–277.
- Bruneaux, M., Johnston, S. E., Herczeg, G., Merilä, J., Primmer, C. R., Vasemägi, A., 2013. Molecular evolutionary and population genomic consequences of RADseq in a fish species with moderate levels of genetic diversity. *Molecular Ecology Resources*, 13, 1036–1047.
- Canestrelli, D., Cimmaruta, R., Nascetti, G., 2007. Phylogeography and historical demography of the Italian treefrog, *Hyla intermedia*, reveals multiple refugia, population expansions and secondary contacts within peninsular Italy. *Molecular Ecology*, 16, 4808–4821.
- Carroll, E. L., Bruford, M. W., DeWoody, J. A., Funk, W. C., 2024. Incorporating genetic and genomic data into conservation planning. *Annual Review of Animal Biosciences*, 12, 1–23.

- Castella, V., Ruedi, M., Excoffier, L., 2001. Contrasted patterns of mitochondrial and nuclear structure among nursery colonies of the bat *Myotis myotis*. *Journal of Evolutionary Biology*, 14, 708–720.
- Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., Cresko, W. A., 2013. Stacks: an analysis tool set for population genomics. *Molecular Ecology*, 22, 3124–3140.
- Centeno-Cuadros, A., Razgour, O., García-Mударra, J. L., Mingo-Casas, P., Sandonís, V., Redondo, A., Ibáñez, C., de Paz, O., Martínez-Alós, S., Pérez Suarez, G., Echevarría, J. E., Juste, J., 2019. Comparative phylogeography and asymmetric hybridization between cryptic bat species. *Journal of Zoological Systematics and Evolutionary Research*, 57, 1004–1018.
- Cheng, H., Edwards, R. L., Broecker, W. S., Denton, G. H., Kong, X. G., Wang, Y. J., Zhang, R., Wang, X. F., 2009. Ice age terminations. *Science*, 326, 248–252.
- Clement, M., Posada, D., Crandall, K. A., 2000. TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659.
- Cosson, J. F., Hutterer, R., Libois, R., Sarà, M., Taberlet, P., Vogel, P., 2005. Phylogeographical footprints of the Strait of Gibraltar and Quaternary climatic fluctuations in the western Mediterranean: a case study with the greater white-toothed shrew, *Crocidura russula* (Mammalia: Soricidae). *Molecular Ecology*, 14, 1151–1162.
- Çoraman, E., Dundarova, H., Dietz, C., Mayer, F., 2020. Patterns of mtDNA introgression suggest population replacement in Palaearctic whiskered bat species. *Royal Society Open Science*, 7, 191805.
- DaCosta, J. M., Sorenson, M. D., 2014. ddRAD-seq phylogenetics based on nucleotide, indel, and presence–absence polymorphisms. *Methods in Ecology and Evolution*, 5, 1287–1298.
- Darriba, D., Taboada, G. L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772.
- Deblase, A. F., 1980. The bats of Iran: systematics, distribution, ecology. *Fieldiana Zoology*, New Series, 4, 1–424.
- Dool, S. E., Puechmaille, S. J., Dietz, C., Juste, J., Ibanez, C., Hulva, P., Roué, S. G., Petit, E. J., Jones, G., Russo, D., 2013. Phylogeography and postglacial recolonization of Europe by *Rhinolophus hipposideros*: evidence from multiple genetic markers. *Molecular Ecology*, 22, 4055–4070.

Drummond, A. J., Suchard, M. A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29, 1969–1973.

Dufresnes, C., Dutoit, L., Brelsford, A., Zamora, M., Litvinchuk, S. N., 2023. Inferring genetic structure when there is little: Population genetics versus genomics of the threatened bat *Miniopterus schreibersii* across Europe. *Scientific Reports*, 13, 11314.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14, 2611–2620.

Excoffier, L., Smouse, P. E., Quattro, J. M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes — application to human mitochondrial-DNA restriction data. *Genetics*, 131, 479–491.

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

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

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

Furman, A., Çoraman, E., Çelik, Y. E., Postawa, T., Bachanek, J., Ruedi, M., 2014. Cytonuclear discordance and the species status of *Myotis myotis* and *Myotis blythii* (Chiroptera). *Zoologica Scripta*, 43, 549–561.

Furman, A., Çelik, Y. E., Çoraman, E., Bilgin, R., 2020. Reproductive isolation and morphological discrimination of *Myotis myotis macrocephalicus* and *M. blythii* s.l. (Chiroptera: Vespertilionidae) in Turkey. *Acta Chiropterologica*, 22, 21–28.

Gippner, S., Strowbridge, N., Sunje, E., Veith, M., 2024. The effect of hybrids on phylogenomics and subspecies delimitation in *Salamandra*, a highly diversified amphibian genus. *Salamandra*, 60, 105–128.

Gómez, A., Lunt, D. H., (Eds.), 2007. *Refugia Within Refugia: Patterns of Phylogeographic Concordance in the Iberian Peninsula*, Springer, Dordrecht.

Graur, D., Li, W. H., 2000. *Fundamentals of molecular evolution*, Second Ed., Sinauer Associates, Sunderland, MA.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696–704.

Gürün, K., Furman, A., Juste, J., Ramos-Pereira, M. J., Palmeirim, J. M., Puechmaille, S. J., Hulva, P., Presetnik, P., Hamidovic, D., Ibáñez, C., Karataş, A., Allegrini, B., Georgiakakis, P., Scaravelli, D., Uhrin, M., Nicolaou, H., Abi-Said, M. R., Nagy, Z. L., Gazaryan, S., Bilgin, R., 2019. An extended study on the social structure and phylogeography of the bent-wing bat *Miniopterus schreibersii* (Mammalia: Chiroptera), using new microsatellite data. *Journal of Mammalogy*, 100, 1865–1878.

Habbe, K. A., 2007. Stratigraphische Begriffe für das Süddeutsche Alpenvorland. *Eiszeitalter und Gegenwart (Quaternary Science Journal)*, 56, 66–83.

Habel, J. C., Dieker, P., Schmitt, T., 2009. Biogeographical connections between the Maghreb and the Mediterranean peninsulas of southern Europe. *Biological Journal of the Linnean Society*, 98, 693–703.

Habel, J. C., Rödder, D., Scalercio, S., Meyer, M., Schmitt, T., 2010. Strong genetic cohesiveness between Italy and the Maghreb in four butterfly species. *Biological Journal of the Linnean Society*, 99, 818–830.

Habel, J. C., Lens, L., Rödder, D., Schmitt, T., 2011. From Africa to Europe and back: refugia and range shifts cause high genetic differentiation in the Marbled White butterfly *Melanargia galathea*. *BMC Evolutionary Biology*, 11, 215.

Harpending, H. C., Sherry, S. T., Rogers, A. R., Stoneking, M., 1993. Genetic structure of ancient human populations. *Current Anthropology*, 34, 483–496.

Harrison, D. L., Bates, P. J. J., 1991. *The Mammals of Arabia*, Second Ed., Harrison Zoological Museum, Sevenoaks.

Heinrich, G., 1936. *Myotis bureschi* n. sp. aus Bulgarien. *Mitteilungen aus den Königlichen Naturwissenschaftlichen Instituten in Sofia*, 9, 23–28.

Hewitt, G., 1999. Postglacial recolonization of European Biota. *Biological Journal of the Linnean Society*, 68, 87–112.

Husemann, M., Schmitt, T., Zachos, F. E., Ulrich, W., Habel, J. C., 2014. Palaearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. *Journal of Biogeography*, 41, 81–94.

Hutson, A. M., Spitzenberger, F., Aulagnier, S., Juste, J., Karataş, A., Palmeirim, J., Paunović, M., 2008. *Myotis capaccinii*. In *IUCN Red List of Threatened Species*, Version 2012.1. <http://www.iucnredlist.org> (accessed October 2012).

Ibanez, C., Garcia-Mudarra, J. L., Ruedi, M., Stadelmann, B., Juste, J., 2006. The Iberian contribution to cryptic diversity in European bats. *Acta Chiropterologica*, 8, 277–297.

Jeffries, D. L., Copp, G. H., Handley, L. L., Olsén, K. H., Sayer, C. D., Hänfling, B., 2016. Comparing RADseq and microsatellites for estimating genetic diversity and relatedness—implications for brown trout conservation. *Ecology and Evolution*, 6, 2086–2100.

Johnson, L. N., Hohoff, C., Fenton, M. B., 2014. Genetic structure and sex-biased dispersal in the northern long-eared bat (*Myotis septentrionalis*). *Conservation Genetics*, 15, 1305–1315.

Kerth, G., Mayer, F., König, B., 2000. Mitochondrial DNA (mtDNA) reveals that female Bechstein's bats live in closed societies. *Molecular Ecology*, 9, 793–800.

Kerth, G., Mayer, F., Petit, E., 2002. Extreme sex-biased dispersal in the communally breeding, nonmigratory Bechstein's bat (*Myotis bechsteinii*). *Molecular Ecology*, 11, 1491–1498.

Kerth, G., 2008. Causes and consequences of sociality in bats. *Bioscience*, 58, 737–746.

Koopman, K. F., 1994. Chiroptera: Systematics. In *Handbook of Zoology, 8: Mammalia*, Walter de Gruyter, New York.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.

Kumerloeve, H., 1975. Die Säugetiere (Mammalia) der Türkei. *Veröffentlichungen der Zoologischen Staatssammlung München*, 18, 69–158.

Leigh, J. W., Bryant, D., 2015. PopART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6, 1110–1116.

Lemopoulos, A., Uusi-Heikkilä, S., Huusko, A., Vasemägi, A., Vainikka, A., 2019. Comparison of RADseq and microsatellites for estimating population structure and genetic diversity in wild brown trout (*Salmo trutta*). *Conservation Genetics Resources*, 11, 247–259.

Litt, T., Behre, K. E., Meyer, K. D., Stephan, H. J., Wansa, S., 2007. Stratigraphische Begriffe für das Quartär des norddeutschen Vereisungsgebietes. *Eiszeitalter und Gegenwart (Quaternary Science Journal)*, 56, 7–65.

- Medail, F., Diadema, K., 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *Journal of Biogeography*, 36, 1333–1345.
- Nabholz, B., Glemin, S., Galtier, N., 2008. Strong variations of mitochondrial mutation rate across mammals—the longevity hypothesis. *Molecular Biology and Evolution*, 25, 120–130.
- Nagy, M., Günther, L., Knörnschild, M., Mayer, F., 2013. Female-biased dispersal in a bat with a female-defence mating strategy. *Molecular Ecology*, 22, 1733–1745.
- Nei, M., Tajima, F., 1981. DNA polymorphism detectable by restriction endonucleases. *Genetics*, 97, 145–163.
- Papadatou, E., Butlin, R. K., Altringham, J. D., 2008. Seasonal roosting habits and population structure of the long-fingered bat *Myotis capaccinii* in Greece. *Journal of Mammalogy*, 89, 503–512.
- Papadatou, E., Butlin, R. K., Pradel, R., Altringham, J. D., 2009. Sex-specific roost movements and population dynamics of the vulnerable long-fingered bat, *Myotis capaccinii*. *Biological Conservation*, 142, 280–289.
- Paris, J. R., Stevens, J. R., Catchen, J. M., 2017. Lost in parameter space: a road map for STACKS. *Methods in Ecology and Evolution*, 8, 1360–1373.
- Peterson, A. T., Soberón, J., Sánchez-Cordero, V., 1999. Conservatism of ecological niches in evolutionary time. *Science*, 285, 1265–1267.
- Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., Hoekstra, H. E., 2012. Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE*, 7, e37135.
- Petit, E., Excoffier, L., Mayer, F., 1999. No evidence of bottleneck in the postglacial recolonization of Europe by the noctule bat (*Nyctalus noctula*). *Evolution*, 53, 1247–1258.
- Prugnolle, F., de Meeus, T., 2002. Inferring sex-biased dispersal from population genetic tools: a review. *Heredity*, 88, 161–165.
- Puechmaille, S. J., Allegrini, B., Boston, E. S., Dubourg-Savage, M. J., Evin, A., Knochel, A., Le Bris, Y., Lecoq, V., Lemaire, M., Rist, D., 2012. Genetic analyses reveal further cryptic lineages within the *Myotis nattereri* species complex. *Mammalian Biology*, 77, 224–228.

Putman, A. I., Carbone, I., 2014. Challenges in analysis and interpretation of microsatellite data for population genetic studies. *Ecology and Evolution*, 4, 4399–4428.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., Suchard, M. A., 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, 67, 901–904.

Razgour, O., Juste, J., Ibanez, C., Kiefer, A., Rebelo, H., Puechmaille, S. J., Arlettaz, R., Burke, T., Dawson, D. A., Beaumont, M., 2013. The shaping of genetic variation in edge-of-range populations under past and future climate change. *Ecology Letters*, 16, 1258–1266.

Razgour, O., Forester, B., Taggart, J. B., Bekaert, M., Juste, J., Ibáñez, C., Puechmaille, S. J., Novella-Fernandez, R., Alberdi, A., Manel, S., 2019. Considering adaptive genetic variation in climate change vulnerability assessment reduces species range loss projections. *Proceedings of the National Academy of Sciences USA*, 116, 10418–10423.

Rossiter, S. J., Benda, P., Dietz, C., Zhang, S., Jones, G., 2007. Range wide phylogeography in the greater horseshoe bat inferred from microsatellites: Implications for population history, taxonomy and conservation. *Molecular Ecology*, 16, 4699–4714.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., Sánchez-Gracia, A., 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 34, 3299–3302.

Ruedi, M., Mayer, F., 2001. Molecular systematics of bats of the genus *Myotis* (Vespertilionidae) suggests deterministic ecomorphological convergences. *Molecular Phylogenetics and Evolution*, 21, 436–448.

Ruedi, M., Castella, V., 2003. Genetic consequences of the ice ages on nurseries of the bat *Myotis myotis*: a mitochondrial and nuclear survey. *Molecular Ecology*, 12, 1527–1540.

Salicini, I., Ibanez, C., Juste, J., 2013. Deep differentiation between and within Mediterranean glacial refugia in a flying mammal, the *Myotis nattereri* bat complex. *Journal of Biogeography*, 40, 1182–1193.

Schmitt, T., 2007. Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology*, 4, 11.

Scott, D. D., Mayor, S. P., Fenton, M. B., 2018. Relatedness and parentage in a population of Natterer's bat (*Myotis nattereri*) in southern Ontario, Canada. *Canadian Journal of Zoology*, 96, 19–25.

Shackleton, J. C., Vanandel, T. H., Runnels, C. N., 1984. Coastal paleogeography of the central and western Mediterranean during the last 125 000 years and its archaeological implications. *Journal of Field Archaeology*, 11, 307–314.

Smith, M. F., Patton, J. L., 1993. The diversification of south-American murid rodents: Evidence from mitochondrial DNA sequence data for the akodontine tribe. *Biological Journal of the Linnean Society*, 50, 149–177.

Sommer, R. S., Nadachowski, A., 2006. Glacial refugia of mammals in Europe: evidence from fossil records. *Mammal Review*, 36, 251–265.

Stadelmann, B., Lin, L.-K., Kunz, T. H., Ruedi, M., 2007. Molecular phylogeny of New World *Myotis* (Chiroptera, Vespertilionidae) inferred from mitochondrial and nuclear DNA genes. *Molecular Phylogenetics and Evolution*, 43, 32–48.

Stöck, M., Sicilia, A., Belfiore, N. M., Buckley, D., Lo Brutto, S., Lo Valvo, M., Arculeo, M., 2008. Post-Messinian evolutionary relationships across the Sicilian channel: mitochondrial and nuclear markers link a new green toad from Sicily to African relatives. *BMC Evolutionary Biology*, 8, 56.

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., Rambaut, A., 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution*, 4, vey016.

Swenson, N. G., Howard, D. J., 2005. Clustering of contact zones, hybrid zones, and phylogeographic breaks in North America. *The American Naturalist*, 166, 581–591.

Tajima, F., 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics*, 105, 437–460.

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

Veith, M., Schmidtler, J. F., Kosuch, J., Baran, I., Seitz, A., 2003. Palaeoclimatic changes explain Anatolian mountain frog evolution: A test for alternating vicariance and dispersal events. *Molecular Ecology*, 12, 185–199.

Viglino, A., 2012. Study of variability and genetic structure of European populations of *Myotis emarginatus* and *Myotis capaccinii* (Chiroptera, Vespertilionidae). Doctoral thesis, Alma Mater Studiorum - Università di Bologna.

Walker, M. J. C., 1995. Climatic changes in Europe during the last glacial/interglacial transition. *Quaternary International*, 28, 63–76.

Worthington Wilmer, J., Barratt, E., 1996. A non-lethal method of tissue sampling for genetic studies of chiropterans. *Bat Research News*, 37, 1–3.

Zhang, D. X., Hewitt, G. M., 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, 12, 563–584.

Zimmermann, T., Junker, R. R., Müller, J., 2020. Microsatellites versus SNPs: a comparative study of genetic diversity and population structure in a forest tree. *Tree Genetics & Genomes*, 16, 1–12.

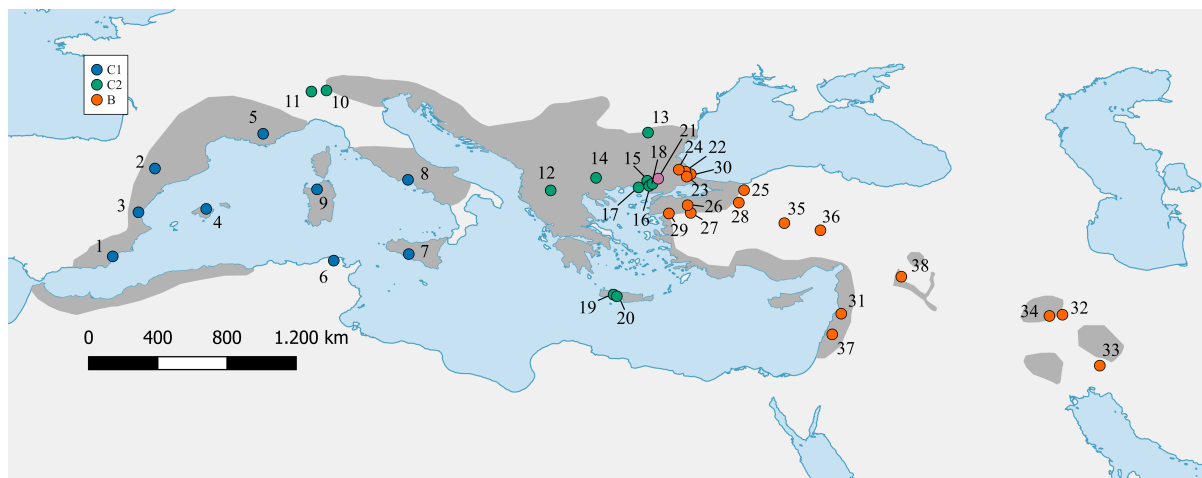
FIGURES

Figure 1 – Map of sampling locations. The map and color codes correspond to those in Table 1.

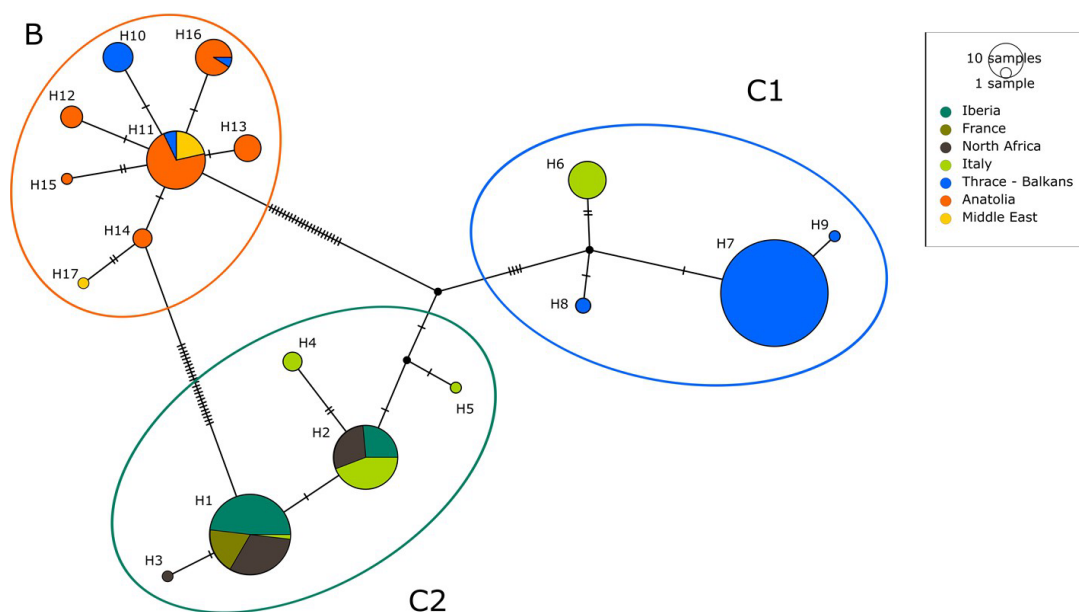


Figure 2 – The haplotype network for 265 cyt-b sequences (692 bp) and their distribution based on geographical regions. The sizes of the circles are proportional to the frequency of the haplotypes they represent. The small dots stand for missing haplotypes. Another version of this network, which is based on 38 populations instead of geographical regions, is given in Supplementary Figure X.

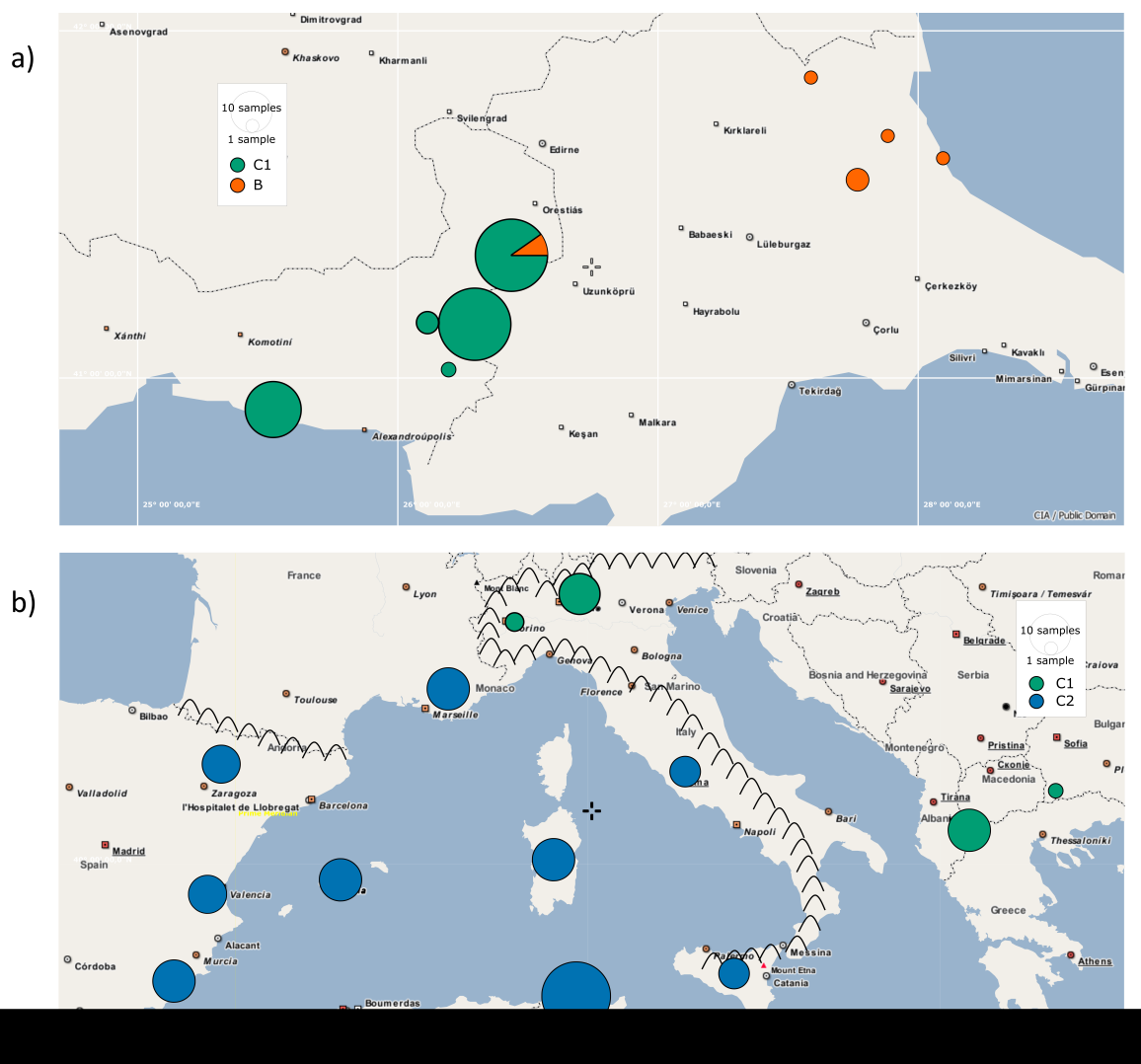


Figure 3 – The sizes of the circles are proportional to the population sizes. a) The split zone between haplogroups B and C1. b) The split zone between haplogroups C1 and C2. The mountains extending along the France-Italy border, as well as the northern borders of Italy are the Alps, whereas the Apennine Mountains stretch from the northwest to the southeast of Italy. The Apennines, along with the Alps, act as a geographic barrier separating haplogroups C1 and C2. The mountains separating France from Iberia are the Pyrenees.

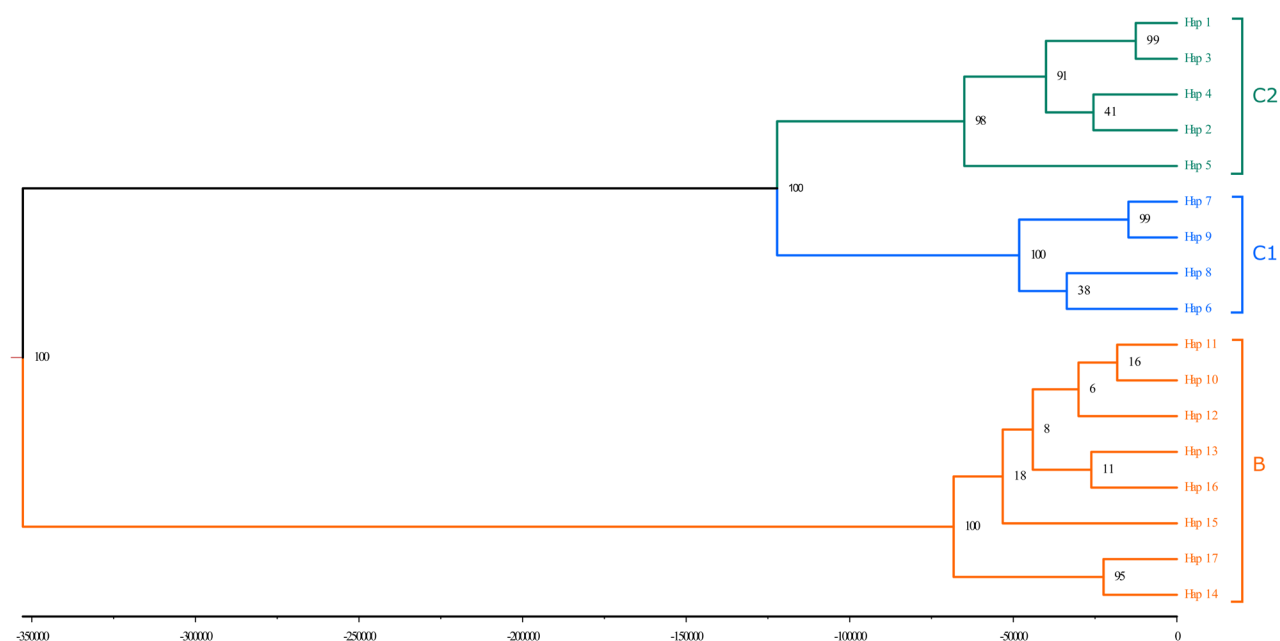


Figure 4 – The Bayesian phylogenetic tree of 17 cyt-b haplotypes of *M. capaccinii*. The posterior probabilities are given as percentages on the nodes. The units on the reverse scale axis are years.

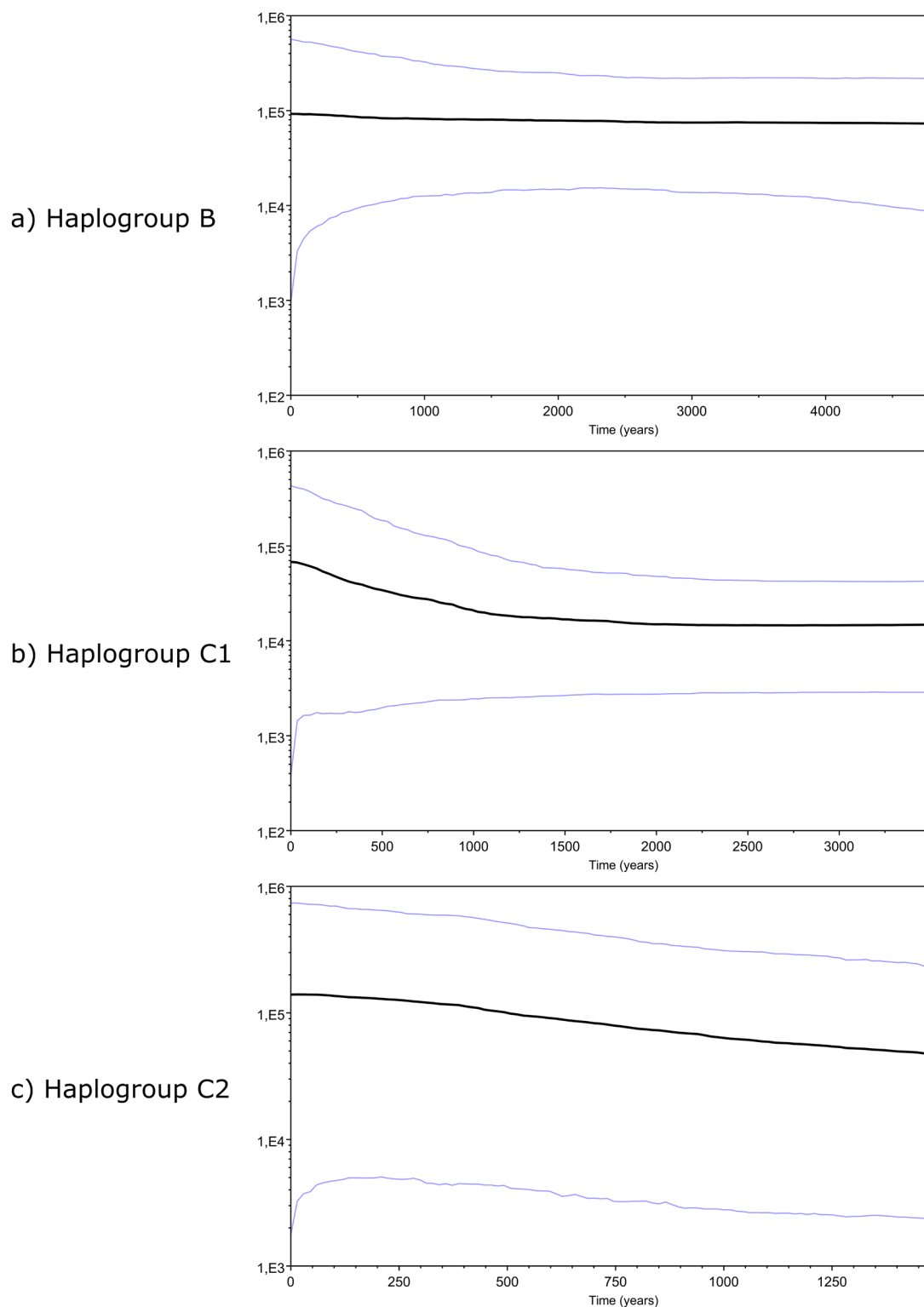


Figure 5 – Bayesian skyline plots for *M. capaccinii* haplogroups (a) B, (b) C1 and (c) C2.

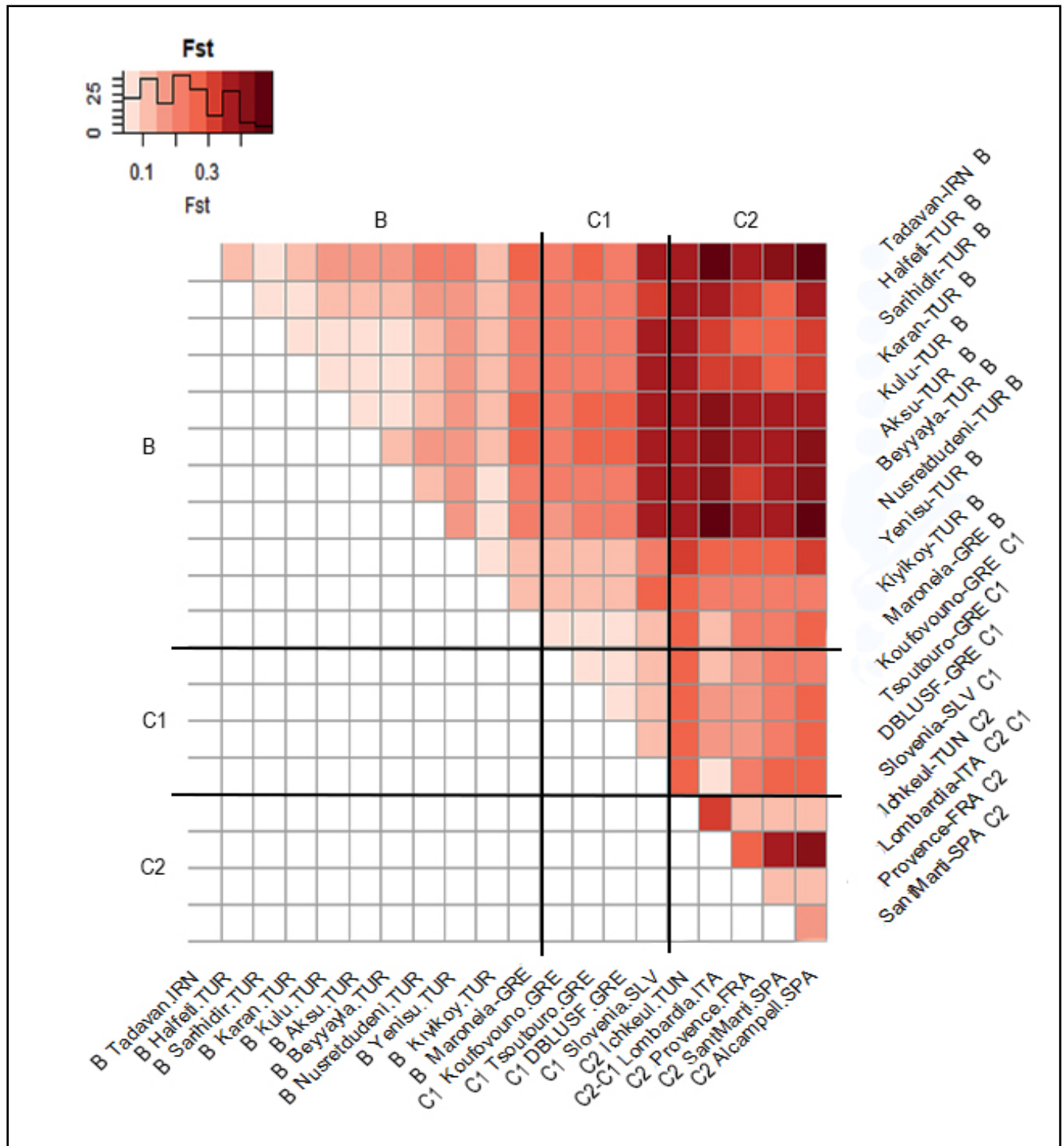


Figure 6. Matrix of Pairwise F_{ST} for 20 localities with their corresponding geographical mtDNA clade zone information.

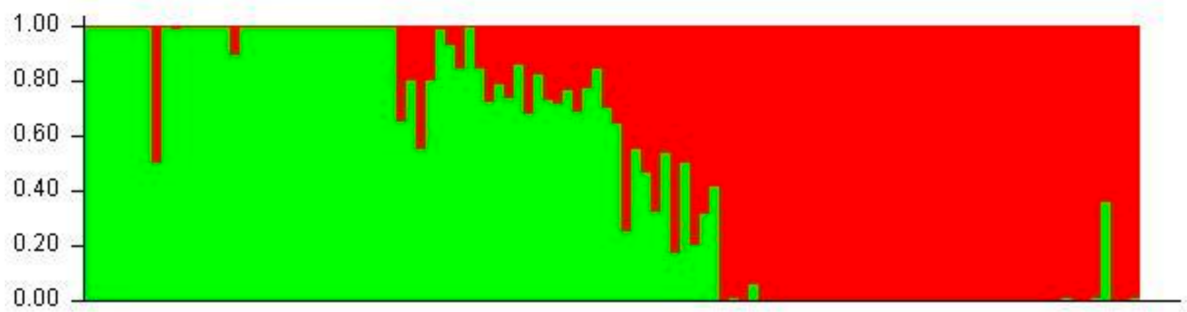


Figure 7. The Structure plot of K=2 with individuals sorted by increasing longitude on the x-axis.

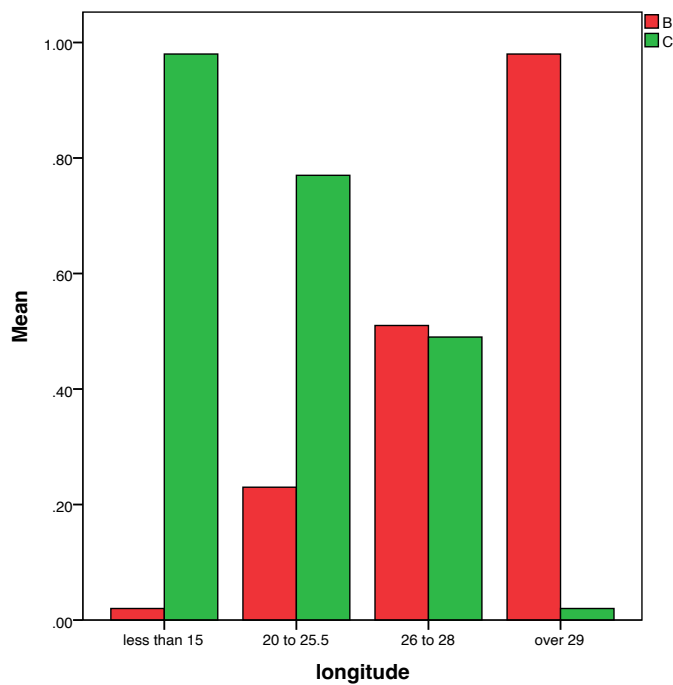


Figure 8. The Structure assignment probabilities to either of the populations (at $K=2$), sorted by longitude categories.

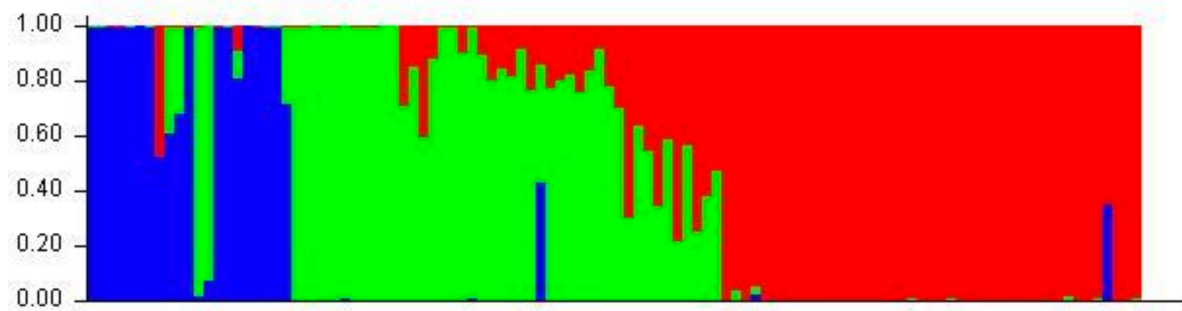


Figure 9. The Structure plot of K=3 with individuals sorted by increasing longitude on the x-axis.

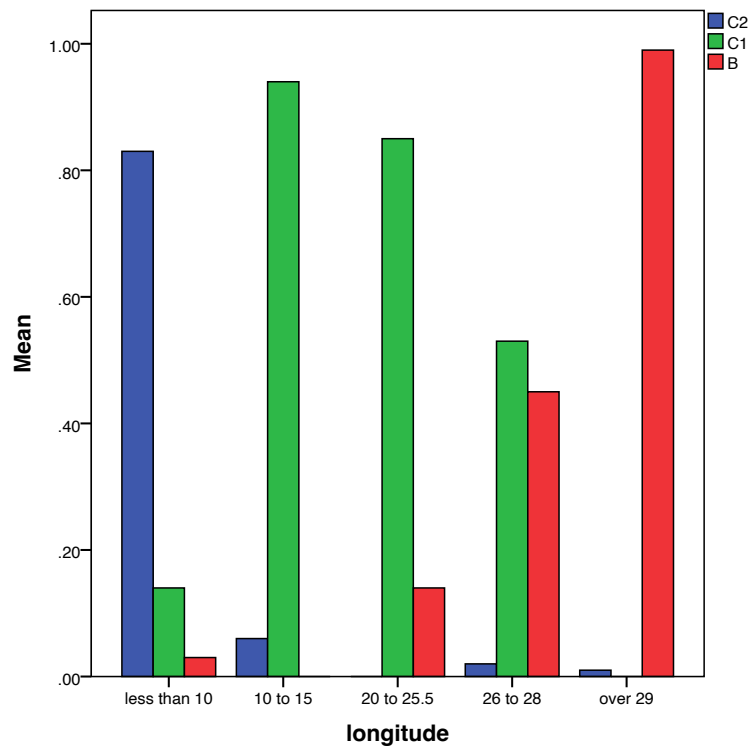


Figure 10. The Structure assignment probabilities to either of the populations (at $K=3$), sorted by longitude categories.

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