



Circum-Mediterranean phylogeography of a bat coupled with past environmental niche modeling: A new paradigm for the recolonization of Europe?



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ABSTRACT

The isolation of populations in the Iberian, Italian and Balkan peninsulas during the ice ages define four main paradigms that explain much of the known distribution of intraspecific genetic diversity in Europe. In this study we investigated the phylogeography of a wide-spread bat species, the bent-winged bat, *Miniopterus schreibersii* around the Mediterranean basin and in the Caucasus. Environmental Niche Modeling (ENM) analysis was applied to predict both the current distribution of the species and its distribution during the last glacial maximum (LGM). The combination of genetics and ENM results suggest that the populations of *M. schreibersii* in Europe, the Caucasus and Anatolia went extinct during the LGM, and the refugium for the species was a relatively small area to the east of the Levantine Sea, corresponding to the Mediterranean coasts of present-day Syria, Lebanon, Israel, and northeastern and northwestern

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Egypt. Subsequently the species first repopulated Anatolia, diversified there, and afterwards expanded into the Caucasus, continental Europe and North Africa after the end of the LGM. The fossil record in Iberia and the ENM results indicate continuous presence of *Miniopterus* in this peninsula that most probably was related to the Maghrebian lineage during the LGM, which did not persist afterwards. Using our results combined with similar findings in previous studies, we propose a new paradigm explaining the general distribution of genetic diversity in Europe involving the recolonization of the continent, with the main contribution from refugial populations in Anatolia and the Middle East. The study shows how genetics and ENM approaches can complement each other in providing a more detailed picture of intraspecific evolution.

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1. Introduction

The three peninsulas in southern Europe, the Iberian, the Italian and the Balkan, comprise refugia which not only made it possible for populations of species to survive during the ice ages (Taberlet et al., 1998), but also acted as “engines for speciation” (Hewitt, 2011). During the ice ages, isolated populations of many widespread species became genetically differentiated in the peninsulas and later repopulated the rest of Europe after the last glacial maximum (LGM). Based on the role played by these peninsulas, four paradigms of intraspecific genetic evolution in Europe have been defined (Habel et al., 2005; Hewitt, 1999). Under the grasshopper paradigm, the Balkan populations populated continental Europe more quickly than the Iberian and/or the Italian populations, which were confined to their own peninsulas by the Pyrenees and the Alps, respectively. Under the hedgehog paradigm, species dispersed out of their respective peninsulas in tandem and the differentiated Iberian, Italian and Balkan populations populated regions corresponding roughly to western, central and eastern Europe, respectively. The bear paradigm outlines a pattern where the Alps acted as a barrier to dispersal of the Italian populations after the LGM, whereas the Iberian and Balkan populations populated rest of Europe, meeting around central Europe (Hewitt, 1999). The most recently defined paradigm, the butterfly paradigm, outlines a scenario in which expansion took place out of differentiated populations in the Italian and Balkan/Anatolian peninsulas, but those in the Iberian peninsula were confined by the Pyrenees (Habel et al., 2005).

However, these paradigms are likely to be oversimplifications of the origins of intraspecific genetic evolution in Europe, especially at smaller geographic scales. For instance, smaller “refugia within refugia” have been suggested to exist within Iberia (Gómez and Lunt, 2007), Italy (Canestrelli et al., 2007), the Balkans (Previšić et al., 2009; Surina et al., 2011), and Anatolia (Bilgin, 2011). Additionally, local continental glacial refugia in central Europe have been suggested for many species, including the Carpathian Basin, Black Forest region, Pyrenean region, and Higher Tatras (Schmitt, 2007).

Recolonization of European populations may also have origins in glacial refugia farther to the east. For instance, in addition to the Balkan refugium, Anatolia has been shown to be a potential refugium, from where populations subsequently colonized Europe (Bilgin, 2011; Rokas et al., 2003). For some species, especially those inhabiting the taiga (e.g. Russian flying squirrel *Pteromys volans*), dispersal centers are found even farther to the east, in central Asia (Oshida et al., 2005). Recolonization of the Mediterranean basin by eastern source populations is also supported by a meta-analysis of species belonging to six phyla around the Mediterranean basin, showing that intra-specific genetic diversity generally decreases from east to west (Conord et al., 2012).

The details of the postglacial history of species found in Europe are far from simple, and for widely distributed species, reconstructing historical dispersal patterns requires sampling both at

continental and regional/local geographic scales. In this study we examined the phylogeography of a cave-dwelling bat species, the bent-winged bat, *Miniopterus schreibersii*, using a dense sampling strategy from around the Mediterranean basin and the Caucasus. The evolutionary history of *M. schreibersii* has been studied locally in different parts of Europe and Anatolia, yet a comprehensive study has not previously been conducted. The work at a regional level in and around the vicinity of Anatolia, including Bulgaria and Greece indicates the presence of two mitochondrial DNA clades (Bilgin et al., 2008a, 2006; Furman et al., 2009), which were recently shown to represent two different species (Bilgin et al., 2012; Furman et al., 2010b). Within *M. schreibersii*, Bilgin et al. (2008a) found that the southeastern Balkans could be a refugial region from which the rest of Anatolia was populated, while Furman et al. (2010a) suggested a Transcaucasian refugium for the recolonization of the eastern Black Sea coast by *M. schreibersii*. In a more continental perspective, although refugial populations have been suggested to exist in Iberia or Northern Africa (Ramos Pereira et al., 2009) or Thrace (the European part of Turkey) (Furman et al., 2010a), the issue has not been properly addressed using samples from throughout Europe, including the Italian peninsula, and North Africa. To better understand the details of the evolutionary history of *M. schreibersii* in the western Palearctic, we have collected and undertook genetic analyses on samples from 15 countries spanning North Africa, southern and central Europe, Anatolia, the Middle East and the Caucasus. We also coupled genetics data with Environmental Niche Modeling (ENM) of the current time frame and the LGM to investigate whether the hypothetical refugia determined by genetics data were supported by inferred LGM niche distributions. We were also able to formulate testable hypotheses for future investigations, showing the strength of combining genetics and ENM approaches.

2. Materials and methods

2.1. Field methods

For the study, sequence data available from GenBank from Turkey ($n = 217$), Greece ($n = 11$), Bulgaria ($n = 17$) and Portugal ($n = 302$) (Bilgin et al., 2008a, 2006; Furman et al., 2009, 2010a, 2010b; Ramos Pereira et al., 2009) were used, and additional samples ($n = 337$) were collected from 28 locations in 15 countries including Russia, Lebanon, Cyprus, Greece (including Crete), Romania, Slovakia, Slovenia, Croatia, Italy, France, Serbia, Spain, Morocco and Tunisia (Fig. 1, Table 1). For the new samples, wing-punches (3–4 mm) were placed in 80% ethanol or dried in silica gel in the field (Puechmaille et al., 2011), and subsequently stored at $-20\text{ }^{\circ}\text{C}$ until further processing.

2.2. Laboratory methods

Total DNA from each sample was extracted using a Roche High Pure PCR Template Preparation Kit, following the manufacturer's

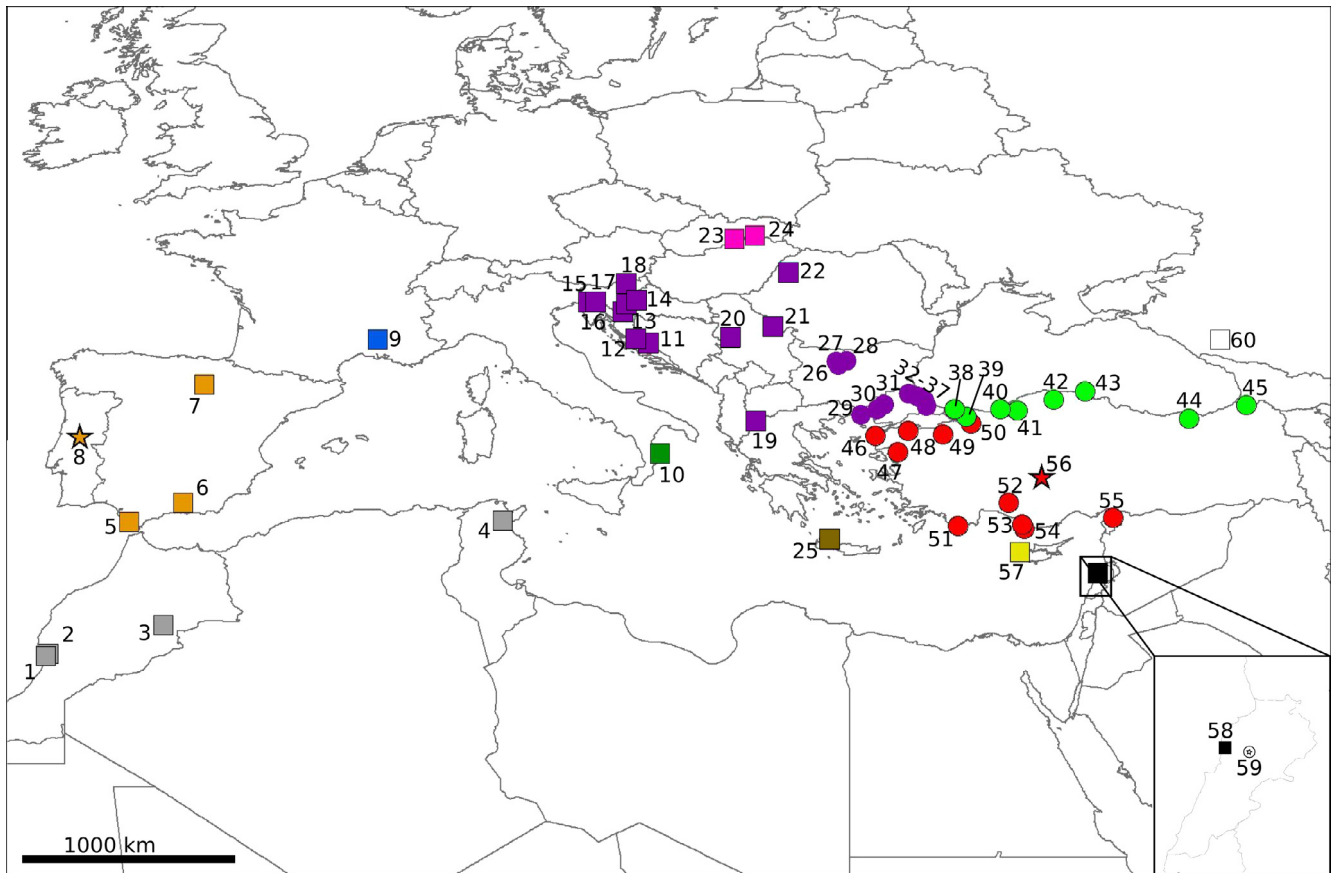


Fig. 1. Sample collection locations. The squares designate new sampling locations for this study; the circles denote locations for which sequence data was obtained from previous studies, with the stars indicating sets of locations from Furman et al. (2010b) and Ramos Pereira et al. (2009). The numbers and colors correspond to the location definitions in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protocols (Roche, Indianapolis, USA). For mitochondrial DNA (mtDNA) analysis, a partial cytochrome *b* (*cyt-b*) fragment, tRNA proline and tRNA threonine genes, and a partial fragment of the hyper-variable region I (HV1) of the control region (these fragments are referred to as HV1 from this point on forward, as it is the longest fragment among the four) were amplified and sequenced using primers C and E and the PCR conditions described in Wilkinson and Chapman (1991). In addition, for a subset of newly collected samples representing the main geographical regions, another partial sequence of the *cyt-b* gene was amplified using the primers Molcit-F (Ibáñez et al., 2006) and MVZ-16 (Smith and Patton, 1993), as described in Ibáñez et al. (2006). Sequencing was commercially done at Macrogen Inc. (South Korea), and the obtained sequences were edited and aligned using Sequencher 4.7 (Gene Codes Corp.). To confirm species identity of samples from Morocco ($n = 36$) and Spain ($n = 22$), where two distinct mtDNA clades were found, six nuclear microsatellite loci, *Mschreib2*, *Mschreib4*, *CH37*, *CH87*, *CHB12*, *CHD2*, were amplified using the primers and conditions as described in Miller-Butterworth et al. (2002) and Han et al. (2008). These six loci were chosen for their informativeness based on the results of studies undertaken by Bilgin et al. (2012) and Furman et al. (2010b). The microsatellite PCR products were also sent to Macrogen Inc. to size the fragments. Allele sizes were determined using the software program Peak Scanner V.1.0 (Applied Biosystems) and scored manually.

2.3. Analyses and statistical methods

2.3.1. Phylogeographic analyses

The 830 *M. schreibersii* HV1 sequences were used to construct a statistical parsimony network using TCS v. 1.21, with a 95% limit

(Clement et al., 2000). To further characterize relationships between haplotypes, we constructed phylogenetic trees by concatenating HVI and *cyt-b* for a subset of representative individuals ($n = 155$). Analyses were done using neighbor-joining and maximum likelihood methods as implemented in Mega v.5 (Tamura et al., 2011), and using the Bayesian method as implemented in BEAST v.8.1.2 (Drummond and Rambaut, 2007). For neighbor-joining and maximum likelihood trees, maximum composite likelihood distances and the general time reversible (GTR) model were used, respectively. The robustness of the phylogenies was tested by 1000 bootstraps for both methods. For the Bayesian tree a strict molecular-clock, Yule process and GTR model with gamma and invariant sites were used, and starting with 10,000,000 generations, the priors and number of generations was adjusted after each run until the effective sample size (ESS) for all of the traces was over 200 (this approach was also used for calculation of the timing of the split for the two *Miniopterus* species and Bayesian Skyline plots, see below). The final analysis was run for 750 million generations, logging every 10,000 states, and the first 10% of the states were discarded as burn-in. BEAST v.8.1.2 was also used to date the divergence between the two species in Northern Africa, using the HV1 data set, *Miniopterus pallidus* as an outgroup, and a strict clock with a rate of 20% change per million years (Petit et al., 1999). The analysis was run for one billion generations, logging every 10,000 states, and the first 10% of the states were discarded as burn-in.

Individual populations were grouped into geographical regions based on their geographic proximity, general geographic definitions and previous research. Regions were defined as North Africa (Morocco, Tunisia), Iberia (Portugal, Spain), France, Italy, Balkans

Table 1

The sampling sites, with number of samples and color codes corresponding to the defined regions and matching Fig. 1 for all samples (*M. schreibersii* and *M. maghrebenensis*). N (HV1) and N (cb) designate the number of samples of *M. schreibersii* sequenced for HV1 and cytochrome *b* regions, respectively. For the presented N values that are separated by a dash (/), those before and after the dash correspond to *M. schreibersii* and *M. maghrebenensis*, respectively. New sites sampled and sequenced in this study are shaded in gray. *M. pallidus* samples were from Rouais Cave in Lebanon (location #59).

Region	Code	Country	Location Name	N (HV1)	N (cb)	Latitude	Longitude
North Africa	1	Morocco	Talmat	0/4	0/4	30.6129	-9.4671
	2	Morocco	Cave Win-Timdouine, Agadir	12/10	8/4	30.680578	-9.346463
	3	Morocco	Keff Aziza, Errachidia		0/10	30.6805	-9.3449
	4	Tunisia	Mines de Zaghouan	0/1	0/1	36.3997	10.1472
Iberia	5	Spain	Mesas de Algar Cave	1	5	36.3561	-5.9102
	6	Spain	Silo del Carmen de los Catalanes	7	5	37.1767	-3.5905
	7	Spain	Jorge's Cave	5	12	42.2230	-2.6596
	8	Portugal	Ramos Pereira et al. (2009)	302			
France	9	France	Piolenc	19	7	44.1790	4.7681
Italy	10	Italy	Verzino	5		39.2817	16.8616
Balkans	11	Croatia	Ćulumova	24	3	43.9892	16.3772
	12	Croatia	Topla	20		44.1920	15.8481
	13	Croatia	Dragina	24		45.3470	15.2949
	14	Croatia	Veternica	22		45.8410	15.8786
	15	Slovenia	Jama v Doktorjevi ogradi	14		45.7793	13.7901
	16	Slovenia	Predjamski sistem	16		45.7793	14.1313
	17	Slovenia	Cerkev v Završah	10	2	46.2260	15.4475
	18	Slovenia	Cerkev v Puščavi	7	1	46.5526	15.4262
	19	Albania	Treni Cave	16	6	40.6721	20.9869
	20	Serbia	Deguricka	1		44.2352	19.8830
	21	Romania	Astileu Cave	19	15	47.0330	22.3830
	22	Romania	Gaura cu Musca cave	10	5	44.7200	21.7100
	26	Bulgaria	Parnitzite	10	3	24.4321	43.2003
	27	Bulgaria	Mandrata	2		24.5247	43.0620
	28	Bulgaria	Devetashkata	5		24.8852	43.2337
	29	Greece	Maronia	2		25.5041	40.9321
	30	Greece	Tsoutouro	3		26.1912	41.1523
	31	Greece	Koufovouno	6		26.4862	41.3514
	32	Turkey	Kuru	1		27.5574	41.8397
	33	Turkey	Kız	4		27.5573	41.8363
34	Turkey	Kovantaşı	4		27.9112	41.7072	
35	Turkey	Kiyikoy	3		28.1124	41.6132	
36	Turkey	Çilingoz	5	3	28.2216	41.5238	
37	Turkey	Kocakuyu	1	1	28.3180	41.2885	
Slovakia	23	Slovakia	Veľká Drienčanská	16	14	48.4891	20.0640
	24	Slovakia	Drienovská	12		48.6250	20.9524
Crete (GR)	25	Greece	Agios Ioannis Cave	1		35.5911	24.1494

Tabl 1 (continued)

Region	Code	Country	Location Name	N (dL)	N (cb)	Latitude	Longitude
Black Sea	38	Turkey	Sofular	1	1	29.5121	41.1853
	39	Turkey	Gökçeören	2	1	29.9956	40.8496
	40	Turkey	Hüseyinli, Zonguldak	1		31.4722	41.1906
	41	Turkey	Karabük	2	1	32.2000	41.1300
	42	Turkey	Kastamonu	1		33.7673	41.5795
	43	Turkey	Sinop	1	1	35.0947	41.9383
	44	Turkey	Çatak	1	1	39.5630	40.7648
	45	Turkey	Cehennemdere	8	8	42.0126	41.3703
Anatolia without Black Sea	46	Turkey	Çanakkale	1	1	26.1123	40.0241
	47	Turkey	Balıkesir	1	1	27.5244	40.2314
	48	Turkey	Havran	1	1	27.0900	39.3480
	49	Turkey	Doğancı Köyü, Bursa	1		29.0042	40.1058
	50	Turkey	Şahmelek Köyü, Sakarya	1		30.2042	40.5743
	51	Turkey	Kaş	14	6	29.6445	36.1728
	52	Turkey	Çatdere	13	2	31.8120	37.1596
	53	Turkey	Yalandünya Cave	5	4	32.4022	36.2198
	54	Turkey	Anamur	1		32.4928	36.0509
	55	Turkey	Hatay	1		36.3113	36.5121
	56	Turkey	Furman et al. (2009, 2010a)	129			
Cyprus	57	Cyprus	Akamas	10	5	35.0500	32.3300
Lebanon	58	Lebanon	Amchite Cave	7	5	34.1500	35.6700
	59	Lebanon	Rouais Cave	4	4	34.1166	35.9167
Russia	60	Russia	Svetlaya Cave	19	10	44.1700	40.9200

(Croatia, Slovenia, Serbia, Albania, Greece, Romania, Bulgaria, Turkish Thrace), Slovakia, Crete, Lebanon, Cyprus, Black Sea (Anatolian Black Sea coast), Anatolia without Black Sea (“Anatolia w/o BS”) from this point forward – all of Anatolia excluding Black Sea coast), and Russia (Fig. 1). Anatolian populations were subdivided as Black Sea and “Anatolia w/o BS”, due to previously observed genetic differentiation (Bilgin et al., 2008a; Furman et al., 2010b).

For each geographical region, the HV1 data set was evaluated using DnaSP v.5 (Librado and Rozas, 2009) to estimate the number of haplotypes, haplotype diversity (h_d), nucleotide diversity (π), Tajima’s D and Harpending’s raggedness index (r). Significance of Tajima’s D and r parameters was tested using coalescent simulations with 1000 replicates. Tajima’s D (Tajima, 1989) is used to determine if a set of DNA sequences is evolving neutrally or not, with a value of D significantly smaller than zero being indicative of a population expansion. The time of the onset of the expansion was also determined using the HV1 sequences, with τ and its 95% confidence interval as calculated by Arlequin v. 3 (Excoffier et al., 2005), and assuming a mutation rate of 20% per million years (Petit et al., 1999). In a similar vein, Bayesian skyline plots were prepared using BEAST v.8.1.2, separately for Anatolia w/o BS (the final analysis was run for 100 million generations), Balkans (250 million generations) and Iberia (100 million generations), using a strict clock with a rate of 20% change per million years, logging every 10,000 generations and discarding the first 10% of the states as burn-in.

As the number of detected haplotypes in a population depends on the number of sampled individuals, it can be challenging to compare the number of haplotypes across populations sampled with different number of individuals. Therefore, to compare haplotype numbers between three potential refugia, Anatolia w/o BS ($n = 168$), the Balkans ($n = 229$) and Iberia ($n = 315$), we used a resampling method to calculate a median and 95% confidence

interval for the number of unique haplotypes in each geographical region given a pre-defined sample size. As Anatolia w/o BS was the putative refugial region with the lowest sample size, we randomly picked 100,000 times (with replacement) 168 individuals from the Balkans and Iberian data sets. Re-sampling was done in R v 3.0.2 (R Development Core Team, 2013) with a custom script available upon request.

The pairwise genetic differentiation between the geographical regions was calculated as ϕ_{st} using the HV1 sequences in Arlequin v. 3, and the significance was evaluated with 100 replications. In addition, the geographical distances of the centroids of regions were measured from “Anatolia w/o BS”, the most probable source (see Section 3), using the shortest connections over land (as a bat flies), as opposed to calculating direct geographic distances between any two given coordinates, and plotted against haplotype and nucleotide diversity in each region with the significance of the Spearman’s correlation tested with SPSS v. 12, for a test of “expansion from Anatolia”.

2.3.2. Differentiation in North Africa

The sequence analyses (cf. Section 3) indicated differentiation between a subset of individuals from North Africa and the rest of the sampled individuals (including other individuals from North Africa). Our subsequent analyses provide evidence that these divergent individuals represent *Miniopterus maghrebensis*, while we use *M. schreibersii* to refer to *Miniopterus schreibersii sensu stricto* from this point forward (Puechmaile et al., 2014) (please note that Puechmaile et al. (2014) used microsatellite and mtDNA data from this study, along with those from Bilgin et al. (2012) to describe *M. maghrebensis*, and they refer to the results that they used from this study as “Data presented in the current study are based on the work by Bilgin et al. (submitted) and further analyses of those data.”. Even though it was submitted later, as Puechmaile

et al. (2014) was published before this study (referred to as Bilgin et al. (submitted) in Puechmaile et al. (2014)), we find it necessary to make this explanation to clear the issue). We used microsatellites to investigate if the mtDNA differentiation between these two putative species was also seen at the nuclear level. The microsatellite data were generated for *M. maghrebensis* ($n = 22$) and for individuals of *M. schreibersii* from N. Africa ($n = 14$) and Spain ($n = 22$). Descriptive statistics of observed heterozygosity, the number of alleles (N_a), and effective number of alleles (N_e) were calculated, and the plots of allele frequencies for *M. maghrebensis* and *M. schreibersii* were made using Genalex v.6 (Peakall and Smouse, 2006). Linkage disequilibrium between loci was tested using Genepop on the web (<http://genepop.curtin.edu.au/>). To confirm the presence of two distinct genetic clusters (i.e. species) a frequency-based assignment test (Paetkau et al., 1995, 2004) was made using microsatellites and Genalex v.6 to determine if the individuals identified as *M. maghrebensis* and *M. schreibersii* based on mtDNA were also differentiated in their nuclear DNA.

2.3.3. Environmental niche modeling

For the constructions of the environmental niche models, we downloaded all available locations of *M. schreibersii* in Europe ($n = 857$) that had a resolution higher than 0.1° (ca. 10 km), from the Global Biodiversity Information facility (<http://data.gbif.org/welcome.htm>), and from the personal databases of the authors of this study. The location data for North Africa were from individuals that were genetically confirmed to be *M. schreibersii*. The modeling area covered the majority of the species range in Europe and the Mediterranean, located between $13\text{--}52^\circ\text{E}$ and $30\text{--}53^\circ\text{N}$. The initial dataset presented high levels of spatial autocorrelation (nearest neighbor ratio = 0.53, $z = -37.88$, $P < 0.001$), therefore records were deleted until no more clusters were detected (nearest neighbor ratio = 1.22, $z = -0.72$, $P = 0.77$); as a result, the final dataset for analyses included 191 non-autocorrelated records. We used a maximum entropy modeling technique (Maxent species distribution modeling, v.3.3.3k; <http://www.cs.princeton.edu/>

~schapire/maxent) that estimates the probability densities of presence data in a covariate space calculated using the environmental predictors, i.e. estimates the distribution of a species across geographic space (Elith et al., 2011) (see Supplementary Material S1 for further detail).

3. Results

3.1. Niche modeling

3.1.1. Predicted distribution for the present and LGM

Models showed a very good predictive ability (training data AUC = 0.90 ± 0.01) without over-fitting presence data (test data AUC = 0.84 ± 0.02). For the present conditions, the predicted range (Fig. 2a) is largely in agreement with what is known to be the species occurrence in the western Palearctic (Dietz and Kiefer, 2014), mainly restricted to southern Europe, northern Africa, Turkey and coastland of the Middle East. Regarding predicted LGM distributions, the species was predicted to occur mainly in southwest Iberia, the islands of Crete, (parts of) Sardinia and Sicily, northern Africa, and eastern and southern coasts of the Levantine Sea (along the Mediterranean coasts of present-day Syria, Lebanon, Israel, and northeastern and northwestern Egypt). Low levels of suitability were predicted for western coasts of Greece and Albania, western and southern coasts of Anatolia, southern Italy, and the northern coast of the Adriatic Sea (Fig. 2d). These ENM results, which were obtained by excluding collinear climatic variables, were somewhat different from those obtained without excluding them (in the models with all the collinear variables included, parts of Anatolia and Greece & Albania showed higher suitability, see Supplementary Material, Fig. S6).

3.1.2. Variables limiting the range

Variables associated with extreme cold events seem to limit the distribution of *M. schreibersii*. More specifically, precipitation of the coldest and wettest quarters, minimum temperature of the coldest

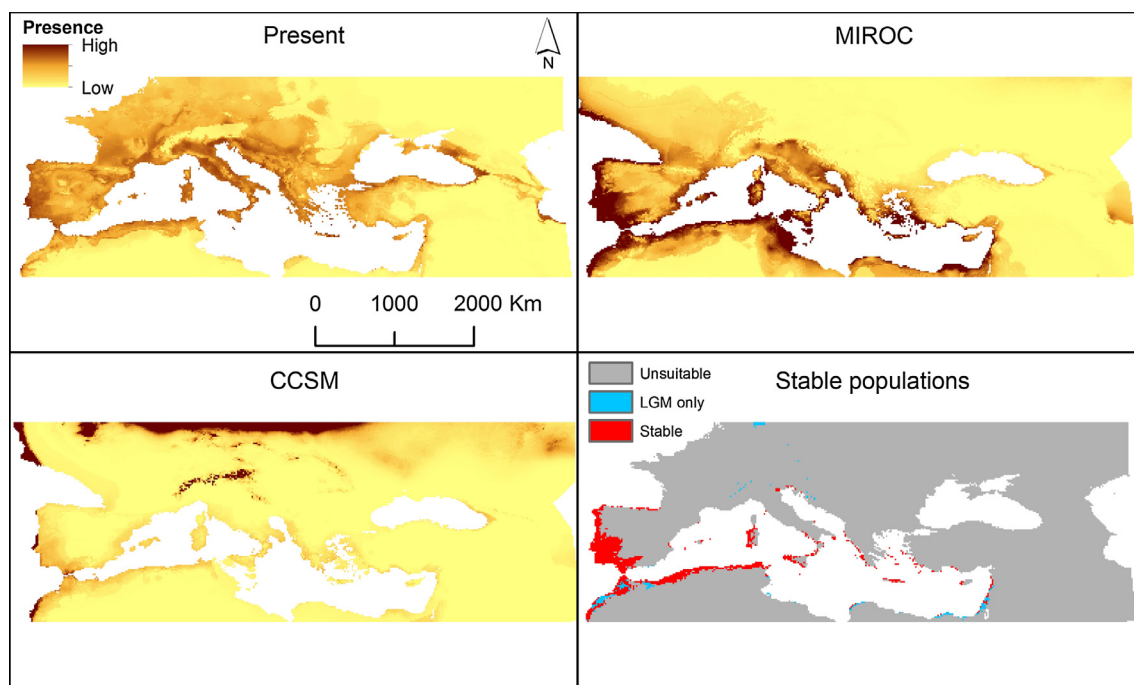


Fig. 2. Three Maxent models and a reclassified map of the potential distribution of *Miniapterus schreibersii*. (a) Map obtained from the present conditions. (b) and (c) Maps calculated for the last glacial maximum (LGM) employing two different general circulation models [Model for Interdisciplinary Research on Climate (MIROC) and Community Climate System Model (CCSM)], respectively. (d) Reclassified map indicating areas where suitable conditions existed in the LGM alone and in both glacial and interglacial periods (here called stable), obtained from the present and both LGM models.

month and mean diurnal temperature range were the most relevant variables selected for models' calculations. The values of these variables within the known range of the species suggest an avoidance of colder temperatures and arid conditions (see Fig. S5, Supplementary Material S2).

3.2. Phylogeography of range expansion

The total data comprised 849 HV1 sequences (830 *M. schreibersii*, 15 *M. maghrebensis*, and four *M. pallidus*) and 164 *cyt-b* sequences (142 *M. schreibersii*, 18 *M. maghrebensis*, and four *M. pallidus*, all from this study). The trimmed sequences corresponded to 254 and 533 base pairs for the HV1 and *cyt-b* regions, respectively. The sequences obtained in this study have been submitted to GenBank with accession numbers KX008522–KX008566 and KU842048–KU842052.

Various lines of evidence from phylogeographic analyses of *M. schreibersii* (excluding *M. maghrebensis*), including descriptive

statistics, mismatch distributions, Bayesian skyline plots, the phylogenetic trees and the haplotype networks, suggest that Anatolia was the most likely refugium from which the rest of the western Palearctic was populated. For the HVI data set, Anatolia w/o BS showed a relatively high diversity of haplotypes, having the highest value for the corrected number of haplotypes (19, compared to 17 [IC95%: 14–20] for Balkans and 5 [IC95%: 4–6] for Iberia), and second highest number of private haplotypes (13) even though this region had the smallest sample size of all potential refugial regions ($n = 168$ in Anatolia w/o BS vs. $n = 229$ in Balkans and $n = 315$ in Iberia). Anatolia w/o BS also had the highest nucleotide diversity (0.00607), and the second highest haplotype diversity (0.757) following the Black Sea region (0.879). Plots of haplotype and nucleotide diversity show a clear pattern of decrease as one moves away from Anatolia (Fig. 3; Spearman's $r = 0.651$ and 0.601 , respectively, $P < 0.05$ for both cases). Additionally, mismatch distributions (Fig. 4) and Harpending's r values indicate Anatolia w/o BS as the only putative refugial region without the signature of an expanding

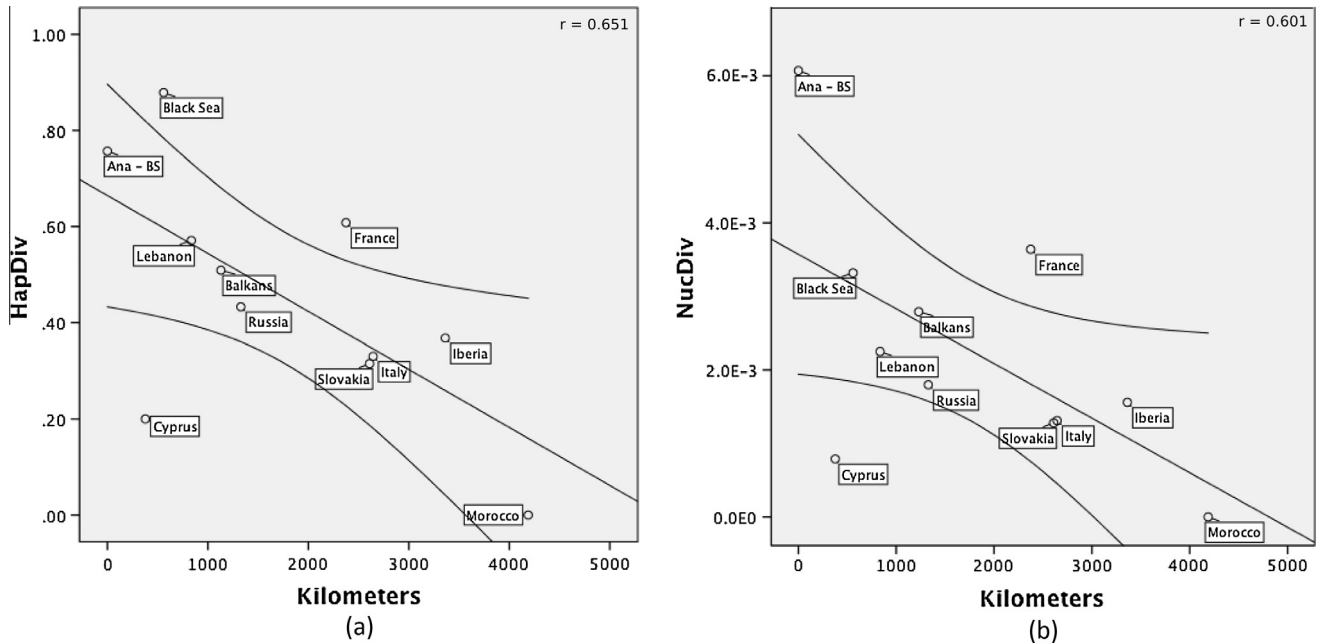


Fig. 3. A regression of distance from Anatolia against (a) haplotype diversity (b) nucleotide diversity for the regions as defined in Table 1. 'Ana - BS' refers to the Anatolia w/o Black Sea region.

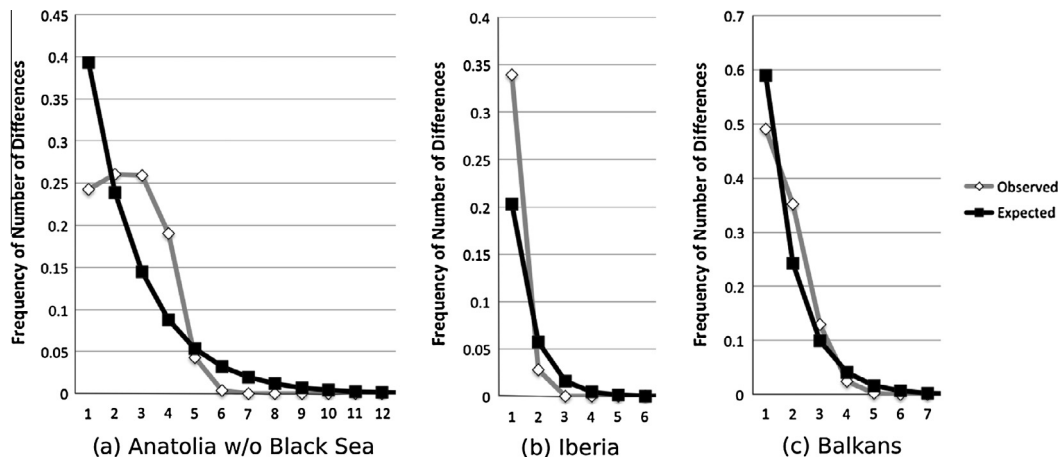


Fig. 4. The observed and expected mismatch distributions under expansion for the populations in a. Anatolia w/o Black Sea b. Iberia c. Balkans. The x-axis shows the number of base differences between HV1 haplotypes, and the y-axis represents the frequency of these base differences.

Table 2

Number of HV1 haplotypes analyzed for each region for *M. schreibersii* and *M. maghrebensis*. 'Ana. w/o BS' refers to 'Anatolia without Black Sea' region. Crete was not included due to having a single sample only. V/P/S designate number of variable/parsimony informative/singleton variable sites. The Tajima's D and Harpending's raggedness index (*r*) values significantly different from zero ($P < 0.05$) are shown in bold and marked with an asterisk.

Region	N (# of individuals)	# of haplotypes	# of private haplotypes	Haplotype diversity	Nucleotide diversity	V/P/S	Tajima's D	<i>r</i>
Cyprus	10	2	1	0.200	0.00079	1/0/1	-1.112	0.4000
Balkans	229	20 (17) ^a	14	0.509	0.00278	20/11/9	0.000	0.0804
Black Sea	17	7	4	0.879	0.00332	5/4/1	0.216	0.1210
Ana. w/o BS	168	19	13	0.757	0.00607	15/13/2	-1.034	0.0283*
France	19	3	2	0.608	0.00364	3/3/0	0.208	0.1225
Iberia	315	6 (5) ^a	3	0.368	0.00156	5/4/1	-0.902	0.1830
Italy	5	2	0	0.333	0.00131	1/0/1	-0.933	0.1043
Lebanon	7	2	0	0.571	0.00225	1/1/0	1.342	0.3469
Morocco	12	1	0	0	0	0/0/0	N/A	N/A
Russia	19	3	0	0.433	0.0018	2/1/1	-0.485	0.2058
Slovakia	28	3	0	0.315	0.00128	2/1/1/1	-0.768	0.2315
Total (<i>M. schreibersii</i>)	830 ^b	45	37	0.659	0.00444	33/29/4	-1.914*	0.0377
<i>M. maghrebensis</i>	15	3	NA	0.615	0.00272	2/1/1	-0.0303	0.2426

^a The corrected number of haplotypes based on sample size of Anatolia w/o BS.

^b Includes the one sample from Crete.

population (Table 2). The ancestry of the Anatolian populations was also suggested by the longer coalescence time observed (ca. 8500 years), when compared to the Balkans (ca. 3500 years) and Iberia (ca. 250 years) (Fig. 5a–c).

The haplotype network constructed using the HV1 sequences (Fig. 6) can be separated into two sub-networks with (1) the top part centered around the most common haplotype (No. 1, shared among 479 individuals from across Europe and North Africa) with most of the derived haplotypes arranged in a star-like pattern and differing by 1–2 bp, and (2) the more diverse part with haplotypes found at more similar frequencies and clustered around haplotypes Nos. 5 and 7, but without a clear star-like pattern, suggesting more stable populations. A large majority of the haplotypes (37/45) were unique to regions (and sometimes to sites) while the few largely shared haplotypes were the most common ones (Nos. 1, 4–5) (Fig. 6). These results indicate that the populations expanding out of Anatolia dominantly carried the HV1 haplotype No. 1, which subsequently diversified into the numerous haplotypes that formed the star-like pattern typical of expanding populations. The timing of the start of the expansion was calculated as 11,752 years ago (95% C.I.: 2844–21,181 years).

The concatenated phylogenetic trees (see Bayesian tree in Fig. 7, and neighbor-joining and maximum likelihood trees that gave similar topologies as Supplementary Material S2 Fig. S1 and S2, respectively) supported the patterns observed in the haplotype network and provided subsequent information regarding ancestral/derived haplotypes. First, the clade basal to all the other *M. schreibersii* haplotypes contained samples from Anatolia w/o BS, Black Sea, Lebanon and Cyprus, demonstrating the most basal and ancestral character of sequences from populations in these regions relative to those in Europe, the Caucasus and North Africa. Second, some haplotypes and sometimes subclades were geographically restricted to regions and sometimes to individual caves, indicating philopatry of individuals in extant populations. The pairwise ϕ_{st} measures corroborate this local differentiation with 36 out of the 45 comparisons being significantly different from zero (Table 3).

3.3. Differentiation in North Africa

The phylogenetic trees demonstrate the presence of another strongly supported monophyletic clade (*M. maghrebensis*) sister to *M. schreibersii*, and strictly found in N. Africa, including Tunisia (see Supplementary Material S2 Fig. S3 for the geographic

distribution of the samples in each clade). The time of the divergence was dated to 72,041 years ago (95% HDP Interval = 5637–139,570 years). Individuals of both clades were found syntopically

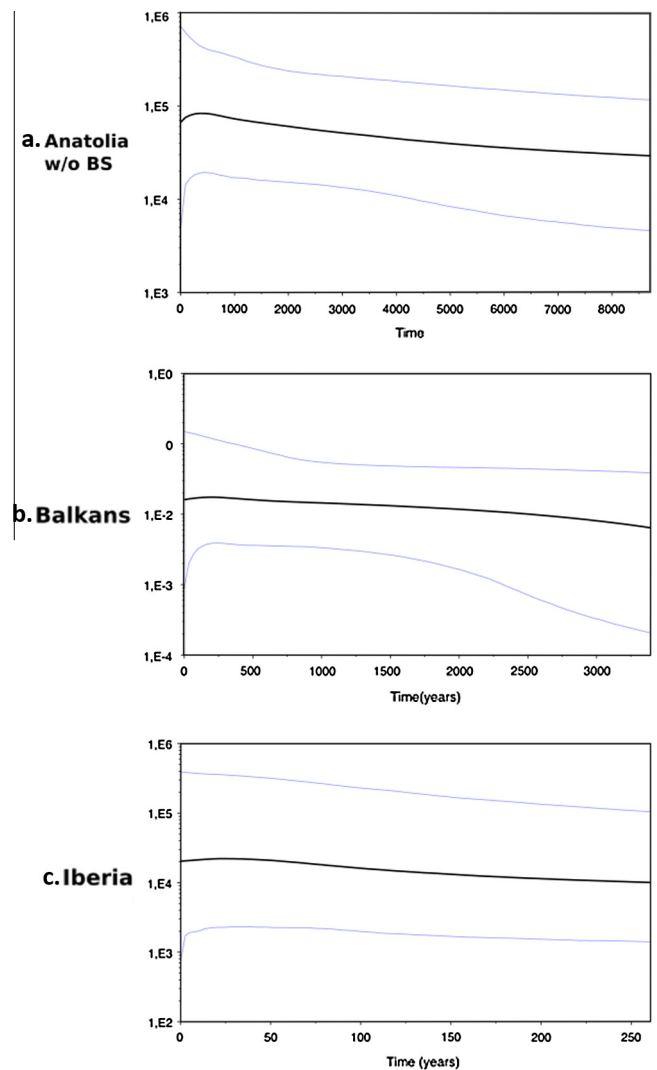


Fig. 5. Bayesian Skyline plots for a. Anatolia w/o Black Sea b. Balkans c. Iberia.

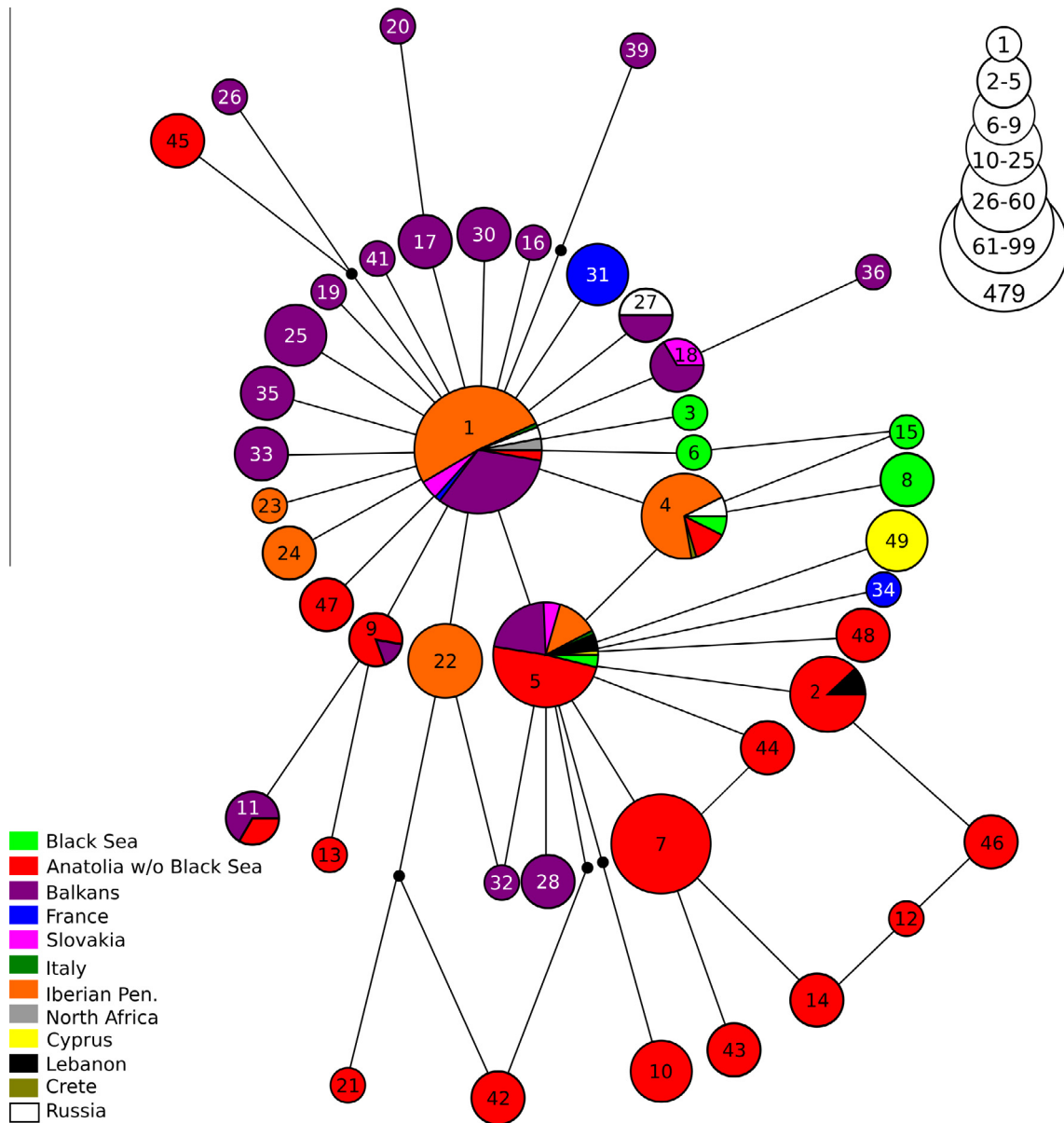


Fig. 6. Haplotype network for HV1 sequences (254 bp) and their distribution based on regions. The sizes of the circles are proportional to the frequency of a particular haplotype. Numbers inside circles are the haplotype codes. Missing haplotypes are represented as small black dots.

in the cave Win-Timdouine, in Morocco. For the microsatellites, all colonies were in Hardy–Weinberg equilibrium and no linkage disequilibrium was detected between loci at any colony (Fisher's method; $P > 0.05$). Descriptive parameters for nuclear loci are displayed in [Supplementary Material S3 Table S2](#). The pairwise assignment tests showed that individuals of *M. schreibersii* from Spain and Morocco could not be differentiated ([Fig. 8a](#)), while the samples of *M. maghrebensis* were clearly differentiated from *M. schreibersii* from Morocco ([Fig. 8b](#)) and from Spain ([Fig. 8c](#)). The examination of private alleles revealed that half of the alleles (19/38) typed at the six loci were not shared between *M. schreibersii* and *M. maghrebensis* ([Supplementary Material S2 Fig. S4a–f](#)).

4. Discussion

4.1. Patterns of post glacial expansion based on genetic data

The currently accepted paradigms of genetic differentiation in Europe outline four scenarios ([Hewitt, 1999](#); [Schmitt, 2007](#)), and

the common denominator is the isolation and differentiation of populations in the Iberian, Italian and Balkan peninsulas during the LGM. The differences among these paradigms relate to the geographical distributions of genetic diversity following post-glacial range expansions. The phylogeography of a number of bat species around the Mediterranean basin and North Africa, such as *Nyctalus noctula* ([Petit et al., 1999](#)), *Rhinolophus ferrumequinum* ([Rossiter et al., 2007](#)), *Rhinolophus hipposideros* ([Dool et al., 2013](#)), *Plecotus austriacus* ([Razgour et al., 2013](#)) and *Myotis myotis* ([Ruedi and Castella, 2003](#)), and *Myotis nattereri* complex ([Puechmaille et al., 2012](#); [Salicini et al., 2013](#)) supports LGM isolation in these refugia. In the case of *M. schreibersii*, we present a scenario that differs from these paradigms and from the patterns previously observed in bats, and globally other mammals. Based on genetic data, we suggest extinction of this species throughout Europe during the LGM, including populations in the three refugial peninsulas (Iberian, Italian and Balkan), and subsequent re-colonization from the highly diverse Anatolian populations. Contrary to previous suggestions, genetic data did not provide evidence of refugial populations for

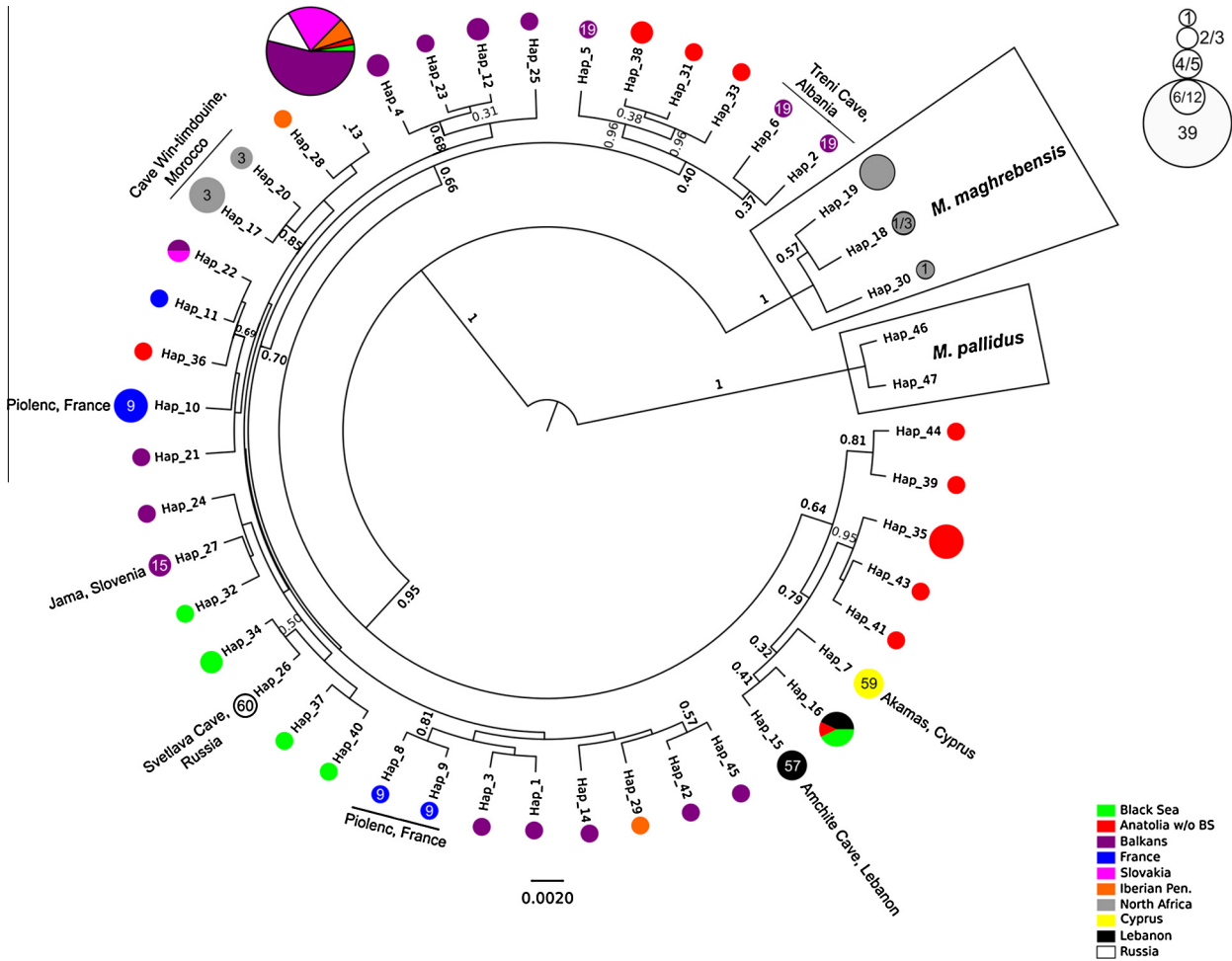


Fig. 7. The Bayesian phylogenetic tree of the concatenated HV1 and cyt-*b* data, with their frequency and geographic location defined by the circles next to the haplotype names. The posterior probabilities those are greater than 0.30 are shown. The numbers inside circles indicate the actual site number (as in Table 1) when the haplotype is unique to the site. Note that haplotype names from Fig. 5 are independent from haplotype numbers presented here.

Table 3
The pairwise ϕ_{st} values between regions based on HV1 for *M. schreibersii*, with those significantly greater than zero ($P < 0.05$) shown in bold (lower diagonal), and pairwise Dxy values between regions (upper diagonal).

	Cyprus	Balkans	Black Sea	ANA-BS	France	Iberia	Italy	Lebanon	Morocco	Slovakia
Cyprus		0.005	0.006	0.004	0.004	0.004	0.003	0.001	0.004	0.005
Balkans	0.68		0.001	0.003	0.001	0	0	0.003	0	0
Black Sea	0.61	0.28		0.004	0.002	0.001	0.001	0.004	0.001	0.001
ANA-BS	0.41	0.41	0.367		0.004	0.004	0.003	0.001	0.004	0.003
France	0.73	0.28	0.31	0.43		0.001	0.001	0.005	0.001	0.001
Iberia	0.83	0.07	0.35	0.55	0.42		0	0.004	0	0
Italy	0.86	-0.08	0.12	0.30	0.20	0.01		0.003	0	0
Lebanon	0.73	0.54	0.43	0.15	0.59	0.74	0.64		0.005	0.003
Morocco	0.96	0.01	0.27	0.41	0.33	0.01	0.15	0.87		0
Slovakia	0.84	-0.01	0.27	0.36	0.32	0.06	-0.11	0.70	0.04	

M. schreibersii in North Africa (Ramos Pereira et al., 2009), nor in Thrace (Furman et al., 2010a) or southeastern Europe (Bilgin et al., 2008a).

In the present study, the combination of the haplotype network and classical population genetics approaches is helping to elucidate the colonization history of *M. schreibersii*. This includes an initial postglacial migration out of Anatolia into Europe where *M. schreibersii* had likely gone extinct during the Pleistocene (LGM). This initial expansion started around 11,750 years ago, which is congruent with the onset of a demographic expansion during the Younger Dryas in the bats *N. noctula* in central and

Eastern Europe (Petit et al., 1999), and *Myotis capaccinii* in Anatolia and southeastern Europe (Bilgin et al., 2008b). It appears that once these expanding populations were established in Europe, individuals carrying newly evolved haplotypes did not disperse freely but mostly remained geographically restricted. This is evidenced by the distribution of the derived and closely related haplotypes, which are geographically limited by region and even sometimes individual sites, and despite the high flying capacity of this bat. Although *M. schreibersii* is physically capable of long distance flight due to its long and slender wings with a high-aspect ratio (Norberg and Rayner, 1987), it appears to exhibit strong philopatric behavior

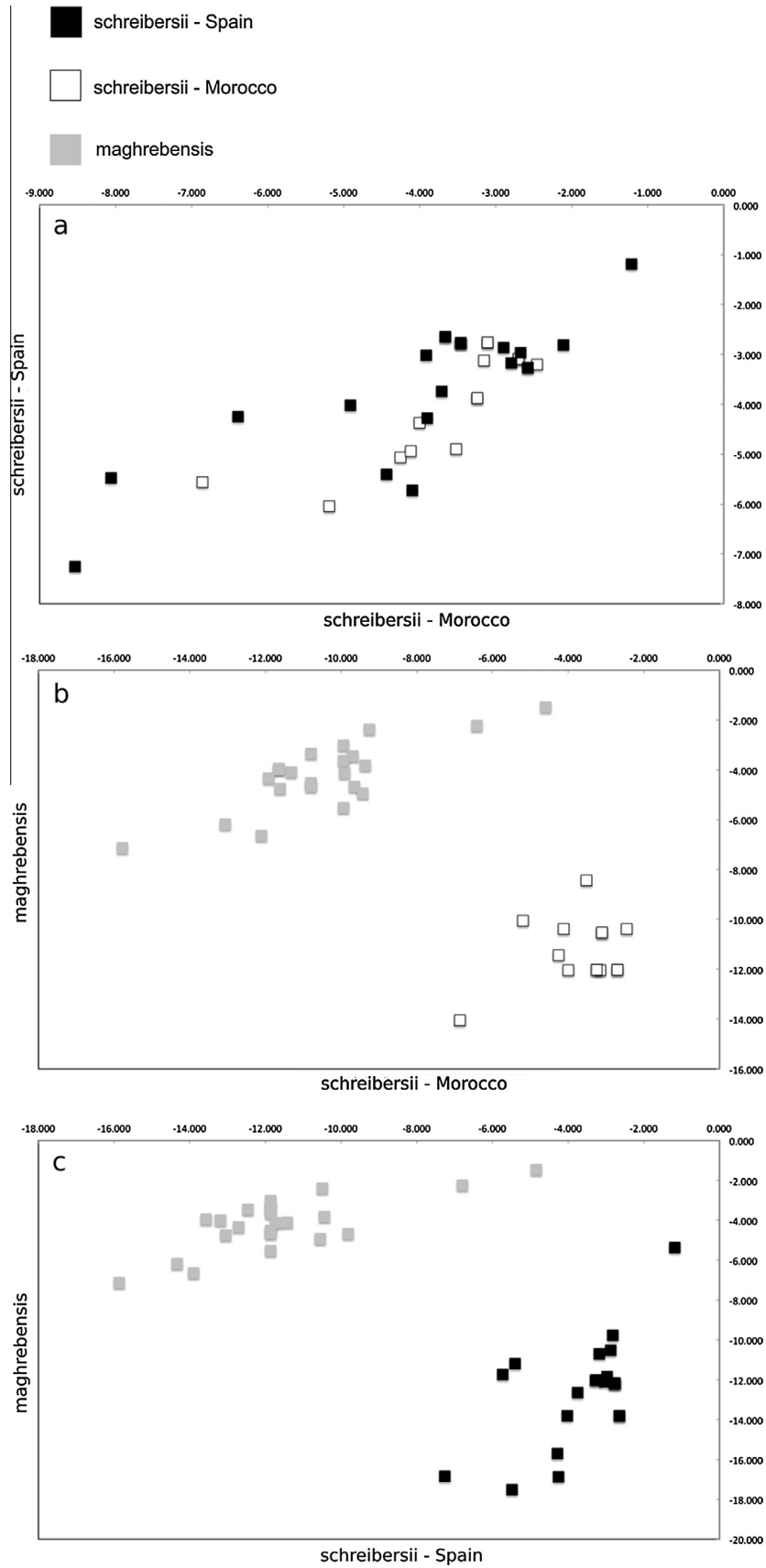


Fig. 8. The frequency-based pairwise assignment tests between populations (a) between *M. schreibersii* from Spain and *M. schreibersii* from Morocco, (b) between *M. maghrebensis* and *M. schreibersii* from Morocco, and (c) between *M. maghrebensis* and *M. schreibersii* from Spain.

or restricted dispersal. This is fully consistent with results from large banding studies demonstrating natal philopatry in both sexes in the species (Rodrigues et al., 2010) and a recent genetic study on host-parasite evolution of *M. schreibersii* (Witsenburg et al., 2015), which showed the genetic differentiation of the species in seven countries in continental Europe.

4.2. Differentiation in North Africa

In addition to the classical refugia that are known to exist in Iberia, Italy and the Balkans, the Maghreb region of North Africa is known to have acted as a glacial refugium during the Pleistocene (Dool et al., 2013). A recent review investigating a large diversity of animals (mammals, amphibians, reptiles and arthropods) concluded that northward colonization from North Africa to Europe was more common than colonization in the opposite direction (Husemann et al., 2014). However, in bats, it has been suggested that *Eptesicus isabellinus* colonized North Africa from Iberia (Juste et al., 2009) and we present here a second bat species, *M. schreibersii*, with a similar pattern. The presence of *Miniopterus* as one of the most common bats found in Iberian Upper Pleistocene deposits (Cuenca-Bescós et al., 2010; López-García et al., 2012; Sevilla, 1988) seems to contradict our proposed pattern of recolonization of Europe (including Iberia) from Anatolia. Given the close proximity of Iberia to North Africa, we hypothesize that this Upper Pleistocene Iberian population most likely belonged to *M. maghrebensis*, now exclusively found in Northern Africa. The reasons for the shrinking and possible extinction of *M. maghrebensis* in Iberia and Northern Morocco are unknown, and may be due to competitive exclusion following the recolonization of these regions by *M. schreibersii*. Evidence for competitive exclusion in other bat species have already been suggested, either occurring during post-glacial recolonization (Bruyndonckx et al., 2010) or in extant species (Santos et al., 2014).

4.3. Genetics combined with Environmental Niche Modeling (ENM)

The genetics and ENM methods have been successfully combined in studies of a number of taxa, including bats (Flanders et al., 2011; Rebelo et al., 2012). The ENM results of this study, evaluated just by themselves, indicate a number of potential local glacial refugia (especially in the southwestern Iberian peninsula, northern Africa, and the southern and eastern coasts of the Levantine Sea) that were suitable to accommodate stable populations through the LGM and the present. These results support the idea of “refugia within refugia”, in which each of the major refugial peninsulas hosted a number of regions supporting different refugial populations (Bilgin, 2011; Canestrelli et al., 2007; Gómez and Lunt, 2007; Medail and Diadema, 2009).

Considering Anatolia, its Black Sea coast not being suitable for *M. schreibersii* during the LGM is parallel to the results of the genetic analyses. With their nucleotide diversity being nearly half the value as for the rest of Anatolia, and their high haplotype diversity mostly explained by similar frequencies of the different haplotypes, the genetic data suggest that the Black Sea population could have gone extinct during the LGM. Our genetic and modeling findings, therefore, do not support a Transcaucasian refugium for *M. schreibersii* along the eastern Black Sea coast, a hypothesis suggested by Furman et al. (2010a).

Together, the genetic, ENM and fossil data available provide greater resolution for understanding the evolutionary history of the species in the Italian and Iberian peninsulas as well and underlie the necessity of combining different approaches. In terms of the distribution of stable populations based on the results of the ENM, the general unsuitability of the Italian peninsula is concordant with the patterns observed in the genetic analyses.

On the other hand, although ENM models support stable and suitable habitats in Iberia for *Miniopterus* since the LGM to present, we have found the lowest levels of genetic diversity among the investigated regions in Iberia. These results, together with a decreasing pattern in genetic diversity from east to west, point to the absence of an Iberian refugial population for *M. schreibersii*. Fossil records are in agreement with the ENM results and suggest a possible continuous presence of *Miniopterus* in this peninsula. With these results, we hypothesize that the populations present in Iberia were most probably related to the species *M. maghrebensis* (or even to a third species/lineage that went extinct after the LGM).

Considering the Balkans peninsula, the ENM results show very low levels of suitability for it to have acted as a refugium, consistent with the structure of the haplotype network for the populations in Anatolia w/o BS and the Balkans for this species. Anatolia w/o BS shows a more structured and older network of haplotypes as demonstrated by phylogenetic reconstruction, whereas the Balkans shows more of a star-like network indicative of a recent population expansion. This is reflected in the frequency of the haplotypes as well: Anatolian haplotypes have more balanced frequencies, whereas Balkan haplotypes have a high frequency for a main haplotype (1), surrounded by many low frequency haplotypes with only one base difference from haplotype 1. This is probably the reason that the Balkans has relatively low haplotype and nucleotide diversities, despite having a higher number of private haplotypes than Anatolia w/o BS. The dominance of haplotype 1 in most regions outside of Anatolia (representing between 37% and 100% of haplotypes) is likely the result of the surfing phenomenon whereby haplotypes present in the wave front of expanding populations can by chance alone reach high frequency away from their place of origin (here most likely Anatolia where the haplotype is present but in low frequency) (Excoffier and Ray, 2008). Due to the gradually and linearly decreasing patterns of genetic diversity out of Anatolia and the lack of genetic breaks between the Anatolia and the Balkans, we hypothesize that Anatolian populations populated the Balkan Peninsula.

On the other hand, the ENM results suggest almost complete unsuitability for Anatolia w/o BS (except for about 20 pixels in western and southern coasts). However, the ENM results suggest suitability during the LGM for eastern and southern coasts of the Levantine Sea. Interestingly, the haplotypes from Lebanon, along with those from Anatolia and Cyprus are basal to the rest of the haplotypes of *M. schreibersii* from Europe, Caucasus and North Africa. Hence, this region to east and west of the Levantine Sea is the only one that shows support for refugial populations based on both ENM and genetics data, a hypothesis that should be confirmed with more extensive sampling in the area.

Assuming that the area to the east and south of the Levantine Sea has been the refugium that made it possible for *M. schreibersii* to survive the LGM, the high levels of diversity in Anatolia suggest subsequent diversification of *M. schreibersii* in this peninsula, prior to its expansion into the Caucasus and the rest of Europe, and ultimately North Africa. It should also be noted that, ENMs based on different circulation models can give conflicting results for predicting LGM distributions (Varela et al., 2015), and as was the case in this study for *M. schreibersii*, the CCSM model was observed to give conservative estimates of the LGM distributions of species (Rebelo et al., 2012). On the other hand, the ENM constructed using the MIROC circulation model for *M. schreibersii* shows the suitability of parts of Anatolia as a potential refugium. Anatolia is considered to be a “center of endemism” from which neighboring regions, including Europe and the Middle East, have been colonized. A number of studies show this pattern in both terrestrial (e.g. *Lepus europaeus* Stamatis et al., 2009) and aquatic organisms (e.g. *Salmo trutta* Canestrelli et al., 2007). Studies on multiple

species, including bats (Çoraman et al., 2013), indicate that compared to other regions in Europe, Anatolia harbored elevated levels of genetic diversity during the LGM (Bilgin, 2011). A recent review shows a similar pattern of longitudinal decrease in genetic diversity across six phyla from east to west (Conord et al., 2012), although most of the species used in the assessment did not include samples from Anatolia.

Specifically, studies on the oak gallwasp, *Andricus quercustozae* (Rokas et al., 2003), the meadow grasshopper, *Chorthippus parallelus* (Korkmaz et al., 2014), and Alpine rock-cress, *Arabis alpina* (Ansell et al., 2011; Koch et al., 2006), indicate that European populations originated in Anatolia and were the source for the pre-Pleistocene colonization of Europe, with genetic diversity decreasing with distance from Anatolia. In this perspective, we propose a new paradigm (the oak gallwasp), so as to generalize the post-glacial recolonization history and distribution of genetic diversity in Europe. Under this paradigm, we refer to the species that found refuge in Anatolia and/or Middle East during the LGM, and subsequently reseeded the rest of Europe as the climate became more hospitable. We use the oak gallwasp as the flagship species for this paradigm as the Rokas et al. (2003) study was the first to demonstrate this “out-of-Anatolia/Middle East” pattern for the recolonization of Europe.

In summary, after a very dense sampling strategy with a broad distribution spanning Europe, Anatolia, the Middle East and the Caucasus, we can conclude that: (1) either all European populations of *M. schreibersii* went extinct during the LGM or some populations remained in localized refugia (e.g. non-sampled parts of the Italian or Balkans peninsula) but did not contribute to the recolonization of Europe post LGM. (2) The Eastern and Southern coasts of the Levant acted as the oldest refugial area for *M. schreibersii*. (3) Anatolia emerges as an intermediate area in a stepping stone repopulation process of the Caucasus and mainland Europe from this Levantine refugium. In this context, we hypothesize that additional sampling in this region will reveal high levels of genetic diversity, although the fieldwork for this would be extremely difficult in the short run due to the political instability of the region.

The study also highlights the importance of methodological approaches with regards to the results and conclusions of phylogeographical/modeling studies. For instance, whether we used collinear variables had an important effect on the suitability of Anatolia and Balkans as LGM refugia, resulting in different phylogeographic conclusions. Thinking about sampling strategies, for species with wide geographical distributions like *M. schreibersii*, conclusions based on samples from a narrow geographical range can give inaccurate results, even if inferences are made just for that partial distribution. The study also highlights the importance of using multiple approaches at the same time; for instance, had we used genetics data or ENMs only, our conclusions would have been different. We also acknowledge that the main phylogeographical inferences of the study are based on mtDNA only, and analyses using biparentally inherited markers (e.g. microsatellites) from the entire range of the species will be necessary to confirm its findings. In addition, further studies sampling across these potential refugial areas and including ancient DNA analyses from fossils would greatly contribute to a better understanding of re-colonization patterns and the role of species interactions in shaping actual species distributions.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2016.03.024>.

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