

Ecology, biogeography and evolutionary history of the alpine long-eared bat *Plecotus macrobullaris*

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This article is a comprehensive summary of the PhD presented by Antton Alberdi at the University of the Basque Country (UPV/EHU), on december 5th, 2014, under the supervision of Dr. Inazio Garin and Dr. Joxerra Aihartza. The thesis was awarded by the Spanish Society of Mammalogist (SECEM) in 2015, as the best thesis about Iberian mammals.

Introduction

The alpine long-eared bat *Plecotus macrobullaris* is one of the mammals most recently recognised as species in Europe. It was first described by Kuzjakin in 1965 (Kuzjakin 1965), but it did not transcend to the international scientific community, presumably because the description was flimsy and was written in Russian language only (Kiefer 2008). For the following almost 40 years the bat was ascribed either as *P. auritus* or *P. austriacus* in Europe, until the presence of a different taxon ultimately emerged in the Alps between 2001 and 2003 (Spitzenberger *et al.* 2002, Kiefer & Veith 2002, Spitzenberger *et al.* 2003). Its high morphological resemblance to other *Plecotus* species explains this late discovery. European *Plecotus* exhibit a high morphological similarity and a considerable biometric overlap (Tvrtkovic *et al.* 2005, Ashrafi *et al.* 2010), which complicates species identification in the field. Additionally, echolocation characteristics are also very similar, making identification by echolocation signals barely reliable (Barataud 2014). Therefore, in many cases a molecular analysis of DNA is necessary to ensure correct identification.

Information about its general ecology, behaviour and evolutionary history were barely known before the beginning of this doctoral thesis, and besides, some of the few published studies had reported conflicting results. For example, in several studies performed in the Swiss Alps (Rutishauser *et al.* 2012,

Ashrafi *et al.* 2013), *P. macrobullaris* was reported to select deciduous forest habitats for foraging and roosting, which led them to assume a high ecological resemblance to the forest specialist *P. auritus*. However, Preatoni *et al.* (2011) stated that in the Italian Alps the species avoids woodlands while Dietrich *et al.* (2006) described the echolocation characteristics of *P. macrobullaris* as closer to those of *P. austriacus* than *P. auritus*. To further blur the picture, the alpine long-eared bat had been captured in forests in Croatia (Tvrtkovic *et al.* 2005) but also in totally deforested areas in the Pyrenees (Garin *et al.* 2003), in Crete (Benda *et al.* 2008) and in Iran (Benda *et al.* 2012). Regarding dietary preferences of *P. macrobullaris*, the high importance of moths in its diet was reported by two authors (Whitaker & Karatas 2009, Ashrafi *et al.* 2011), although the details about the consumed species, as well as the foraging behaviour and hunting strategy remained unrevealed.

The evolutionary history and intraspecific taxonomic status of *P. macrobullaris* were also open to discussion. The species most closely related to the alpine long-eared bat is *P. auritus* (Juste *et al.* 2004, Spitzenberger *et al.* 2006). Juste *et al.* (2004) estimated that the split between *P. macrobullaris* and *P. auritus* lineages occurred within the last 5 Myr, but that dating was criticized by Spitzenberger *et al.* (2006) for considering that the approach for time-calibrating the phylogenetic analyses was inappropriate. Moreover, several authors have identified two main lineages within *P. macrobullaris*,

but the conclusions about the evolutionary history and intraspecific taxonomic status of the species have been controversial, as while some authors defended the existence of two subspecies (Kiefer & Helversen 2004), others reported almost no population structure (Juste *et al.* 2004).

Considering all these controversies, which are expected when the research on the ecological and molecular characteristics of a newly described species are in its initial steps, this Doctoral Thesis aimed to give insight into the ecological preferences, the biogeography and the evolutionary history of the alpine long-eared bat *Plecotus macrobullaris*. Specifically, (1) we obtained a detailed view of the geographical and elevational distribution of *Plecotus macrobullaris*; (2) we described the diet of *P. macrobullaris* at consumed species level to infer whether *P. macrobullaris* use alpine environments for foraging; (3) we characterized the roosting behaviour of *P. macrobullaris*; (4) we modelled the ecological niche of the species to identify and assess the main factors shaping its distribution pattern; and, finally (5) we reconstructed the genetic structure of populations and the demographic history of *P. macrobullaris* during the Pleistocene.

Materials and methods

Bat captures in alpine environments

Bat trappings were performed using mist-nets in the Pyrenees, Alps, Pindos Mountains, and Caucasus between 2009 and 2012 to get presence records of *P. macrobullaris*, tissue samples and faecal pellets. Overall, mist-nets were placed in 54 sites above the treeline, at 1450-2400 m amsl (above mean sea level). For further details of the sampling methods read Alberdi *et al.* (2013). Presence records of *P. macrobullaris* were complemented by revisiting information published in scientific articles, atlases, and books, and by contacting researchers, nature conservation organizations, and naturalists.

Molecular diet analysis

Faecal samples of 40 individuals captured in alpine environments in the Pyrenees were molecularly analysed. After extracting the DNA from faecal pellets, a 157-bp fragment of the mitochondrial COI gene was amplified using ZBJ-ArtF1c and ZBJ-ArtR2c primers developed by Zeale *et al.* (2011). PCR products were cloned using the pGEM-T

Easy Vector System, and 20 colony-forming units (cfu) were selected from each plate, for subsequent steps and sequencing. The obtained sequences were collapsed into Molecular Operational Taxonomic Units (MOTUs) using the software jMOTU (Jones *et al.* 2011), with the threshold-value set to 1,3% (2 nucleotides). The most common sequences from each MOTU were used for species identification. BLAST was used for sequence similarity analysis, comparing query sequences to reference sequences stored in GenBank most of which were derived from BOLD (Ratnasingham & Hebert 2007). Dietary diversity was determined at the MOTU level using the Shannon's diversity index. Moths consumed by *P. macrobullaris* were grouped according to their altitudinal preference as well as their known foraging habitat preferences. Further details of the employed methods can be found in Alberdi *et al.* (2012).

Roosting ecology analysis

The fieldwork was performed in July and August 2012, in eight valleys scattered throughout the Pyrenees. Bats were fitted with radio-transmitters and tracked to diurnal roosts for eight days. Radio-tracked bats were grouped into three classes based on sex and reproductive condition: breeding (lactating and pregnant) females, nulliparous females, and males. Roost types were classified into four categories: crevices, caves, scree deposits, and buildings. We analysed eight habitat variables to test for habitat selection, and compared our results with the published literature. We calculated the relative area of each habitat type in two predefined radii around each roosting site: $r = 1,300$ m to obtain comparable results with that of Rutishauser *et al.* (2012), and $r = 2,900$ m, which is the average distance recorded between the capture sites and roosting sites in this study. All roost locations were compared to a random set of locations generated within the study area. The habitat selection analysis was developed using generalized linear models (GLM) with a binomial error distribution and a logit link function (logistic regression models). Roost fidelity (FR) was calculated using an equation that reflects the probability of roost switching each day, with the highest values indicating high lability (1= switches every day) and low values indicating high fidelity (0= same roost everyday). Further details of the employed methods can be found in Alberdi *et al.* (2015a).

Ecological niche modeling

We used a two-scale modelling approach to define the ecological niche of *P. macrobullaris*. The extent of the broad-scale models was set as the Western Palearctic region (20-70° N, -20-60° E), while fine-scale models were limited to the Pyrenees and the surrounding area (40-42.1° N, -1.1-2.2° E). All predictive models were generated with the software Maxent 3.3.3 (Phillips *et al.* 2006, Phillips & Dudík 2008). For the broad-scale modelling we used presence location records from all of the known distribution area of *P. macrobullaris*. After filtering highly correlated predictors, we used three types of variables (6 climatic, 2 topographic and 1 habitat-related) for generating 90 different models (each one including 50 replicates) using different variable combinations and regularization parameters. For the fine-scale modelling, we used roost location data for modelling the fine-scale environmental niche of *P. macrobullaris*. Roosts were identified using radio-tracking. We used six variables (3 topographic and 3 habitat-related) to generate 45 different models. Models were evaluated using both threshold-independent and dependent means that deal with several aspects of model performance (MPA), including model accuracy (AUC), model complexity (AICc) and prediction success (validation using all *P. macrobullaris* records) (Murphy & Winkler 1987, Pearce & Ferrier 2000, Warren & Seifert 2011). The relative importance of each variable was checked using heuristic (percent contribution), permutation (permutation importance) and jackknife approaches (Yost *et al.* 2008, Moratelli *et al.* 2011). Response curves for the best explanatory variables were plotted in order to determine the response of suitability values to changes in specific predictors, and thus identify the most suitable conditions for the species. Detailed information of the employed methods can be found in Alberdi *et al.* (2014).

Evolutionary history analyses

Wing biopsies of 66 bats were used for the study. After extracting the DNA from wing biopsies, we used two technical approaches to prepare the DNA extracts for high-throughput sequencing. Initially we attempted on all samples a long-range PCR coupled to Illumina sequencing protocol PCR, modified from Morin *et al.* (2010).

Mitogenomes from the samples we were not able to amplify using LR-PCR (n= 38) were generated through target enrichment hybridisation capture, following Maricic *et al.* (2010). The LR-PCR and DNA-capture datasets were sequenced on independent lanes of an Illumina HiSeq 1000 platform, using 100bp SR chemistry. In order to estimate the changes of environmental suitability that could account for the demographic history of *P. macrobullaris* populations during the Pleistocene, we generated geographical suitability maps for the whole Palearctic region based on climatic and topographic variables with the software Maxent 3.3.3 (Phillips *et al.* 2006). Suitability maps were created based on the reference broad-scale model for other eight climatic conditions extrapolating data from three climatic envelopes: present, last glacial maximum (LGM ~ 21,000 yBP) and last interglacial (LIG ~ 130,000 yBP, (Otto-Bliesner 2006)), available from WorldClim (www.worldclim.com). Based on the ENMs generated for different paleoclimatic situations and the preliminary molecular information, we set one null and three alternative paleodemographic scenarios that could explain the current genetic structure of *P. macrobullaris* populations. The conflicting paleodemographic scenarios were compared using the ABC approach implemented in DIYABC v2.0.4 (Cornuet *et al.* 2014). To estimate the time when the ancestral split of the two main lineages occurred, given the absence of reliable and informative fossil records in the lineage of plecotine bats, we opted to build several Bayesian phylogenetic trees using complete mitochondrial genomes of nine vespertilionid species and one mitogenome of each lineage of *P. macrobullaris* with different molecular clock frameworks and data partitioning. The posterior distributions for divergence times between both main *P. macrobullaris* lineages were used as prior tree-root calibration to generate the intraspecific phylogeny of *P. macrobullaris*. We also computed several metrics of genetic diversity within and between the two main lineages identified in this study using Arlequin 3.5 and DnaSP 5.10, and further detailed information about the relationship between the *P. macrobullaris* haplotypes was obtained by median-joining network using NETWORK (v4.610, Fluxus Technology). Details of the molecular and bioinformatics methods employed are available in Alberdi *et al.* (2015b).

Results

Bat captures in alpine environments

Overall, we captured 285 bats in 48 of the 54 sampled sites, at an elevation range of 950 m, between 1,450 and 2,400 m amsl. Bats were caught in subalpine and alpine meadows as well as bare rock areas with scarce vegetation. Captured individuals belonged to ten species (Table 1), with the alpine long-eared bat predominant: 197 *Plecotus macrobullaris* constituted 70% of all captures, followed by *Myotis nattereri* sensu lato (12%; see Salicini *et al.* (2011)) and *M. myotis* (6%). The gender ratio for *P. macrobullaris* was biased toward females (58% females, 42% males), whereas 65% of the specimens of other species were males.

Geographical and elevational distribution

We gathered 198 published and 113 unpublished records, and the mist-netting in alpine environments resulted in 41 new supraforestral localities of *P. macrobullaris*. Additionally, we captured the species at four other localities below the treeline. The overall compilation resulted in 356 presence localities throughout its geographic distribution (see Alberdi *et al.* 2013, for details). The alpine long-eared bat is present in the main mountain ranges of Southern Europe, the Near East, and the Middle East (Fig. 1a). The total compiled data shows that the species' known localities span 0-2,800 m amsl, though the recorded elevational distribution differs among mountain ranges (Fig. 1b).

Diet and foraging ecology

We analysed 102 faecal pellets produced by 29 individuals from 12 different locations. We found 90 different sequences that were collapsed into 54 MOTUs. We successfully identified 28 moth species (539 sequences, 31 MOTUs) and 1 moth genus (*Rhyacia*). The identified prey species occupy very diverse altitudinal ranges. Strictly alpine species were found in 15 samples and orophilous-alpine species in 13 samples. Species with wide altitudinal ranges appeared together with strictly alpine species in five individuals, with orophilous-alpine species in four individuals, and alone in six. Most of the sequences belonged to altitudinally broad species (42.8%), followed by orophilous-alpine species (32.3%) and strictly alpine species (25.0%) (Fig. 2a). The leading habitat types used by moths consumed by *P. macrobullaris* are subalpine meadows, followed by orophilous meadows and alpine meadows (Fig. 2b). Thus, almost all the habitat types used by consumed moth species are open habitats. In addition, 76% of the detected moths use host plants from grasslands.

Roosting ecology

We radio-tagged 51 animals and were able to identify the roosts of 37 (72%): 8 breeding females, 12 nulliparous females, and 17 males for an average of 3.4 ± 0.81 location points during the 8-day sampling period. We identified 54 roosts, averaging 2.2 ± 0.85 roost per bat (several tracked bats shared the same roosts). *P. macrobullaris* used three of the four defined roosts categories: crevices

Table 1. Summary of bats captured in alpine environment. An asterisk (*) indicates species captured in only one place. *P. macrobullaris* values are shown in bold.

Species	Total individuals	Males	Females	Elevation range
<i>Hypsugo savii</i>	4	4	0	1800-2000
<i>Myotis myotis</i>	16	10	6	1600-2050
<i>M. mystacinus</i>	2	2	0	2000*
<i>M. nattereri</i>	34	18	16	1420-2350
<i>Pipistrellus pipistrellus</i>	5	5	0	1700-2100
<i>Plecotus auritus</i>	7	7	0	1420-1900
<i>P. austriacus</i>	9	4	5	1600-2000
<i>P. macrobullaris</i>	197	83	114	1600-2400
<i>Rhinolophus ferrumequinum</i>	6	3	3	1800*
<i>Tadarida teniotis</i>	1	1	0	1800*

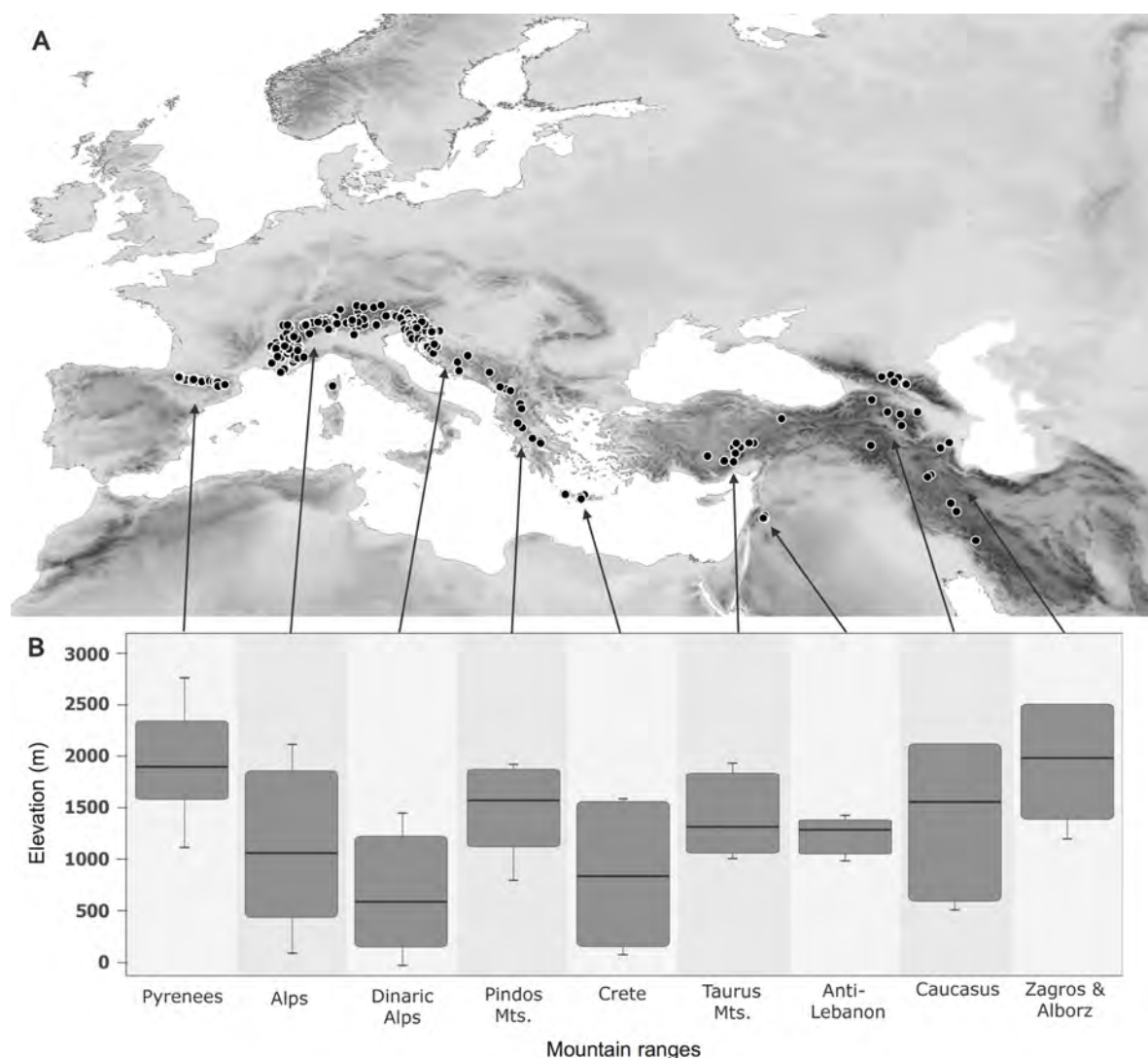


Figure 1. Geographical and elevational distribution of *P. macrobullaris* **A)** Presence of *P. macrobullaris* on an elevation map of the Western Palearctic. **B)** Elevational distribution of *P. macrobullaris* in several mountain chains. Boxes limit the 5% and 95% percentiles; vertical lines show minimum and maximum values and horizontal lines indicate the mean value. Figure originally published in Alberdi *et al.* 2013.

($n = 30$), scree deposits ($n = 21$), and buildings ($n = 3$). Roosts were located between 1,450 and 2,430 m amsl. Males roosted in scree deposits more often than females, yet five females were also tracked to these locations (Table 2). At these deposits, bats were found alone under average-sized stones (Fig. 3). The three building roosts (5.5% of all roosts) housed maternity colonies comprising 10-15 individuals. The logistic regression models showed that roosting sites were located closer to meadows and open forest, and further away from deciduous forest and shrubbery (Table 3). The average elevation of roosts used by breeding bats was lower than the elevation of nulliparous females (Tukey: $p < 0.01$) and males (Tukey: $p = 0.03$) (Table 2). The overall index of roosting fidelity was 0.51, but values

differed considerably among bat classes and roost categories. By class, pregnant and lactating females showed the highest fidelity (FR= 0.11) nulliparous females had intermediate values (FR= 0.35), whereas males exhibited high roosting lability (FR= 0.88). By category, bats roosting in buildings had the highest fidelity (FR= 0), followed by bats roosting in crevices (FR= 0.21). Conversely, bats roosting in scree deposits exhibited low fidelity, switching roosts every day they were tracked (FR= 1).

Ecological niche

Analysed evaluators showed that the broad-scale model with the best predictive ability was the one including one topographic (ABR), five climatic (B4,

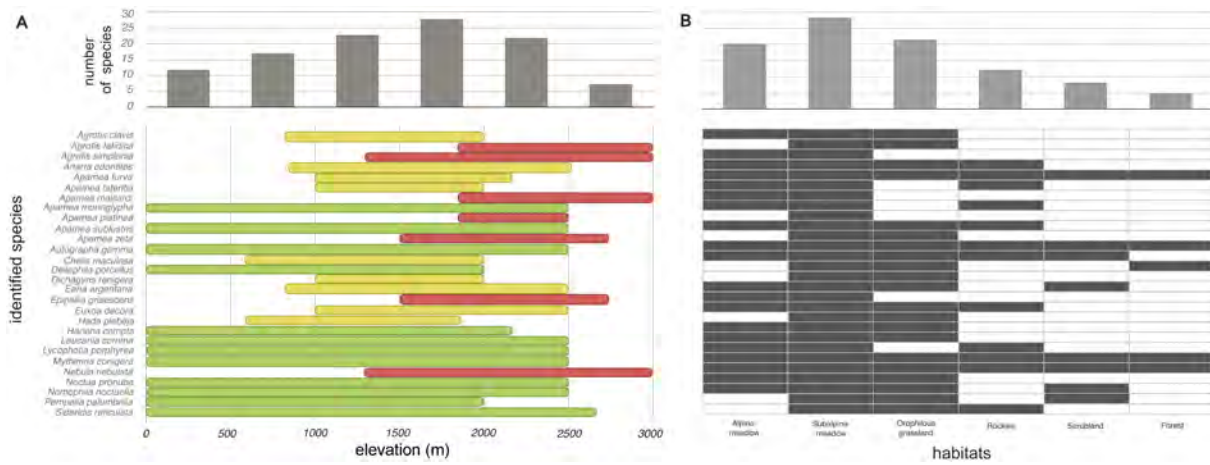


Figure 2. A) Elevational distribution of species consumed by *P. macrobullaris* in South Europe. Species with wide elevational ranges are drawn in light green, orophilous-alpine species in yellow, and strictly alpine species in magenta. Bibliographic sources are given in the main text. B) Habitat types used by each identified prey species in South Europe. Used habitats are drawn in black. Figure originally published in Alberdi *et al.* 2012.

Table 2. Summary statistics for roosting sites of breeding females, nulliparous females and males. The last three columns show the number of roost types used by bats from each category.

	Capture elevation (m)	Roosting elevation (m)	Treeline difference (m)	Forest distance (km)	Capture distance (km)	Crevices	Screenes	Buildings
Breeding females	1,887 ± 225	1,705 ± 208	123 ± 280	0.78 ± 1.02	2.86 ± 2.17	5	1	3
Nulliparous females	1,921 ± 118	2,071 ± 212	510 ± 255	2.74 ± 1.22	3.51 ± 2.39	8	4	0
Males	1,930 ± 291	1,924 ± 234	345 ± 285	1.89 ± 1.37	2.66 ± 2.13	17	16	0
Total	1,850 ± 244	1,921 ± 249	349 ± 297	1.91 ± 1.41	2.88 ± 2.18	30	21	3

Table 3. Used and available habitat features and the coefficients of the GLM in the two analysed scales.

	1300m radius			2900m radius		
	Used	Available	Coefficient	Used	Available	Coefficient
Deciduous forest (%)	2.57±5.62	16.10±20.55	-0.087 ***	3.68±5.21	15.77±16.88	-0.092 ***
Mixed forest (%)	0.51±1.89	5.93±13.56	-0.125 *	2.03±4.83	5.76±10.29	-0.023
Coniferous forest (%)	8.61±16.67	14.70±19.83	-0.014	7.35±11.47	14.69±15.85	-0.023
Open forest (%)	11.01±13.40	8.12±12.98	0.033 **	7.50±9.39	7.94±9.82	0.061 ***
Shrubbery (%)	1.25±3.77	8.03±13.55	-0.124 **	2.18±2.87	7.57±9.94	-0.109 **
Orchards (%)	0	0.04±0.75	-9.461	0	0.04±0.47	-12.906
Meadows (%)	50.59±18.58	22.66±23.45	0.023 ***	51.33±13.33	22.54±19.63	0.045 ***
Richness (1-4)	2.27±0.87	2.37±0.69	0.106	2.81±0.39	2.97±0.62	-0.474

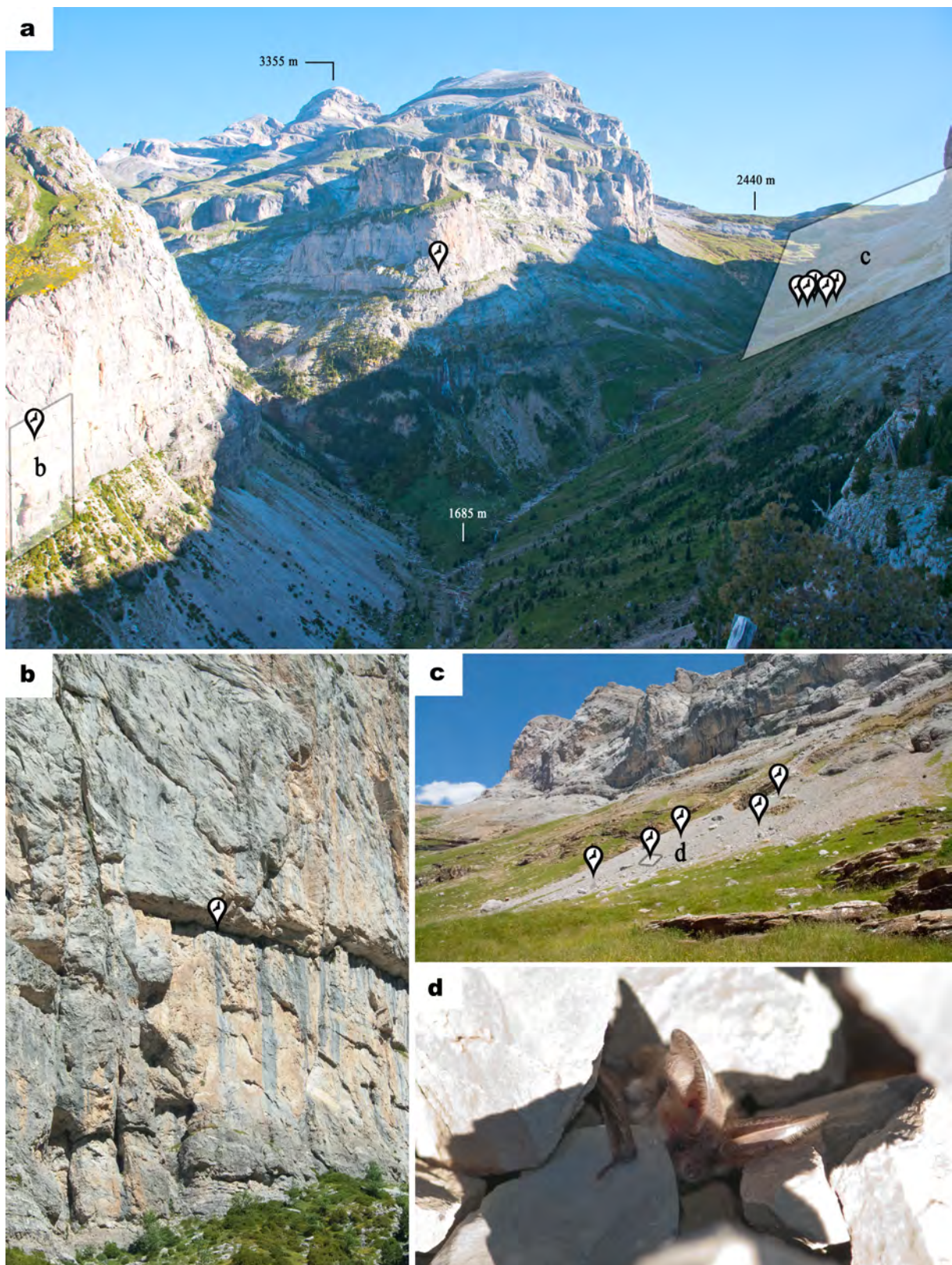


Figure 3. Roost views, with icons showing exact roost locations. **A)** General view of a roost area in Añislo Canyon; the area covered by photos B and C is marked with white insets. **B)** Detail of the limestone cliff with a horizontal crevice (1,880 m) where two breeding females roosted. **C)** The scree deposit (2,140 m) of five roosting points belonging to two male bats; the area shown by photo D is marked with an inset. **D)** A male alpine long-eared bat roosting in a scree deposit sticking out its head after stones had been removed. Figure originally published in Albertdi *et al.* 2015a.

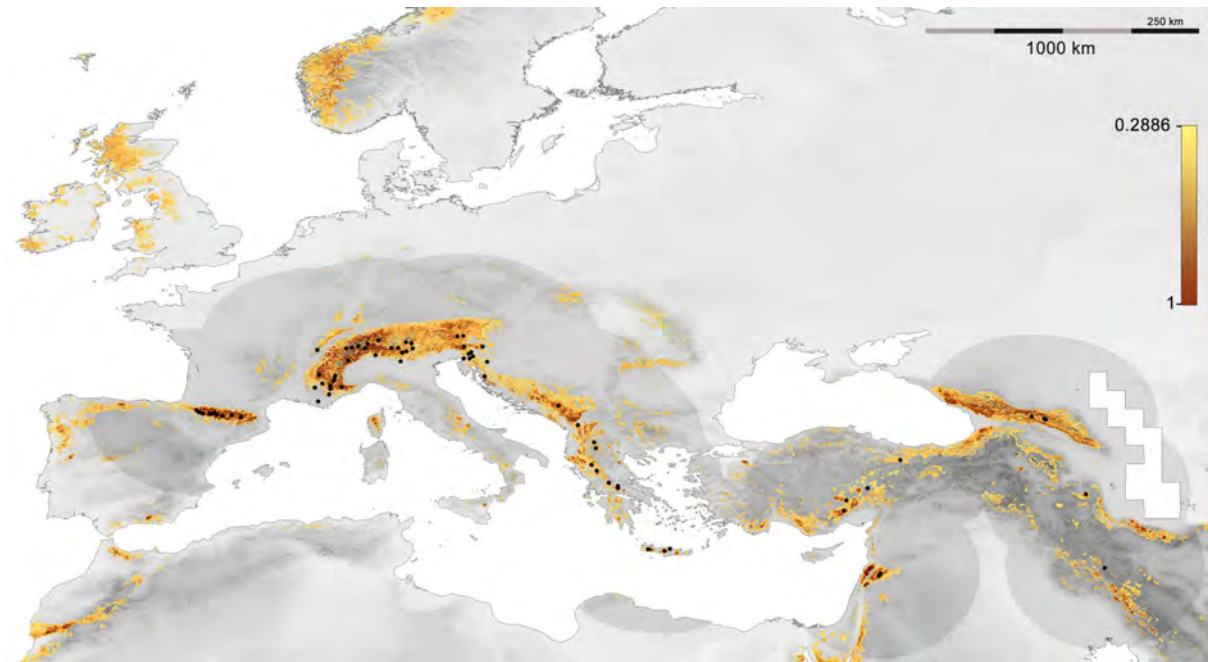


Figure 4. Distribution model for *P. macrobullaris*, presented on a greyscale elevation map. Only suitable areas (SV > 0.2886) are shown in a colour gradient from light yellow (low suitability) to brown (high suitability). Presence location records used for modelling are represented by black dots and the darkened region is the area used for calibrating the model. Figure originally published in Alberdi *et al.* 2014.

B10, B12, B15 and B17) and one habitat-related (LAND) variables, generated with a regularization multiplayer of $\beta=1$ (Table 2). The best model showed suitable areas in the main mountain ranges of the Southern Palearctic, as well as some areas of Northern Europe (Fig. 4). All metrics indicated that abruptness was the variable with the best explanatory power. The second most important variable, based on all analysed metrics, was the mean temperature of the warmest quarter (B10), and the remaining climatic and habitat-related variables obtained lower values in all the evaluated metrics. The response curve of the best explanatory variable showed that suitability increased with abruptness, starting with zero probability of presence in flat regions (Fig. 5a). The most suitable mean temperature of the warmest quarter was 14 °C, but suitable values ranged from 6 to 22 °C (Fig. 5b). The best fine-scale model was built using two habitat (distance to rocky and urban areas) and two topographic variables (elevation and slope) (Table 2). The three main factors shaping roosting habitat suitability for *P. macrobullaris* were the distance to rock areas (DIS-ROCK), slope and elevation. The closer the rocks and higher the slope the greater the probability of finding suitable roosting sites (Fig. 5c, 5e). The response curve for elevation showed

suitable areas for roosting in an elevation range from 1,300 to 2,400 m, with maximum values between 1,500 and 2,000 m (Fig. 5b).

Ancient environmental suitability

The most suitable conditions were predicted for current climatic conditions, and suitability values decreased under other climatic conditions (Fig. 6). However, the specific patterns of suitability variation for each mountain system differed considerably. Mountain ranges in Western Europe (Pyrenees and Alps) tolerated warm conditions better than cold conditions, while the opposite trend was observed in Eastern mountain ranges such as the Taurus or the Zagros Mountains.

Complete mitochondrial genomes

Of the 66 samples obtained across the whole distribution area of the species, we were able to process 28 samples using the LR-PCR shotgun sequencing approach, and the rest were processed using the DNA-capture method. We successfully sequenced and assembled the complete mtDNA genome of 57 individuals. The length of the mitochondrial genome of *P. macrobullaris* was approximately 16,830 bp (16,829-16,835).

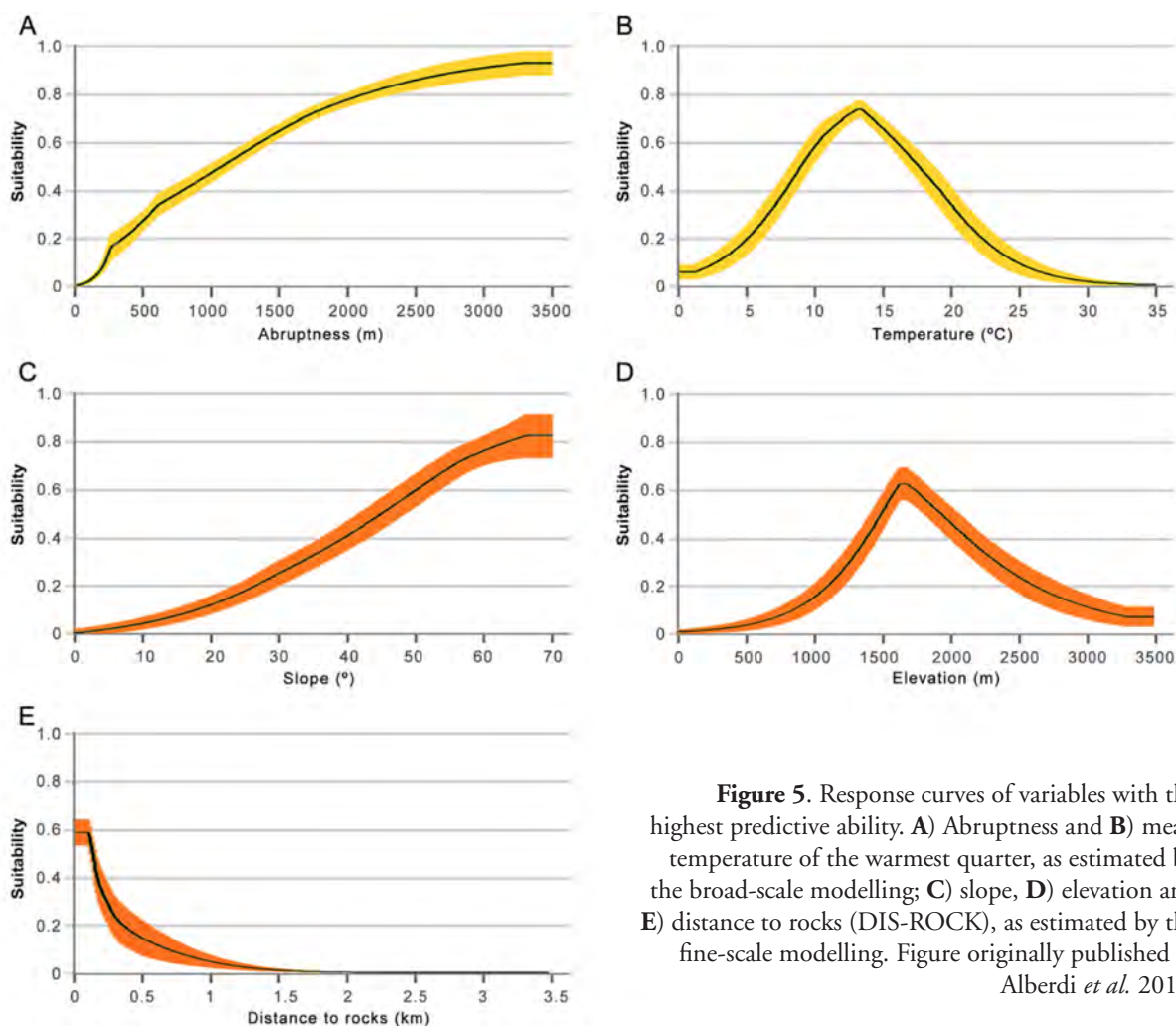


Figure 5. Response curves of variables with the highest predictive ability. **A)** Abruptness and **B)** mean temperature of the warmest quarter, as estimated by the broad-scale modelling; **C)** slope, **D)** elevation and **E)** distance to rocks (DIS-ROCK), as estimated by the fine-scale modelling. Figure originally published in Alberdi *et al.* 2014.

Population structure

The analysis of the molecular variation (AMOVA) depicted a genetic structure divided into two main lineages. The analysis showed that 79.5% of the genetic variation occurred between these two main clades, while inter and intra-population variation explained 13.8% and 6.7% of genetic variation respectively (Table 7.1). The genetic differentiation between both lineages was 3.4 ± 0.07 %. The structure and diversity of both lineages differed considerably, and all diversity indices indicated a lower genetic diversity in the Western Lineage (WL) compared to the Eastern Lineage (EL).

Paleodemography and phylogenetic relationships

The ABC analysis showed that the observed data is consistent with a model in which the two *P.*

macbullaris lineages split long before the LGM, and that the western population size remained small until it expanded substantially (about 700-fold expansion) after the LGM. The split of the two main *P. macbullaris* lineages is estimated to have occurred 1.67 MyBP (95% HPD: 1.18 - 2.15 MyBP) (Fig. 7a). The results indicate that the diversification of the EL began around 899 kyBP (95% HPD: 727 - 1093 kyBP) with the split of the Iranian clade, and continued around 583 kyBP (95% HPD: 472 - 711 kyBP) with the divergence of the Caucasus and Anatolia- Balkan clades. Conversely, the diversification of extant lineages in the WL occurred around 68 kyBP (95% HPD: 45 - 95 kyBP). The haplotype-network analysis supports the more recent diversification of the WL, whereby haplotypes are separated by only a few mutational steps, as opposed to the much deeper divergence across the EL (Fig. 7b).

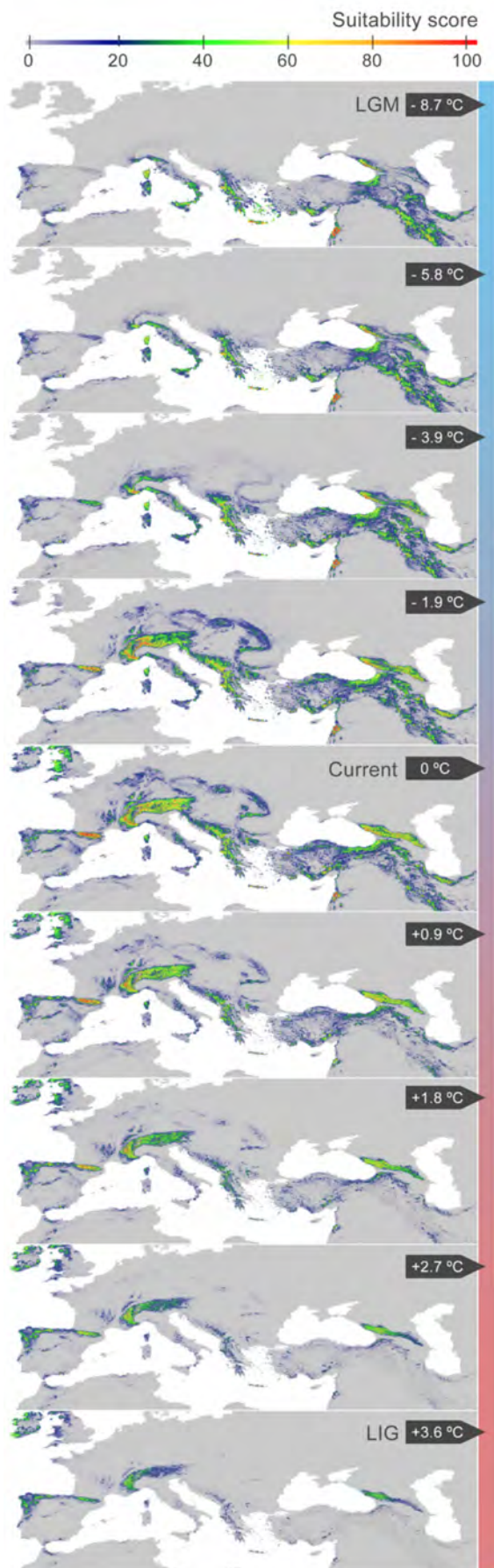
Conclusions

This Doctoral Thesis aimed to address multiple issues regarding the ecology, the biogeography and evolutionary history of *Plecotus macrobullaris*. The work performed during four years contributed to better comprehend the general biology of the alpine long-eared bat, which was poorly understood and full of contradictory evidence when this study was set out in 2010. Additionally, this work has also contributed to unveil diverse aspects of bats in alpine environments and better understand the biogeography of alpine vertebrates.

Distribution, biogeography and ecological niche

- *P. macrobullaris* is a frequent species in European alpine areas. It was captured in all the surveyed alpine environments, comprising 70% of all the captures. Although the IUCN indicates that their population is decreasing, there are not evidences suggesting such a trend. However, climate change and pasture abandonment are two potential threats that would be worth studying in the future.
- There is a nearly unknown chiropteran community in the alpine environments. *P. macrobullaris* was not the only species captured in alpine environments. I captured nine more species by placing nets in alpine meadows and rocky areas (avoiding commuting paths as mountain passes), revealing that bats use alpine resources. Hence, chiropterans should be taken into account when developing inventories and management plans in alpine environments.
- *P. macrobullaris* is widely distributed in the Western Palearctic but restricted to mountain environments. Despite covering an elevational range from sea level to at least 2,800 m depending on the region, *P. macrobullaris* is lacking from

Figure 6. Suitability maps for the nine climatic scenarios affecting the evolutionary history of *Plecotus macrobullaris* in the Western Palearctic. The temperature values indicate the difference between the global mean temperature of the warmest quarter (BIO10) for the Western Palearctic compared with current conditions. LGM, Last Glacial Maximum; LIG, Last Interglacial. Figure originally published in Alberdi *et al.* 2015b.



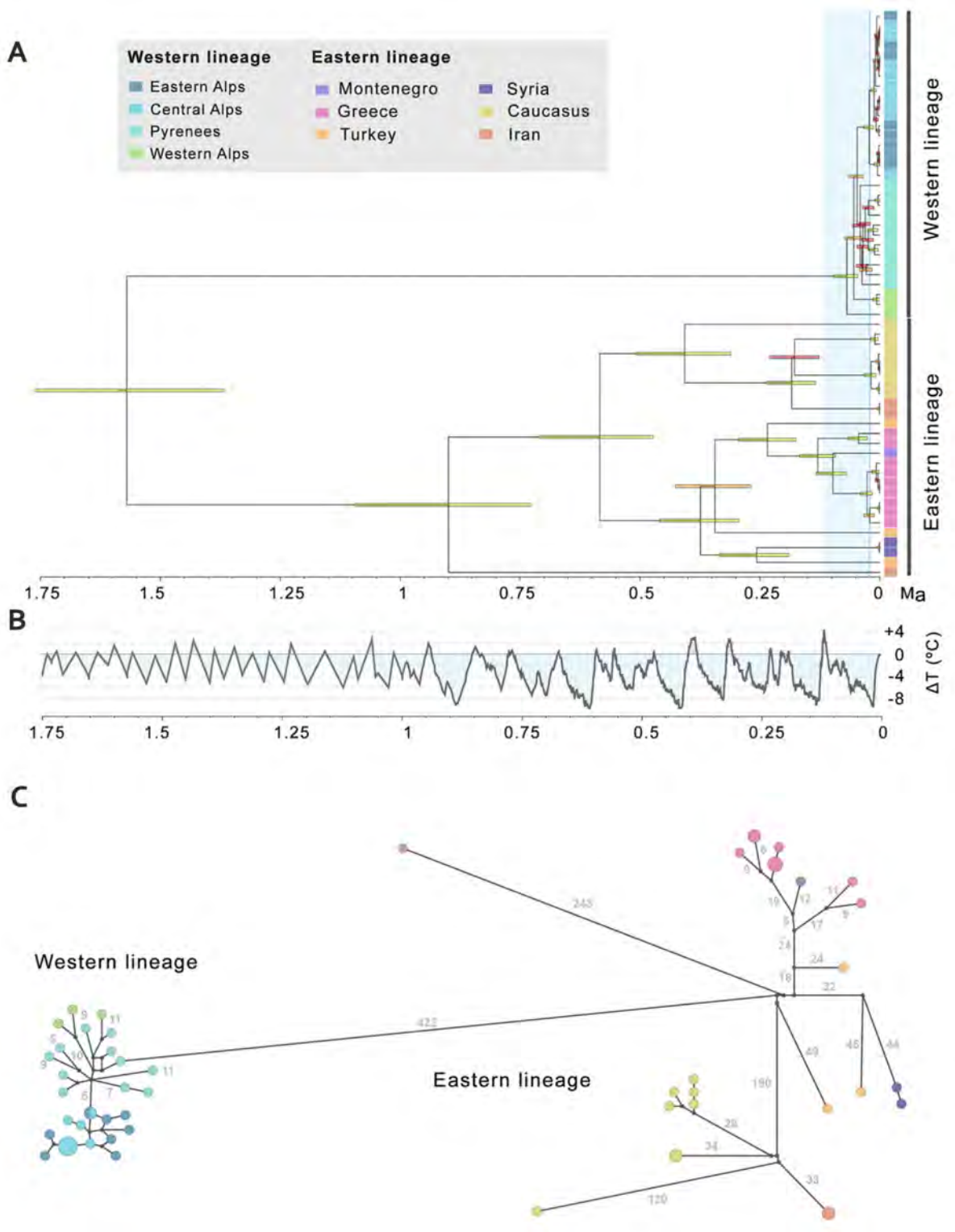


Figure 7. The phylogeny and haplotypes of *Plecotus macrobullaris* in the Western Palaearctic, and temporal temperature variation. **A)** Time-calibrated phylogenetic tree of *P. macrobullaris*. Node bars show the 95% bound of the highest posterior density (HPD) and the colours indicate the posterior probability: green, = 1; yellow, > 0.9; red < 0.9 (exact posterior probabilities are provided in Fig. S5 in Appendix S3). **B)** Approximate global temperature variations relative to current conditions (ΔT), modified from Lisiecki & Raymo (2005) and Huybers (2009). **C)** Haplotype network of the sampled individuals. The haplotypes are shown as circles, with the size indicating the frequency in the sampled populations and the colour showing the geographical location. Black points indicate missing intermediate haplotypes and grey numbers indicate the number of mutations between different haplotypes. Figure originally published in Albertdi *et al.* 2015b..

flatland, being only found in mountain ranges and their close surroundings. Further sampling effort in alpine areas, for example in the Apennines (Italy), might surface new populations of *P. macrobullaris*.

- **The geographical distribution of *P. macrobullaris* is mainly shaped by topographic factors**, while climatic determinants adjust its elevational distribution within mountain ranges. Abruptness likely acts as a surrogate of several ecological variables, such as availability of open spaces and rocky areas.
- ***P. macrobullaris* is not a cold-adapted but a cold-tolerant species**. It finds suitable habitats in open-space areas with abundant rock resources. These requirements are mainly found in alpine environments, which due to its wide thermal tolerance *P. macrobullaris* is able to exploit, but also in abrupt landscapes at lower elevations.
- **Interspecific competition could affect the distribution of the alpine long-eared bat in a regional scale, but does not shape the continental level distribution of *P. macrobullaris***. Geographical and elevational competitive exclusion between *P. macrobullaris* and similar species such as *P. austriacus* probably occurs, but the results we obtained suggest that the general distribution pattern of the alpine long-eared bat is not affected by such processes, as the species is kept linked to mountain environments also in areas where putative competitors are absent.

Diet, foraging and roosting ecology

- ***P. macrobullaris* captured in alpine environments feed mainly on moths and forage in supraforestral open-space areas**. Despite being phylogenetically closer to *P. auritus*, sensorial, morphological and ecological features of *P. macrobullaris* are more similar to *P. austriacus*. While *P. auritus* is mainly a forest species, *P. macrobullaris* tend to forage in open and semi-open areas, similar to *P. austriacus*.
- ***P. macrobullaris* makes full use of the alpine environment: it forages, roosts and breeds in supraforestral areas**. *P. macrobullaris* is the first bat species known to roost and breed commonly in alpine environments, although it is not the only species foraging and roosting there. Roosting up to 1,000 m above the treeline suggests that these bats could have developed some physiological

or behavioural adaptations to cope with adverse climatic conditions. That issue would be worth studying in the future.

- ***P. macrobullaris* captured in alpine environments roost mainly in natural rock resources, such as crevices and talus slopes**. This conclusion significantly differs from the general view of the species in the Alps, where the species has been almost exclusively found in buildings. Nearly all the roosts known in the Pyrenees before performing the radio-tracking study were also located in buildings, suggesting sample bias in the current knowledge of the roosting behaviour of *P. macrobullaris* in the Alps. However, we cannot rule out that the colder climate of the Alps compared to other mountain ranges could have led into a major use of human-made buildings than in other areas. Radio-tracking studies seeking roosts of bats captured while foraging would be desirable in the Alps to solve this issue.
- **The roosts of Pyrenean *P. macrobullaris* are generally located above the treeline**, selecting areas surrounded by meadows and open forests while discarding deciduous forest and shrubbery. This conclusion also contrasts with the information reported in the Alps, which was based on building roosts identified by direct prospection. The buildings identified as roosts in this study were closer to forest environments than natural resources, suggesting that this conclusion could be also the result of a biased dataset.

Evolutionary history

- ***P. macrobullaris* is genetically structured in two geographically separated lineages, which split during the Mid Pleistocene**. The genetic structuration of these two main lineages had been acknowledged since the initial molecular analyses of the species, but neither the level of differentiation was measured using long molecular sequences, nor the splitting date was estimated to date.
- **Both lineages maintained isolated probably because the land bridge connecting their respective distribution areas was environmentally unsuitable for most of the time during the Pleistocene**. The ecological niche modelling approach applied to different climatic conditions allowed identifying the resilience of

different mountain ranges, depicting the Dinaric Alps as the mountain range with the highest decrease of suitability due either to cooling or warming events.

- **The genetic structure of the Eastern and Western lineages is different, probably due to the differential effect of glaciation events in different mountain systems.** Even though a different molecular structuration had been suggested before based on information obtained from mitochondrial Control Region sequences, complete mitogenomes allowed measuring the level of differentiation and estimating the divergence times of different sublineages with higher level of confidence. The Eastern lineage is more diverse because the geographic area kept suitable for most of the time during the Pleistocene, which could have allowed different populations to survive. Ecological niche models projected to past climatic conditions were essential to reach to this conclusion. The Western lineage has lower diversity because the geographic area suffered high suitability drops during cold stages, which could have produced several subsequent bottlenecks. The cold climatic conditions born during the Pleistocene could have exerted positive selection of physiological traits related to bearing cold conditions in bats of the Western lineage, which would be worth checking.
- **Demographic responses of species to events of climate variation were not geographically and temporally homogeneous, but complex processes with population-level differences.** Even though this statement seems obvious, evidences of contrasting responses of populations within single species to climate changes are still scarce. This study provides an empirical evidence of such a process.

References

- Alberdi A., Garin I., Aizpurua O. & Aihartza J. 2013. Review on the geographic and elevational distribution of the mountain long-eared bat *Plecotus macrobullaris*, completed by utilising a specific mist-netting technique. *Acta Chiropterologica*, 15: 451-461.
- Alberdi A., Aihartza J., Albero J.C., Aizpurua O., López-Baucells A., Freixas L., Puig X., Flaquer C. & Garin I. 2012. First records of the parti-coloured bat *Vespertilio murinus* (Chiroptera: Vespertilionidae) in the Pyrenees. *Mammalia*, 76: 109-111.
- Alberdi A., Aihartza J., Aizpurua O., Salsamendi E., Brigham M. & Garin I. 2015a. Living above the treeline: roosting ecology of the alpine bat *Plecotus macrobullaris*. *European Journal of Wildlife Research*, 61: 17-25.
- Alberdi A., Gilbert M.T.P., Razgour O., Aizpurua O., Aihartza J. & Garin I. 2015b. Contrasting population-level responses to Pleistocene climatic oscillations in an alpine bat revealed by complete mitochondrial genomes and evolutionary history inference. *Journal of Biogeography*, 42: 1689-1700.
- Alberdi A., Aizpurua O., Aihartza J. & Garin I. 2014. Unveiling the factors shaping the distribution of widely distributed alpine vertebrates, using multi-scale ecological niche modelling of the bat *Plecotus macrobullaris*. *Frontiers in Zoology*, 11: 77. BioMed Central Ltd.
- Ashrafi S., Beck A., Rutishauser M., Arlettaz R. & Bontadina F. 2011. Trophic niche partitioning of cryptic species of long-eared bats in Switzerland: implications for conservation. *European Journal of Wildlife Research*, 57: 843-849.
- Ashrafi S., Bontadina F., Kiefer A., Pavlinic I. & Arlettaz R. 2010. Multiple morphological characters needed for field identification of cryptic long-eared bat species around the Swiss Alps. *Journal of Zoology*, 281: 241-248.
- Ashrafi S., Rutishauser M., Ecker K., Obrist M.K., Arlettaz R. & Bontadina F. 2013. Habitat selection of three cryptic *Plecotus* bat species in the European Alps reveals contrasting implications for conservation. *Biodiversity and Conservation*, 22: 2751-2766.
- Barataud M. 2014. *Acoustic Ecology of European Bats*. Biotope.
- Benda P., Faizolahi K., Kiefer A., Obuch J., Reiter A., Sevcik M., Uhrin M., Vallo P. & Ashrafi S. 2012. Bats (Mammalia: Chiroptera) of the Eastern Mediterranean and Middle East. Part 10. Bat fauna of Iran. *Acta Societatis Zoologicae Bohemicae*, 76: 163-582.
- Benda P., Georgiakakis P. & Dietz C. 2008. Bats (Mammalia: Chiroptera) of the Eastern Mediterranean and Middle East. Part 7. The bat fauna of Crete, Greece. *Acta Societatis Zoologicae Bohemicae*, 72: 105-190.
- Cornuet J.M., Pudlo P., Veyssier J., Dehne-Garcia A., Gautier M., Leblois R., Marin J.M. & Estoup A. 2014. DIYABC v2.0: a software to make approximate Bayesian computation inferences about population history using single nucleotide polymorphism, DNA sequence and microsatellite data. *Bioinformatics*, 30: 1187-1189. Oxford University Press.
- Dietrich S., Szameitat D.P., Kiefer A., Schnitzler H. U. & Denzinger A. 2006. Echolocation signals of the plecotine bat, *Plecotus macrobullaris* Kuzyakin, 1965. *Acta Chiropterologica*, 8: 465-475.

- Garin I., Garcia-Mudarra J.L., Aihartza J., Goiti U. & Juste J. 2003. Presence of *Plecotus macrobullaris* (Chiroptera: Vespertilionidae) in the Pyrenees. *Acta Chiropterologica*, 5: 243-250.
- Huybers, P. 2009. Pleistocene glacial variability as a chaotic response to obliquity forcing. *Climate of the Past*, 5: 481-488.
- Jones M., Ghoorah A. & Blaxter M. 2011. jMOTU and Taxonator: turning DNA Barcode sequences into annotated operational taxonomic units. *PLoS ONE* 6:e19259.
- Juste J., Ibáñez C., Muñoz J., Trujillo D., Benda P., Karatas A. & Ruedi M. 2004. Mitochondrial phylogeography of the long-eared bats (*Plecotus*) in the Mediterranean Palaearctic and Atlantic Islands. *Molecular Phylogenetics and Evolution*, 31: 1114-1126.
- Kiefer A. 2008. *Phylogeny of Western Palaearctic long-eared bats (Mammalia, Chiroptera, Plecotus) - a molecular perspective*. Johannes Gutenberg-Universität, Mainz.
- Kiefer A. & Veith M. 2002. A new species of long-eared bat from Europe (Chiroptera: Vespertilionidae). *Myotis*, 39: 5-16.
- Kiefer A. & O. von Helversen. 2004. *Plecotus macrobullaris* (Kuzjakin, 1965) - Alpenlangohr. Pages 1051-1058 in F. Krapp (ed). *Handbuch der Säugetiere Europas- Fledertiere II*. AULA-Verlag, Wiebelsheim.
- Kuzjakin A. 1965. Otrjad Rukokrylyje. Ordo Chiroptera. in N. Bobrinskij, B. Kuznetsov, and A. Kuzjakin (ed). *Opredelitel mljekopitajushhtshikh SSSR*. Moscow.
- Lisiecki, L.E. and Raymo, M.E. 2005. A Pliocene-Pleistocene stack of 57 globally distributed benthic δ 18O records. *Paleoceanography*, 20:1-17.
- Maricic T., Whitten M. & Pääbo S. 2010. Multiplexed DNA Sequence Capture of Mitochondrial Genomes Using PCR Products. *PLoS ONE*, 5:e14004.
- Moratelli R., de Andreazzi C.S., de Oliveira J.A. & Cordeiro J. 2011. Current and potential distribution of *Myotis simus* (Chiroptera, Vespertilionidae). *Mammalia*. 75: 227-234.
- Morin P.A. et al. 2010. Complete mitochondrial genome phylogeographic analysis of killer whales (*Orcinus orca*) indicates multiple species. *Genome Research* 20: 908-916.
- Murphy A.H. & Winkler R.L. 1987. A general framework for forecast verification. *Monthly Weather Review*, 115: 1330-1338.
- Otto-Bliensner B.L. 2006. Simulating Arctic climate warmth and icefield retreat in the last interglaciation. *Science, American Association for the Advancement of Science*, 311: 1751-1753.
- Pearce J. & Ferrier S. 2000. An evaluation of alternative algorithms for fitting species distribution models using logistic regression. *Ecological Modelling* 128: 127-147.
- Phillips S.J., Anderson R.P. & Schapire R.E. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, 190: 231-259.
- Phillips S. & Dudík M. 2008. Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecography*, 31: 161-175.
- Preatoni D., Spada M., Wauters L., Tosi G. & Martinoli A. 2011. Habitat use in the female Alpine long-eared bat (*Plecotus macrobullaris*): does breeding make the difference? *Acta Chiropterologica*, 13: 355-364.
- Ratnasingham S. & Hebert P.D.N. 2007. BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecol Notes*, 7: 355-364.
- Rutishauser M.D., Bontadina F., Braunisch V., Ashrafi S. & Arlettaz R. 2012. The challenge posed by newly discovered cryptic species: disentangling the environmental niches of long-eared bats. *Diversity and Distributions*, 18: 1107-1119.
- Salicini I., Ibáñez C. & Juste J. 2011. Multilocus phylogeny and species delimitation within the Natterer's bat species complex in the Western Palaearctic. *Molecular Phylogenetics and Evolution*, 61: 888-898.
- Spitzenberger F., Haring E. & Tvrkovic N. 2002. *Plecotus microdontus* (Mammalia, Vespertilionidae), a new bat species from Austria. *Natura Croatica*, 11: 1-18.
- Spitzenberger F., Strelkov P. & Haring E. 2003. Morphology and mitochondrial DNA sequences show that *Plecotus alpinus* Kiefer & Veith, 2002 and *Plecotus microdontus* Spitzenberger, 2002 are synonyms of *Plecotus macrobullaris* Kuzjakin, 1965. *Natura Croatica*, 12: 39-53.
- Spitzenberger F., Strelkov P., Winkler H. & Haring E. 2006. A preliminary revision of the genus *Plecotus* (Chiroptera, Vespertilionidae) based on genetic and morphological results. *Zoologica Scripta*, 35: 187-230.
- Tvrkovic N., Pavlinic I. & Haring E. 2005. Four species of long-eared bats (*Plecotus*, Geoffroy, 1818; Mammalia, Vespertilionidae) in Croatia: field identification and distribution. *Folia Zoologica*, 54: 75-88.
- Warren D. & Seifert S. 2011. Ecological niche modeling in Maxent: the importance of model complexity and the performance of model selection criteria. *Ecological Applications*, 21: 335-342.
- Whitaker J.O. & Karatas A. 2009. Food and feeding habitats of some bats from Turkey. *Acta Chiropterologica*, 11: 393-403.
- Yost A.C., Petersen S.L., Gregg M. & Miller R. 2008. Predictive modeling and mapping sage grouse (*Centrocercus urophasianus*) nesting habitat using Maximum Entropy and a long-term dataset from Southern Oregon. *Ecological Informatics*, 3: 375-386.
- Zeale M.R.K., Butlin R.K., Barker G.L.A., Lees D.C. & Jones G. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, 11: 236-244.