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RESEARCH

Islands as biodiversity arks: the role of insular populations in preserving bat DNA-barcoding diversity

Leonardo Ancillotto[1,2] · **Mariella Baratti**[1] · **Ricardo Rocha**[3] · **Catarina Rato**[4] · **Vanessa A. Mata**[5,6] · **Andrea Galimberti**[2,7] · **Nicola Tommasi**[2,7] · **Mauro Mucedda**[8] · **Adrià López-Baucells**[9] · **Estel Blanch**[9] · **Andrea Viviano**[1] · **Paolo Agnelli**[10] · **Emiliano Mori**[1,2]

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Abstract

Island ecosystems offer unique opportunities for studying the evolution and ecology of species. Islands may be colonised by propagules whose ecological and evolutionary trajectories will differ from their mainland counterparts, potentially leading to speciation or to the selection and preservation of specific genetic and ecological traits. This study investigates the DNA-barcoding of grey long-eared bats *Plecotus austriacus* across insular and peninsular regions across its entire range, to unveil the species' colonization history. We d revealed distinct genetic lineages, since bats from the Iberian Peninsula, Italian Peninsula, Sicily, Elba Island, Great Britain, Sardinia, and Madeira, were all clearly distinguishable within the phylogenetic tree of *P. austriacus*. Central European samples clustered within a single haplotype. Phylogenetic analyses supported the recognition of a single taxonomic unit for *P. austriacus*. The evolutionary history of *P. austriacus* might have been shaped by past range contractions and expansions to and from glacial refugia, with the Iberian Peninsula likely representing the primary source for European populations and their genetic diversity. Island populations, such as those in Sardinia and Madeira, exhibit unique genetic lineages, most likely resulting from isolation after colonization and independent evolutionary trajectories. Elba Island and Great Britain showed unique haplotypes, yet similar to those from the mainland, suggesting a more recent colonization with respect to Madeira and Sardinia. Island populations, along with southern refugial areas, are particularly vulnerable to the impacts of global change, emphasizing the need for conservation strategies addressing the unique needs of these isolated populations.

Keywords Chiroptera · DNA barcoding · Glacial refugium · Island ecosystems · Plecotus austriacus

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Leonardo Ancillotto and Mariella Baratti contributed equally to this work.

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✉ Andrea Viviano
andreaviviano@cnr.it

[1] Consiglio Nazionale delle Ricerche, Istituto di Ricerca sugli Ecosistemi Terrestri, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy

[2] National Biodiversity Future Center, 90133 Palermo, Italy

[3] Department of Biology, University of Oxford, OX1 3SZ Oxford, UK

[4] Associação Portuguesa de Herpetologia, Porto, Portugal

[5] CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal

[6] BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal

[7] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2, 20216 Milano, Italy

[8] Centro Pipistrelli Sardegna, Via G. Leopardi 1, 07100 Sassari, Italy

[9] BiBio, Biodiversity and Bioindicators Research Group, Natural Sciences Museum of Granollers, Av. Francesc Macià 51, Granollers, Catalonia 08402, Spain

[10] Sistema Museale dell'Università di Firenze, Museo di Storia Naturale, Sede di Zoologia "La Specola", via Romana 17, 50125 Firenze, Italy

Introduction

Island biogeography is key to understanding biodiversity patterns and informing conservation efforts, as islands provide peculiar environments where unique evolutionary processes can occur, leading to the development and speciation of endemic taxa (MacArthur and Wilson 1967; Kallimanis et al. 2010; Gray and Cavers 2014). By studying the factors influencing species diversity on islands, e.g., island size, distance from the mainland, and habitat diversity, scientists gain valuable insights into ecological and evolutionary processes (Whittaker et al. 2017). Because island ecosystems are particularly vulnerable to environmental changes, invasive species and human impacts, understanding their dynamics has also become critical to conservation under accelerating global change (Mori et al. 2024a).

Species with limited dispersal abilities reaching isolated island systems are more likely to evolve into distinct taxa, whereas highly mobile species, larger-sized organisms, and recent human introductions are less likely to diverge significantly from mainland populations (Amori et al. 2008; Mori et al. 2024a). Geographic and demographic evolution is deeply shaped by historical events, ecological preferences and geographic barriers (Bernatchez and Wilson 1999; Querejeta and Castresana 2018). These geographic and ecological mechanisms, in combination with species-specific traits, can promote genetic isolation and drive diversification (Wang and Bradburd 2014). Thus, studying genetically isolated populations in the early stages of speciation provides a powerful framework for examining the mechanisms of diversification, and islands serve as ideal open-air laboratories for such investigations. Comparisons between island and mainland populations can reveal how isolation and demographic history shape phylogeographic patterns (Kalkvik et al. 2018). Because island populations often experience greater isolation and higher vulnerability to habitat disturbances, they frequently display distinct population structures compared to their mainland counterparts, including bats (Ortiz-Ramírez et al. 2018; Mori et al. 2024b; Fichera et al. 2025). Understanding these differences helps identify key processes influencing intraspecific diversification and supports conservation strategies aimed at preserving genetic diversity and evolutionary potential (Ancillotto et al. 2020).

Bats (Chiroptera) constitute a significant portion of mammalian biodiversity and are often the sole native wild mammalian presence on islands. Namely, over 60% of worldwide bat species inhabit both islands and mainland, whereas nearly 25% are endemic to islands, and 8% are restricted to single islands (Jones et al. 2009; Conenna et al. 2017). This proportion deviates markedly from that generally observed in mammals, where a low proportion of species is expected to reside on islands (38%) and to exhibit island endemism (19%) (Jones et al. 2009; Conenna et al. 2017).

While some species migrate among islands or between islands and mainland (e.g., Gili et al. 2025), many bat species are resident never leaving the island, and are more likely to undergo genetic divergence in insular environments (Conenna et al. 2017). Long-eared bats *Plecotus* spp. are known to be mostly sedentary species, with seasonal movements remarkably lower than 10 km, and daily foraging movements around less than 5 km (Horácek et al. 2004; Razgour et al. 2011; Ashrafi et al. 2013; López-Baucells et al. 2021; Ancillotto et al. 2022), besides including several island-endemic species (Mucedda et al. 2002; Pestano et al. 2003). Among widespread species, the grey long-eared bat *P. austriacus* is known to occur in several Mediterranean and Atlantic archipelagos (Razgour 2023). This near-threatened species (following the International Union for the Conservation of Nature red list) exhibits a wide latitudinal range (Fig. 1), stretching from the Mediterranean Basin, where it is considered relatively common, northward to Great Britain, Germany, and Poland, reaching its northernmost limits at latitudes 51°−53° North (Juste et al. 2004). Besides the European mainland, *P. austriacus* has successfully colonized several Mediterranean islands, including the Balearic Islands, Great Britain, Elba Island, Sicily, Sardinia, and Corsica, as well as the Atlantic Island of Madeira (Razgour 2023). Lanza (2012) re-interpreted the specimen recorded in Cape Verde (Maio Island) in the 1960 s (and never reconfirmed: Dorst and de Naurois 1966; Borloti et al. 2020), suggesting that it might belong to *P. gaisleri*.

Within the Iberian Peninsula (i.e., Spain and Portugal), *P. austriacus* is currently the most prevalent *Plecotus* species (De Paz 1984). In Britain, its distribution is restricted to coastal lowland areas, primarily constrained by harsh winter temperatures, conspicuous summer rainfall, and limited grassland availability, all factors that likely contribute to make the species locally very rare. Recent decades have likely witnessed a northward expansion of *P. austriacus* distribution, with individual records now extending up to the Baltic Sea coast (Razgour 2023). The easternmost boundary of its range lies in southwestern Ukraine and western Türkiye (Dietz and Kiefer 2016). Notably, the absence of *P. austriacus* from the Canary Islands can be attributed to the presence of the endemic *P. teneriffae* (Pestano et al. 2003), whereas other islands and archipelagos host different congeneric taxa: *P. gaisleri* occurs in Pantelleria, Lampedusa and Maltese Islands (Ancillotto et al. 2020)d *kolombatovici* in Crete, Aegean and Adriatic Islands, and Cyprus (Benda et al. 2007; Georgiakakis et al. 2023). These species closely resemble one another, exhibiting only subtle and overlapping morphological and morphometric traits, besides being all closely related to *P. austriacus* (Razgour 2023).

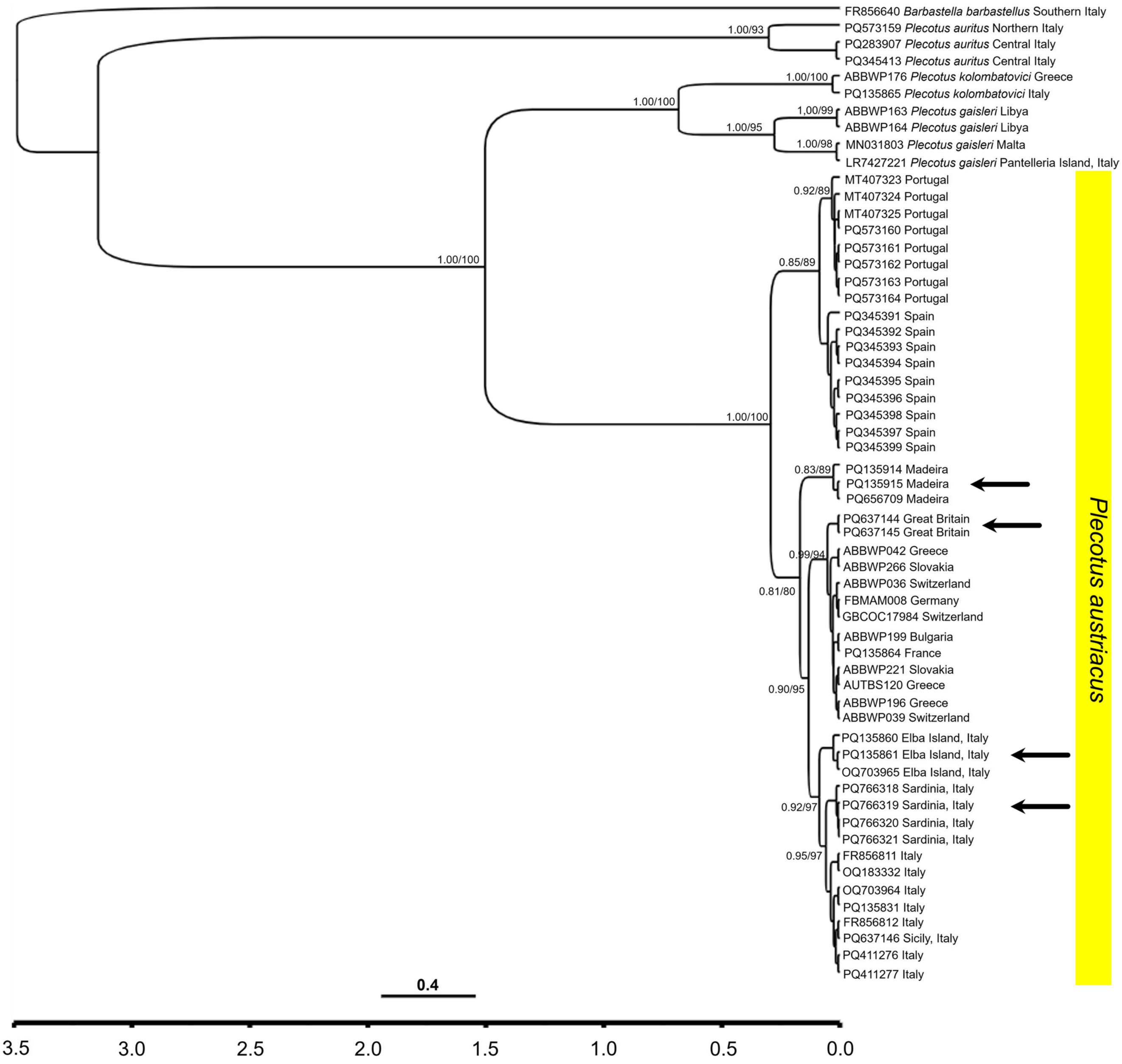

Fig. 1 Geographic distribution of the *P. austriacus* samples used in this study. Different colours refer to different haplotypes (central European haplotype is represented by orange, as Greece in Fig. 3). Main mountain chains are shown as black triangles. Shaded area is the known distribution of *P. austriacus* (Razgour 2023)

Consequently, their accurate identification requires molecular analyses, such as with DNA barcoding (Ancillotto et al. 2019, 2020; Mori et al. 2024b).

Despite its wide distribution across continental Europe, *P. austriacus* exhibits remarkably low levels of genetic variation outside the Iberian Peninsula, with a single mitochondrial haplotype (i.e., a set of alleles or genetic variants on a single chromosome that are inherited together, which is used in genetics to analyze inheritance patterns or population diversity) shared across central Europe (Juste et al. 2004; Razgour et al. 2013). This pattern suggests that, during past Ice Ages, the Iberian Peninsula and, possibly, the Italian Peninsula, may have served as glacial refugia for the species, as also supported by paleontological evidence (Sevilla and Chaline 2011; Galan et al. 2016; López-García et al. 2017). Central and Eastern continental Europe were likely colonized by this species during interglacial periods throughout the Late Pleistocene (Horácek 1975; Horácek et al. 2004), which could explain the low genetic diversity observed in this region (Razgour et al. 2013). Information on the timing of colonization of islands by *P. austriacus* is currently available only for the westernmost islands. Mallorca

(Balearic islands) was most likely colonized during the Late Pleistocene by individuals clustering with coastal Spanish ones (Razgour et al. 2013), whereas Madeira might have been colonized before, in the Middle Pleistocene, by individuals deriving from Central European propagules (Juste et al. 2004). Consistently, Rainho et al. (2000) also suggested an accidental human-mediated introduction from Central Europe to Madeira. Genetic data from other insular populations are still lacking but strongly required to exclude the occurrence of other cryptic species deserving targeted conservation actions (Ancillotto et al. 2020; Razgour 2023).

This study aimed to elucidate the phylogenetic relationships of *P. austriacus* among insular and peninsular populations. We conducted a DNA barcoding survey focused on confirming the taxonomic status of insular populations where *P. austriacus* have not previously been genetically characterized through DNA-barcoding such as Sardinia, Sicily, Elba Island, but also Great Britain and Madeira (where some genetic data on other genes are available: Razgour et al. 2013). We also compared genetic data and variability patterns from this species with other *Plecotus* species of the same phylogenetic group living on islands, i.e. *P. kolombatovici* (occurring in Croatian and Greek islands) and *P. gaisleri* (occurring in Malta, Lampedusa and Pantelleria). Specifically, we aimed to (1) describe the genetic diversity of DNA barcoding sequences across the species' distribution, (2) assess the robustness of the taxonomic cluster within the samples, and (3) describe and detect different haplotypes across the range.

Materials and methods

To obtain *P. austriacus* samples, we contacted universities, national museums of natural sciences, the CNR-IRET collection, wildlife recovery centers, and local researchers, requesting frozen specimens or tissue samples preserved in absolute ethanol. We were able to obtain 29 new samples of *P. austriacus* from various geographic origins (see Table 1; Fig. 1). All samples were collected between 2004 and 2024 and were preserved in absolute ethanol and are currently vouchered and stored at the CNR-IRET in Sesto Fiorentino and University of Milano-Bicocca in Italy.

We extracted total genomic DNA from 25 mg of patagium tissue using the Qiagen Blood and Tissue kit (©Qiagen, Inc, Tokyo, Japan). We amplified a portion (658 bp) of the COI through universal primers already used for *Plecotus* bats (Mori et al. 2024b): LCO 1490 (5'- GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3'). While other genes, such as cytochrome-b and the D-loop, have been extensively studied in *P. austriacus* (e.g., Juste et al. 2004; Razgour et al. 2013), the importance of considering additional genetic markers, such as COI (i.e., the marker commonly used for DNA-barcoding analysis), has been highlighted for inferring phylogenetic relationships of insular populations while ensuring a standardized identification approach, compliant with international standards (e.g., BIOSCAN, iBOL).

We conducted the PCR reactions on an Eppendorf MasterCycler X50 thermal cycler in 25 µL mix including 100 ng of each DNA sample, buffer 10×, 1.2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, and one unit of Taq polymerase (©Life Technologies, Waltham, Massachusetts, USA). PCR conditions were the following: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45″, annealing at 50 °C for 30″, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were run by electrophoresis on 1.5% agarose gels, containing 0.5 mg/mL of SYBR gel staining. Successful amplifications were purified through the ExoSAP-IT PCR clean-up Kit (©Applied Biosystems, Foster City, California, USA) and sequenced via the chain termination method at the BMR Genomics in Padua, Italy. Sequences were corrected by hand and aligned with the Mega XI software (Tamura et al. 2021).

Nucleotide diversity, haplotype diversity and number of polymorphic sites were computed through DNAsp vers. 5 (Librado and Rozas 2009). The software JModelTest version 2.0 (Darriba et al. 2012) was used to test the most accurate model of substitution using the Bayesian Information Criterion (BIC), corrected for the heterogeneity among sites.

Long-eared bat sequences obtained in this study were aligned with previously published COI sequences of the same species available on GenBank (http://www.ncbi.nlm.nih.gov) and BOLD (https://boldsystems.org: Table 1), and with other cryptic European *Plecotus* species (i.e., *P. auritus*, occurring in most of Continental Europe, Sicily and Sardinia, *P. gaisleri*, occurring in Malta, Pantelleria and Lampedusa, and *P. kolombatovici*, occurring in Eastern Mediterranean countries and Central Italy).

To assess the phylogenetic and diversification events, we carried out a phylogenetic reconstruction by Bayesian Analysis (BI) and Maximum Likelihood (ML).

To perform a time-calibrate Bayesian phylogeny for the COI mitochondrial dataset, we employed the software BEAST v.2.7.5 (Bouckaert et al. 2019), using *Barbastella barbastellus* (accession number: FR856640) as an outgroup. Because the COI mutation rate for bat species is unknown, we opted not to use a fixed substitution rate. Instead, the analysis was performed by setting up a minimum and maximum divergence times, respectively 2% and 5% per million years, derived from cyt-*b* rate estimates in bats (Nabholz et al. 2008), and as suggested by Clare et al. (2013). A strict molecular clock was applied, a TN93+F+I nucleotide

Table 1 Samples of *Plecotus* spp. Used in this study, corresponding geographic origin and COI accession numbers from NCBI and BOLD

Species	Origin	Reference	Accession number
Plecotus austriacus	Belvi, Sardinia, Italy	this work	PQ766318
Plecotus austriacus	Orgosolo, Sardinia, Italy	this work	PQ766319
Plecotus austriacus	Cumbida Prantas, Sardinia, Italy	this work	PQ766320
Plecotus austriacus	Badde Tureddu, Sardinia, Italy	this work	PQ766321
Plecotus austriacus	Messina, Sicily, Italy	this work	PQ637146
Plecotus austriacus	Elba Island, Italy	Mori et al. 2024b	OQ703965
Plecotus austriacus	Elba Island, Italy	this work	PQ135860
Plecotus austriacus	Elba Island, Italy	this work	PQ135861
Plecotus austriacus	Cassino, Southern Italy	this work	PQ411276
Plecotus austriacus	Avellino, Southern Italy	this work	PQ411277
Plecotus austriacus	Sila Massif, Southern Italy	this work	PQ135831
Plecotus austriacus	Tolfe, Siena, Central Italy	Mori et al. 2024b	OQ183332
Plecotus austriacus	Orgia, Siena, Central Italy	Mori et al. 2024b	OQ703964
Plecotus austriacus	Southern Italy	Galimberti et al. 2012	FR856811
Plecotus austriacus	Central Italy	Galimberti et al. 2012	FR856812
Plecotus austriacus	Northern Portugal	unpublished	MT407324
Plecotus austriacus	Northern Portugal	unpublished	MT407325
Plecotus austriacus	Northern Portugal	this work	PQ573160
Plecotus austriacus	Northern Portugal	this work	PQ573161
Plecotus austriacus	Northern Portugal	this work	PQ573162
Plecotus austriacus	Northern Portugal	this work	PQ573163
Plecotus austriacus	Northern Portugal	this work	PQ573164
Plecotus austriacus	Madeira Island	this work	PQ135914
Plecotus austriacus	Madeira Island	this work	PQ135915
Plecotus austriacus	Madeira Island	this work	PQ656709
Plecotus austriacus	North Eastern Spain	this work	PQ345391
Plecotus austriacus	North Eastern Spain	this work	PQ345392
Plecotus austriacus	North Eastern Spain	this work	PQ345393
Plecotus austriacus	North Eastern Spain	this work	PQ345394
Plecotus austriacus	North Eastern Spain	this work	PQ345395
Plecotus austriacus	North Eastern Spain	this work	PQ345396
Plecotus austriacus	North Eastern Spain	this work	PQ345397
Plecotus austriacus	North Eastern Spain	this work	PQ345398
Plecotus austriacus	North Eastern Spain	this work	PQ345399
Plecotus austriacus	Switzerland	unpublished	ABBWP036
Plecotus austriacus	Switzerland	unpublished	ABBWP039
Plecotus austriacus	Switzerland	unpublished	GBCOC17984
Plecotus austriacus	Greece	unpublished	AUTBS120
Plecotus austriacus	Greece	unpublished	ABBWP042
Plecotus austriacus	Greece	unpublished	ABBWP196
Plecotus austriacus	Bulgaria	unpublished	ABBWP199
Plecotus austriacus	Slovakia	unpublished	ABBWP221
Plecotus austriacus	Slovakia	unpublished	ABBWP266
Plecotus austriacus	Germany	unpublished	FBMAM008
Plecotus austriacus	Southern France	unpublished	PQ135864
Plecotus austriacus	Great Britain	this work	PQ637144
Plecotus austriacus	Great Britain	this work	PQ637145
Plecotus auritus	Oasi San Michele (Venezia), Northern Italy (continental)	this work	PQ573159
Plecotus auritus	Oasi Dynamo (Pistoia), Central Italy (continental)	this work	PQ283907
Plecotus auritus	Gubbio (Perugia), Central Italy (continental)	this work	PQ345413
Plecotus gaisleri	Pantelleria, Italy	Ancillotto et al. 2020	LR742722
Plecotus gaisleri	Benghazi, Libya	unpublished	ABBWP163

Table 1 (continued)

Species	Origin	Reference	Accession number
Plecotus gaisleri	Benghazi, Libya	unpublished	ABBWP164
Plecotus gaisleri	Malta	Mifsud and Vella 2019	MN031803
Plecotus kolombatovici	Follonica, Central Italy	Ancillotto et al. 2019	PQ135865
Plecotus kolombatovici	Greece	unpublished	ABBWP176

model, and a Coalescent Constant population prior was selected for the trees, with a random starting one. The clock prior was set to a log-normal distribution (mean = 0.035 and standard deviation = 0.2). Two individual runs of 10×10^6 generations were performed with a sampling frequency of 1,000. Convergence for all model parameters was assessed by examining trace plots and histograms in Tracer v.1.7.1 (Rambaut et al. 2018) after obtaining an effective sample size (ESS) > 200. Runs were combined using LogCombiner (discarding 10% of the initial runs), and maximum credibility trees with divergence time means and 95% highest probability densities (HPDs) were produced using Tree Annotator (both part of the BEAST package). Trees were visualized using FigTree v.1.4.4 (Rambaut 2018).

The ML analysis was performed using PhyML version 3.1.5 (Guindon et al. 2010) with 1,000 bootstrap replicates under the SeaView version 5 multiplatform for molecular phylogeny (Gouy et al. 2020). We selected optimized choices, and we obtained the tree-searching operations by Nearest-Neighbour Interchange (NNI) and Subtree Pruning–Regrafting (SPR). Trees were visualized and edited using the software FigTree version 1.4 (https://github.com/rambaut/figtree/releases/tag/v1.4.4: Edinburgh, UK).

Then, to describe haplotypes across the sampled regions, TCS network (Clement et al. 2000) connecting haplotypes was obtained with the software "Hapsolutely", to visualise the relationship between the new and the previously described haplotypes of *P. austriacus* (Vences et al. 2021, 2024).

And finally, to infer *P. austriacus* species delimitation criteria based on a partial COI gene, molecular operational taxonomic unit (MOTU) estimations were performed by employing the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), ran on the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html: accessed on 22.12.2024). The ABDG separates the species based on a range of maximum intraspecific distance. Parameters were steps = 10, X (relative gap width) = 1.5, number of bins = 20.

Results

We successfully amplified the COI fragment for the 29 new samples of *P. austriacus* (Table 1). Adding the already available sequences, we aligned a total of 48 *P. austriacus* COI sequences. The obtained amplicon had a size of 543 bp, and 15 polymorphic sites. The nucleotide diversity (π) of pooled data from all sampled populations was 0.011, and the haplotype diversity (h) was 0.858, whereas we recorded single haplotypes in single islands.

The obtained Bayesian topology showed fairly robust support for all nodes (Fig. 2), and the observed phylogenetic relationships match with the ones from the ML analysis (which also included *Tadarida teniotis* as an outgtroup [Accession Number: FR856844] Supplementary Material). The first divergence event within *P. austriacus* occurred during the Middle Pleistocene (ca. 300 kyr), when the Iberian Peninsula population became separated from the remaining European populations. A subsequent divergence took place around 170 kyr, when the Madeira Island population split from the mainland European lineage. Currently, Elba Island, Sardinia, continental Italy plus Sicily, and eastern Europe plus Great Britain represent monophyletic lineages, sharing a common ancestor which started to diverge 132 Kyr.

Similar results are observed in the haplotype network, with all Central and Eastern European samples clustering within a single haplotype (Fig. 3). In contrast, the Iberian Peninsula exhibited two distinct haplotypes, one associated with Mediterranean Spain and the other with Portugal. Sicily and peninsular Italy shared a common haplotype, while unique haplotypes were observed on Elba Island, in Great Britain, Sardinia, and Madeira.

Also results obtained by ABGD suggested the occurrence of a single taxonomic unit within *P. austriacus* sequences, given their limited Kimura 2-parameter (K2P) distance (Kimura 1980; Figure S2 in Supplementary Material 1). The same K2P distances between haplogroups of *P. austriacus* were all lower than the Optimum Threshold of divergence between species (Galimberti et al. 2012; Figure S2 in Supplementary Material 1).

Discussion

The evolutionary trajectory of *P. austriacus* has been profoundly shaped by historical climatic fluctuations during the Pleistocene and by vicariant events associated with major geographical barriers such as the European mountain ranges (e.g., Alps, Balkans, and Pyrenees; Razgour et al. 2013).

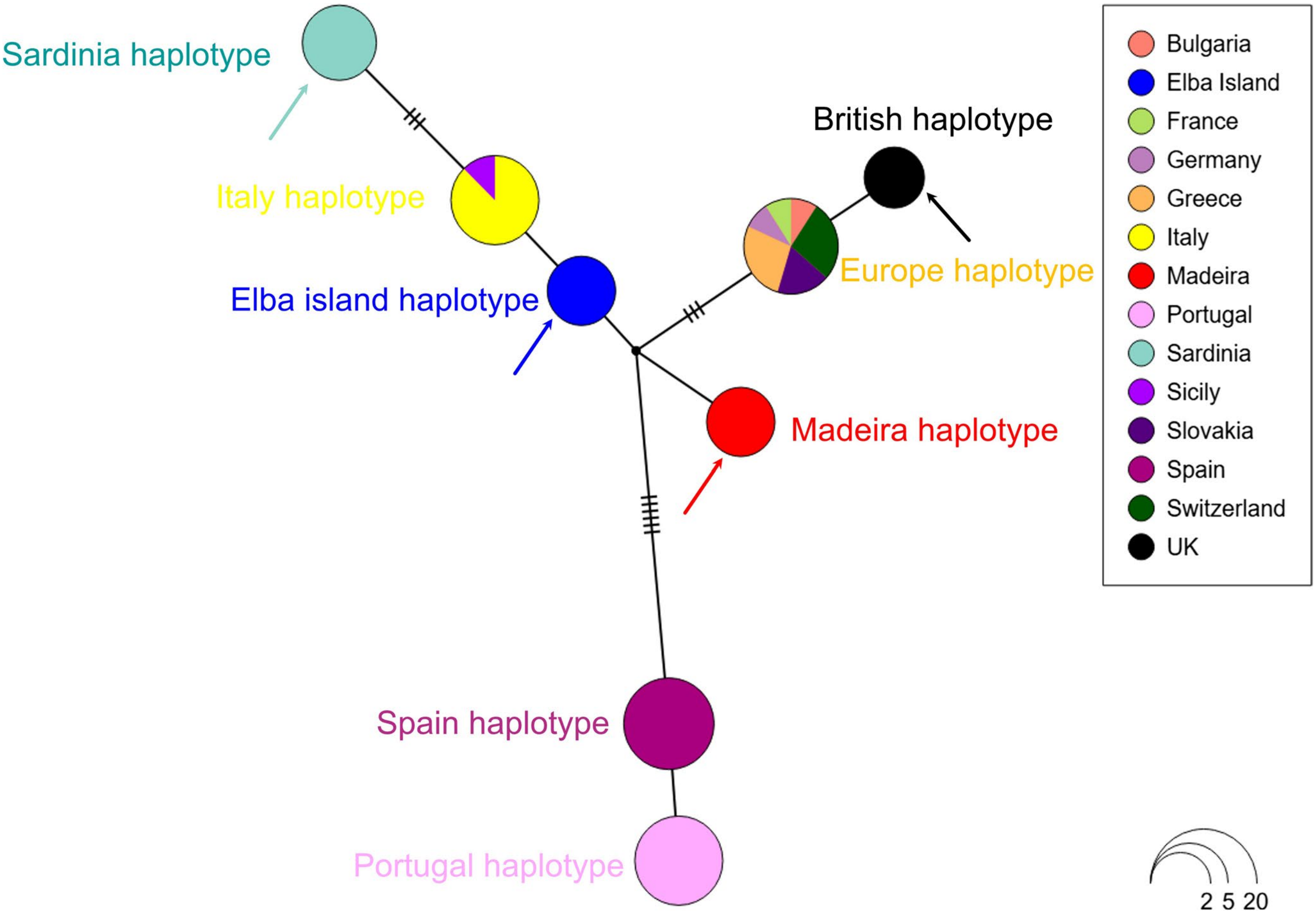

Fig. 2 Bayesian time-calibrated phylogenetic tree of *Plecotus* sp. COI sequences. Numbers above the nodes represent Bayesian Posterior Probabilities (BPP) and Maximum Likelihood (ML) bootstrap values, respectively. DNA analysis supports the monophyly of *P. austriacus*. Below the tree, is represented the time axis in million years before present. Arrows indicate haplotypes unique to islands

These processes, together with the species wing morphology that restricts long-distance flight (Norberg and Rayner 1987), its preference for lowland habitats, and its broad distribution range (Pavlinić and Tvrtković 2004; Starik et al. 2021), make *P. austriacus* an ideal model for investigating evolutionary patterns across the European continent and its islands.

Our analysis suggests a clear geographical differentiation among genetic clades across the species' range, and a low number of mitochondrial haplotypes (Razgour et al. 2013), although more interspersed samples would be needed to determine actual nucleotide and haplotype diversity for single locations. Nevertheless, genetic diversity among island bat populations tends to be low, even in studies which analyzed larger sample sizes (e.g., a single mtDNA haplotype for *Rhinolophus hipposideros* in Pantelleria: Cistrone et al. 2025; very low diversity for island populations of *Carollia perspicillata* in South America: Meyer et al. 2009; see Jones et al. 2009 for a review). This pattern likely reflects founder effects and small effective population sizes, both of which constrain genetic variation. Furthermore, the COI gene is reliable for determining sample origin and detecting geographic structuring, whereas being less suitable for assessing intrapopulation genetic diversity. Namely, all islands in our dataset (but Sicily) represented monophyletic clades and featured unique COI haplotypes, as well as glacial refugia. Surprisingly, the Madeira population did not seem to be the sister clade of the geographically closest Portuguese mainland, but instead the populations from Central Europe, although further samples from the Iberian Peninsula are needed to ascertain this pattern. Further analyses, e.g. using microsatellite markers or DIYABC scenarios are needed to correctly assess the origin of this island population.

As for many vertebrate taxa across Europe, we also found evidence of southern glacial refugia for *P. austriacus*, whose populations likely retreated in the Iberian Peninsula during the glacial periods (Juste et al. 2004; Razgour 2023). The obtained phylogenetic topology suggests that the diversification of the *P. austriacus* bats started during the middle Pleistocene. Considering the existence of fossil records for this bat species during the middle Pleistocene in Spain (Juste et al. 2004), our results may provide further evidence that

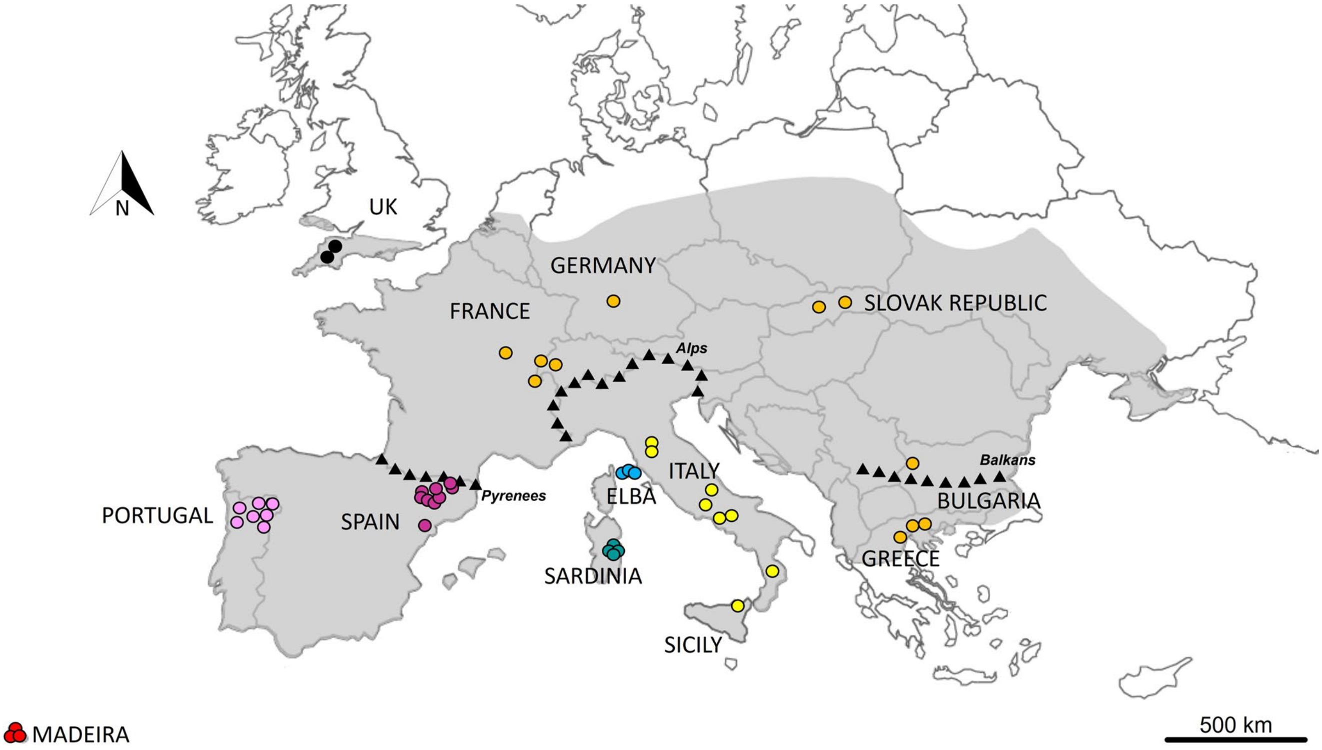

Fig. 3 TCS network showing the relationship among new and previously described haplotypes of *P. austriacus*. Circles represent different haplotypes. Circle sizes are proportional to the number of specimens analysed for each haplotype, whereas bars indicate mutational steps. Coloured arrows indicate haplotypes unique to islands

the Iberian Peninsula could have been a glacial refugium, but also the cradle of diversification of *P. austriacus*. Conversely, and differently from other taxa - including many Palearctic bats (Rebelo et al. 2012; Budinski et al. 2019; Moulistanos et al. 2023) - we found no evidence that either Italy or the Balkans might have been glacial refugia for *P. austriacus*. These results are mainly due to the observed recent diversification and lack of phylogenetic monophyly of both Italian and Balkan populations. Moreover, our time estimates suggest that the diversification of these populations (132 Kya) result from an amelioration of the climate during the Last Interglacial (~130 Kyr). Our phylogenetic analyses confirmed that Greek populations of *P. austriacus* cluster with those from central Europe. This finding might be attributed to the presence of the cryptic sibling species *P. kolombatovici* within the region (Juste et al. 2004; Razgour et al. 2013), which may have elicited past competitive exclusion at small geographical scales. Although featuring a single and unique haplotype throughout the Italian Peninsula, such Italian haplotype is shared by an individual from Sicily (Bogdanowicz et al. 2015). Considering the closeness of Sicily to mainland Italy (3.14 km), it is likely that even grey long-eared bats may easily cross the sea between the two areas. Further samples from this island are needed, particularly for its southern part, to determine the occurrence of other haplotypes and/or species, such as *P. gaisleri*, for which the habitat suitability model predicted potential occurrence in Sicily (Ancillotto et al. 2020).

A more recent colonization of Italy by *P. austriacus* with respect to the Iberian Peninsula is also supported by paleontological data (*Plecotus* fossil records from the start of Holocene: Salari et al. 2019). Samples from Elba island (approximately 9 km from the closest mainland) were included in the same haplotype, which was similar to the haplotype from mainland Italy. Similarly, British samples (approximately 30 km from mainland) were grouped in a unique haplotype similar to the haplotype of the rest of Europe, suggesting a recent colonization (i.e., possibly in Mid-Holocene: Spitzenberger et al. 2006) or a moderate animal movement between islands and mainland. The divergence of the Sardinian and Madeiran haplotypes can be attributed primarily to the prolonged isolation of these islands, which allowed for genetic differentiation. Based on the timing of the colonization of Europe from Iberia during the middle Pleistocene (~300 Kyr) and the relative ages of the nodes in the phylogenetic tree, bats likely arrived first to Madeira (170 Kyr) and subsequently to Sardinia (59 Kyr) (cf. Mucedda et al. 2002). This pattern is consistent with the geographic distances between islands and the mainland (approximately 200 km for Sardinia and 860 km for Madeira - which is 520 km far from North Africa), which further contributed to their genetic isolation. We also obtained a genetic sample from Capraia Island (Tuscan

Archipelago, central Italy), but due to the low-quality DNA typically retrieved from bat guano, this resulted in only a very short DNA minibarcoding sequence (Gili et al. 2025), which was insufficient for inclusion in the phylogenetic tree. Nevertheless, this fragment indicates the presence of the same Sardinian haplotype on Capraia (Accession number: PV744372), suggesting that further investigation would be of considerable interest. The observed phylogenetic position of Madeira Island (where the species is particularly rare: Ferreira et al. 2022; Gonçalves et al. 2024) may rule out the hypothesis from Rainho et al. (2000) that the origin of this population was human-mediated, although further samples from the Iberian Peninsula are needed. *Plecotus austriacus* may have colonised Madeira islands from both continental Europe and North Africa (where the species is not known to currently occur), by using other small islands (including those currently submerged, and others where the species does not occur anymore) as stepping stones (Triantis et al. 2016; Nóbrega et al. 2023). Future research should address current sampling limitations by expanding geographic and population coverage (for instance, from all unsampled islands, e.g., Corsica and Balearic islands). Moreover, integrating genomic approaches, such as reference genome assembly and whole-genome resequencing, would enable higher-resolution insights into demographic history and adaptive variation. Additionally, combining genetic data with improved environmental niche modeling could provide a more comprehensive understanding of phylogeographic patterns and their ecological drivers (Razgour et al. 2013). Previous work on *P. austriacus* (Razgour et al. 2013) predicted a northward range expansion (where the low genetic variability may also be linked to a local bottleneck event, once outside Iberian Peninsula), and a southern contraction for this species (where most unique haplotypes occur), driven by increasing temperatures and aridity in southern Europe, a pattern which may pose at risk the southern refugial and island populations.

Our results highlight the crucial role of islands in preserving unique haplotypes and maintaining a significant portion of the intraspecific diversity of *P. austriacus*. However, island bat populations face severe threats from invasive species, such as free-roaming feral cats (Soto et al. 2023), which prey on bats in exposed roosting sites (Rocha 2015; Woinarski 2018; Mori et al. 2019), as well as from land-use changes and wildfires that destroy habitats and roosting areas (Ancillotto et al. 2021; Bosso et al. 2018; Ferreira et al. 2022). These challenges suggest the urgent need for conservation actions to mitigate the impacts of invasive species and protect fragile island ecosystems from destructive events such as fires (Jones et al. 2009). Additionally, the potential loss of genetic diversity, especially in source populations that historically served as glacial refugia (Juste et al. 2004), remains a major concern. The Iberian peninsula stands out as a key evolutionary hotspot for *P. austriacus*, serving as the main Pleistocene refugium and source for current European populations (Juste et al. 2004; Razgour et al. 2013), with fossil and genetic evidence supporting its status as a long-term “stable rear-edge” population (Hewitt 2000; Juste et al. 2004; Hampe and Petit 2005; Razgour et al. 2013; De Paz 1984; Razgour 2023). Predictive models indicate a northward range expansion and southern contraction of *P. austriacus* due to rising temperatures and aridity (Razgour et al. 2013), threatening southern refugial and island populations where most unique haplotypes occur. Given the species limited dispersal ability (Norberg and Rayner 1987; Safi and Kerth 2004), its near-threatened IUCN status, and low northern genetic diversity (Razgour et al. 2011; Festa et al. 2023), conservation strategies must prioritise the protection of *refugia*, island and leading-edge populations, which are vital for the long-term survival and adaptive potential under ongoing habitat loss and climate change (Razgour 2015; Razgour et al. 2021; Ancillotto et al. 2024).

The observed patterns of low genetic diversity across insular populations highlight the importance of maintaining genetic connectivity among islands to prevent further genetic erosion (Jones et al. 2009). Conservation strategies should prioritise protecting existing dispersal corridors and minimizing habitat fragmentation, particularly in archipelagos where gene flow may already be constrained (Gaines et al. 1997). Given projected climatic shifts in the Mediterranean region (Lionello et al. 2014), identifying and preserving potential climate refugia is crucial to allow a long-term population persistence. Integrating phylogeographic data with spatial and environmental modeling could help identify refugial areas and address management actions to preserve both evolutionary potential and ecosystem resilience.

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Data availability All genetic sequences are deposited on GenBank and all samples are available for further analyses.

Declarations

Ethical approval Authors certify that no living animal was used for this research.

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Competing interests The authors declare no competing interests.

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