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Unravelling the evolutionary history and future prospects of endemic species restricted to former glacial refugia

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Abstract

The contemporary distribution and genetic composition of biodiversity bear a signature of species' evolutionary histories and the effects of past climatic oscillations. For many European species, the Mediterranean peninsulas of Iberia, Italy and the Balkans acted as glacial refugia and the source of range re-colonisation, and as a result they contain disproportionately high levels of diversity. As these areas are particularly threatened by future climate change, it is important to understand how past climatic changes affected their biodiversity. We use an integrated approach, combining markers with different evolutionary rates, and combining phylogenetic analysis with Approximate Bayesian Computation and species distribution modelling across temporal scales. We relate phylogeographic processes to patterns of genetic variation in *Myotis escalerai*, a bat species endemic to the Iberian Peninsula. We found a distinct population structure at the mitochondrial level with a strong geographic signature, indicating lineage divergence into separate glacial refugia within the Iberian refugium. However, microsatellite markers suggest higher levels of gene flow resulting in more limited structure at recent time frames. The evolutionary history of *M. escalerai* was shaped by the effects of climatic oscillations and changes in forest cover and composition, while its future is threatened by climatically-induced range contractions and the role of ecological barriers due to competition interactions in restricting its distribution. This study warns that Mediterranean peninsulas, which provided refuge for European biodiversity during past glaciation events, may become a trap for limited dispersal and ecologically-limited endemic species under future climate change, resulting in loss of entire lineages.

Introduction

The contemporary distribution and genetic composition of biodiversity bear a signature of species' evolutionary histories. Quaternary climatic oscillations, in the form of recurring glacial-interglacial cycles, resulted in substantial range shifts, population extinctions and lineage divergences (Hewitt 2000), though effects varied with latitude, topography (Hewitt 2004) and individual species' adaptations and environmental tolerances (Stewart *et al*. 2010).

With the advent of molecular tools, the study of the distribution of biodiversity was extended to include genetic relationships between individuals and the influence of historical processes on the geographic distribution of genetic lineages (Avise 2000). Phylogeography has provided the framework to determine the causal links between geography, climate change, ecological interactions and the evolution of taxa (Hickerson *et al*. 2010). Its integration with ecological niche modelling has helped elucidate the processes and mechanisms shaping genetic variation and the evolutionary trajectories of species and populations (Alvarado-Serrano & Knowles 2014). Understanding the phylogeographic structure of species, and the mechanisms that sustain it, is integral to conserving their full genetic diversity and to managing evolutionary significant units within species according to their differing regional vulnerabilities (Schmitt 2007). Moreover, understanding species' responses to past events may help us better predict the potential consequences of future climatic changes (Hofreiter & Stewart 2009).

During Pleistocene glacial periods, much of northern and central Europe was covered by ice sheets and permafrost. The Mediterranean peninsulas of Iberia, Italy and the Balkans acted as glacial refugia for many European species and as the source of rapid northern range colonisation during interglacial, warmer climatic periods. Cycles of contraction-expansion into and out of glacial refugia resulted in a genetic signature of southern richness with deep divergence between refugial populations versus northern impoverishment and genetic homogeneity (Hewitt 2004). Stable areas that persisted across glaciation cycles harbour particularly high levels of species richness (Araújo *et al*. 2008) and unique genetic diversity (Hampe & Petit 2005), and as a result are of high evolutionary importance (Stewart *et al*. 2010). However these hotspots of genetic diversity are particularly threatened by future climate change (EEA 2012; Razgour *et al*. 2013), and therefore it is important to understand how past climatic changes affected their biodiversity.

The Iberian Peninsula has a rich and well-studied biogeographic history. Its complex topography and geographic position between the Mediterranean and North Atlantic create distinct bioclimatic regions with ecologically and genetically divergent taxa (Gomez & Lunt 2007). Yet this great environmental heterogeneity, combined with relative climatic stability and long-term lineage persistence and divergence without large geographic displacement, makes it more difficult to interpret the genetic population structure and evolutionary history of species within Iberia (Hewitt 2001; Rodriguez-Sanchez *et al*. 2010). The Iberian Peninsula played an important role in the evolutionary history of European bats. Phylogeographic studies of widely-distributed European bat species show that although Iberia was an important glacial refugium for many species, in some cases it did not necessarily contribute to post-glacial range re-colonisation because lineages remained isolated inside the peninsula by the Pyrenees mountain range (e.g. *Barbastella barbastellus,* Rebelo *et al*. 2012; and *Rhinolophus hipposideros,* Dool *et al*. 2013). However for other bat species, the Iberian refugium was the main source of range re-colonisation, while the Alps formed a stronger barrier to range expansion from other Mediterranean refugia (e.g. *Myotis myotis,* Ruedi & Castella 2003).

Here we set to unravel the effect of Quaternary climatic oscillations on the evolutionary history of *Myotis escalerai*, a bat species endemic to the Iberian Peninsula (defined here as the area including Spain, Portugal, the Balearic Islands, Andorra and the French Pyrenees), and to determine factors that limit its distribution and how it will be affected by future climate change. *M. escalerai* is part of the *Myotis nattereri* cryptic species complex (*M. nattereri sensu stricto*, *M. escalerai*, *M. spA*, and *M. spB*; Salicini *et al*. 2011) that has only recently been genetically confirmed as a separated species (Ibáñez *et al*. 2006), but has been described morphologically more than a century ago (Cabrera 1904). Unlike other bat species, the entire evolutionary history of *M. escalerai* took place within Iberia (Salicini *et al*. 2013), and therefore both its present genetic population structure and future survival are closely linked to climate change processes within the Iberian Peninsula. We use an integrated approach, combining markers with different evolutionary rates, and combining phylogenetic analysis with Approximate Bayesian Computation (ABC) model-based inference and species distribution modelling across temporal scales, to relate phylogeographic processes to contemporary and future patterns of genetic variation.

Methods

Sample collection

Genetic samples, in the form of 3mm wing biopsies were collected from 252 *M. escalerai* bats captured in 16 colonies, located mostly in underground sites (caves), distributed across the Iberian Peninsula and the Balearic island of Mallorca (Table S1, Figure 1a). Sequences of two individuals from the Sevilla colony were previously used in Salicini et al. (2011, 2013) (GenBank accessions: JN591489.1 and JX826314.1). In addition to the samples from the 16 colonies, the mitochondrial DNA analysis also included *M. escalerai* sequences downloaded from GenBank that belonged to samples from the French Pyrenees (JF412390 and JF412391 – Puechmaille *et al*. 2012) and 108 *M. escalerai* sequences from across the Iberian Peninsula, including 21 samples from around the Pyrenees (Navarra, Huesca, Lleida and Girona) and 18 samples from adjacent areas (La Rioja, Zaragoza, Teruel and Tarragona). Out of the individual sequences, 24 were taken from previous studies (Salicini *et al*. 2011, 2013). These samples were added to better characterise the range of the species and to provide better coverage of areas of sympatry with *M. spA* (Table S2; Figure 1a).

Laboratory procedures

Genomic DNA was extracted from all samples following the methods described in Salicini *et al*. (2013).We sequenced 750 bp of the mitochondrial DNA (mtDNA) *Cytochrome b* (*Cyt b*) gene, using the primers Molcit-F (Ibáñez *et al*. 2006) and Molcit-R (Salicini *et al*. 2011). PCR conditions and sequencing information are outlined in Salicini *et al*. (2013). Sequences were aligned and edited using Sequencher 4.5 (Gene Codes Corp, MI, USA), and collapsed into unique haplotypes with Dambe v5.2.31 (Xia & Xie 2001).

Samples were genotyped for 10 microsatellite loci previously published for the genus (A13, D9, D15, E24, F19, G25, H29, A24, H23: Castella & Ruedi 2000; and b22: Kerth *et al*. 2002). The forward primer of each locus pair was labelled fluorescently with HEX or 6-FAM (Applied Biosystems). Microsatellites were combined into single or double PCR sets. Each PCR mix contained 0.3µl primer sets at 10µM, 1µl of PCR Buffer 10X, 0.3µl dNTPs, 0.05µl TAQ and 1µl of DNA, adding H_2O up to 10µl total volume. When needed, 0.8 µl of Bovine Serum Albumin was added. PCR amplification was performed using ABI Veriti thermal cycler (Applied Biosystems, USA). We used the following PCR program: initial denaturation at 95°C for 5min, followed by 30-40 cycles of 95°C for 30s, annealing temperature from 55ºC to 60ºC, depending on the primers, for 45s and 72°C for 45s, followed by a final extension at 72°C for 10 min. PCR products were sequenced using ABI 3130 48-well DNA Sequencer. Allele sizes were assigned using the GeneMapper software (Applied Biosystems, USA).

Observed and expected heterozygosity and estimated null allele frequencies were calculated using CERVUS v3.0.3 (Kalinowski *et al.* 2007) and Micro-Checker (Van Oosterhout *et al.* 2004). Tests for departures from Hardy-Weinberg equilibrium and assessment of linkage disequilibrium were performed in GENEPOP v4.0.10 (Raymond & Rousset 1995; Rousset 2008). Loci that were out of Hardy-Weinberg equilibrium and with a high frequency of null alleles in several populations were removed from the analysis.

Genetic data analysis

Mitochondrial dataset

We used jModelTest v2.1.6 (Darriba *et al.* 2012) to select the Hasegawa-Kishino-Yano (HKY) mtDNA substitution model with gamma-distributed rate variation based on the Bayesian Information Criterion (BIC) values. Bayesian phylogenetic trees were constructed in MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003), using two *Myotis spA* sequences as outgroup to root the tree. We ran 5×10^7 generations with four chains, sampled every 500th generation, and two simultaneous runs, discarding the first 25% of trees generated as burn-in. Trees and posterior probabilities were visualised with Figtree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Parsimony haplotype network was constructed with NETWORK (v4.610, Fluxus Technology), employing the median-joining network algorithm and the Greedy FHP distance calculation method. Nucleotide polymorphism, haplotype diversity, genetic divergence and differentiation between populations were calculated in DnaSP v5.10 (Librado & Rozas 2009), with 10000 permutations to obtain probability values.

Microsatellite dataset

Analysis of microsatellite genetic diversity, including allele frequencies, number of private alleles, allelic richness, heterozygosity, gene diversity and population differentiation (Fst), was carried out at the colony level with GenAlEx v6.4 (Peakall & Smouse 2006) and Fstat v2.9.3.2 (Goudet 1995) controlling for differences in sample sizes. To test for levels of relatedness among individuals, we used the Triadic Maximum Likelihood estimator (TrioML; Wang 2007) implemented in Coancestry (Wang 2011) because this measure allows for inbreeding and accounts for genotyping errors in the data.

Population structure in the microsatellite dataset was inferred using individual-based Bayesian assignment tests implemented in STRUCTURE v2.3.3 (Pritchard *et al.* 2000). Number of tested genetic clusters (K) ranged from 1 to 15. We performed ten independent runs for each K, using the general admixture model with correlated allele frequencies and 10^6 Markov Chain Monte Carlo (MCMC) generations following a burn-in phase of 5×10^5 generations. The number of distinct clusters was determined using STRUCTURE HARVESTER (Earl & Von Holdt 2012) based on the number of clusters at which the mean log-likelihood peaked and where variation among runs was minimal. Cluster assignment was visualised with DISTRUCT (Rosenberg 2004).

Because the presence of closely related individuals (in particular full siblings) can bias the number of clusters identified in the STRUCTURE analysis (Rodriguez-Ramilo & Wang 2012), we first ran assignment tests with the whole dataset and then re-ran the analysis removing individuals with TrioML values >0.5. This threshold was selected because below this value most of the pairwise estimations were among individuals from geographically distant colonies (58% for TrioML =0.5, versus 2% for TrioML >0.5).

Species Distribution Modelling Procedures

We used Species Distribution Models (SDMs) to generate phylogeographic hypotheses for testing with ABC inference, to identify environmentally stable areas where the species persisted overtime, to determine the most important environmental variables limiting the distribution of *M. escalerai* and to predict future changes to distribution (Alvarado-Serrano & Knowles 2014). We predicted the potential distribution of suitable conditions for *M. escalerai* under present, past (LGM ~21,000 years before present, and the Last Interglacial period [LIG] ~130,000 ybp) and future (2070) climatic conditions. Study area extent was set as the Iberian Peninsula, the Balearic Islands and France up to latitude 49.5 N and longitude 6.5 E. This extent enabled the inclusion of potentially suitable areas beyond the species' currently know distribution, while limiting problems associated with selecting pseudo-absences at large distances from known location records (VanDerWal *et al*. 2009). Model resolution was ~1km (30 arc seconds).

Models were generated with MaxEnt v3.3.3 (Phillips *et al*. 2006) using 135 location records, including one location record from the French Pyrenees taken from Evin *et al*. (2009). As the whole of Iberia has been sampled extensively for this species, our dataset is not likely to suffer from sampling bias. All location records were genetically confirmed because of potential range overlap with cryptic congeners of the *M. nattereri* species complex. We used the Average Nearest Neighbor tool in ArcGIS v10.2 (ESRI) to remove duplicate and clustered location records in order to minimise spatial autocorrelation between location records.

We ran two types of models, climatic-topographic models (climate model) for all time periods, and a full model that included also habitat variables for the present only. Climatic and topographic layers were downloaded from WorldClim (http://www.worldclim.org), geological layers from One Geology (http://www.onegeology.org/, reclassified into 18 broad categories) or USF Geoportal Data Depository (Karst Regions of the World, http://gisdata.rc.usf.edu/, Hollingsworth *et al*. 2008). Habitat variables were obtained from the European Space Agency (GlobCover 2009, http://due.esrin.esa.int/page_globcover.php) for land cover (reclassified into 10 categories), European Environment Agency (Corine Land Cover 2006, http://www.eea.europa.eu/) for woodland variables (woodland type and distance to woodlands), and Hansen et al. (2013) for percent tree canopy cover. Multicollinearity among environmental variables was tested with ENMtools v1.3 (Warren *et al*. 2010), removing highly correlated variables (correlation coefficients ≥0.8) and variables that did not contribute to the SDMs. The following layers were included in the final models: maximum temperature of warmest month (BIO5), minimum temperature of the coldest month (BIO6), average temperature of the driest quarter (BIO9), temperature seasonality (BIO4), annual rainfall (BIO12), rainfall seasonality (BIO15), rainfall in warmest quarter (BIO18), slope, altitude, distance to karsts (maternity colonies are known to form in caves; Ibáñez *et al*. 2006), land cover, distance to woodlands and percent tree cover.

Models were projected into the past using the CCSM and MIRCO General Circulation Models (GCMs) for the LGM and one LIG model. Future models for 2070 were generated using three European GCMs (HadGEM2_ES, IPSL-CM5A-LR and MPI-ESM-LR), and the IPCC5 +8.5 W/m^2 Representative Concentration Pathways (IPCC 2013), representing the 'worst case' scenario.

Our modelling procedures followed recommendations in Merow *et al*. (2013). We compared several models with different variables and parameter combinations (regularization values, number of features included) in ENMTools, and selected the best models based on Akaike Information Criterion (AIC) scores. The final full and climate models included all features with a regularization value of 1, 10,000 background points and 1500 iterations. When comparing models we used the raw output, but when running the final models we used the cumulative output. Projected output maps generated by the different LGM or future GCMs were multiplied to produce a single map per time period. In order to determine differences in the extent of areas with high relative occurrence probability over time we converted model outputs into binary maps using the thresholding method that maximises the sum of sensitivity and specificity (Liu *et al*. 2013). Range changes were calculated for the Iberian Peninsula alone (including the Pyrenees). Maps were processed in ArcGIS v10.2 (ESRI).

Model predictive ability was tested with five-fold cross validation and compared based on the Area Under the Curve (AUC) of the Receiver Operator Characteristics. To determine whether our models performed significantly better than random, we tested if our models' training and test AUC scores fell outside the 95% Confidence Intervals of the distribution of the AUC scores of 100 null models (Raes & ter Steege 2007), randomly generated in ENMTools with the altitude layer.

ABC Framework

The evolutionary history of *M. escalerai* was reconstructed using the ABC approach implemented in DIYABC v2.0.4 (Cornuet *et al*. 2014) to identify source populations and patterns of colonisation. Phylogeographic hypotheses were generated based on paleo-SDM predictions. We first ran a full analysis (Analysis 1), which included all colonies, divided into three geographical groups (Western, Southern and North-Central-Eastern). The full analysis aimed to identify the source population, LGM refugial populations and patterns of post-LGM range recolonisation. Next we ran separate ABC analyses for the geographically separated Western (Analysis 2) and Eastern (Analysis 3) groups to identify the representative putative source colonies of each group in relation to predicted climatic suitability during the LGM. Finally, in Analysis 4, we assessed the demographic history of the Western and Eastern groups, comparing scenarios of post-LGM population expansion versus pre/post-LGM population declines (Figure S8). Scenarios compared in each analysis and their specific demographic parameters are outlined in Supplementary Materials.

ABC analyses were carried out with the combined microsatellite and mtDNA datasets as well as on each dataset separately. The separate mtDNA analysis also included the 108 individual samples and the two French sequences from Genbank. The remaining analyses only included samples from the 16 colonies. We generated 10^6 simulations for each scenario tested in each analysis. The posterior probability of scenarios was estimated using a weighted polychotomous logistic regression. We checked model performance and empirically evaluated the power of the model to discriminate among scenarios (confidence in scenario choice) by simulating pseudo-observed datasets with the different scenarios and calculating false allocation rates (type1 and 2 errors, Cornuet *et al*. 2010).

Results

Mitochondrial DNA dataset

We identified 50 unique Cyt *b* haplotypes (20 from the 16 colonies). The Bayesian phylogenetic tree showed maximum posterior probability support for the split of *M. escalerai* into two principal lineages, the Western (South-West clade in Salicini *et al*. 2013) and Southern clade, and the remaining haplotypes, which mainly constituted of North-Central-Eastern haplotypes. The Western and Southern clades were further divided (posterior probability=0.85) into the Western and Southern lineages (Figure 1b).

Similarly, the haplotype network divided the haplotypes into three separate haplo-groups: Western, Southern and North-Central-Eastern. Western haplotypes were separated from the remaining haplotypes by >19 mutational steps (percent differences >2.5%). Most southern haplotypes grouped together and were separated by >8 mutational steps from the North-Central-Eastern haplotypes (>1.1%). However one haplotype from the south-eastern colony Granada grouped with the North-Central-Eastern haplotypes, while most of the samples from the southern colony of Sevilla grouped with the Western haplo-group. Samples from the French Pyrenees belonged to the common Eastern haplotype (CasGiIB), as did most samples from around the Pyrenees. However some unique haplotypes were identified in the Pyrenees, all of which were separated by one mutational step from either the common Eastern (CasGiIB) or North-Central (LROurSeg) haplotypes, depending on their geographical location (eastern and central versus western Pyrenees) (Figure 1c, Table S1-2).

Mitochondrial haplotype diversity was highest in the North-Central-Easter group, even after accounting for differences in sample size (32 haplotypes, 0.16 per sample), but nucleotide diversity was highest in the Southern group (Pi=0.016; Table S3). Among the colonies, Cádiz and Illes Balears had the highest haplotype diversity, while Granada, Alacant and Sevilla the highest nucleotide diversity (Table 1). Overall genetic differentiation at the mtDNA level between the Western, North-Central-Eastern and Southern geographic groups was significant (χ^2_{90} =678.5, P<0.001; overall θ_{ST}=0.73), with particularly high levels of differentiation between the Western and North-Central-Eastern groups (θ_{ST} = 0.93; Table S4).

Microsatellite data

Of the ten microsatellite loci, one marker (H29) was removed due to high frequency of null alleles. After removing this marker, all colonies, but Huelva, were overall in Hardy-Weinberg equilibrium. None of the markers were in linkage disequilibrium and all were in Hardy-Weinberg equilibrium in at least 13 out of the 16 colonies. The dataset, excluding H29, contained a total of 103 alleles, with an average of 11.44 ±5.5 alleles per locus (range 4–21), and 10 private alleles.

Genetic diversity (adjusted for sample size) in terms of allelic richness, heterozygosity, gene diversity and number of private alleles, was highest in Granada (southern Iberia) followed by Cáceres (western Iberia), and was lowest in Girona (eastern Iberia) and Illes Balears (Table 1). Levels of relatedness were particularly high within the Girona and Illes Balears colonies (mean TrioML= 0.44±0.1 and 0.25±0.2, respectively), whereby a third of the pair-wise relatedness values between individuals within the Girona colony were > 0.5. Levels of population differentiation were highest between Girona and all other colonies and Illes Balears and all other colonies, even after the removal of close relatives. Particularly low levels of differentiation were found between Cáceres and most southern and western colonies and among the southern colonies (Table S5).

Individual-based Bayesian assignment tests detected genetic population structure in *M. escalerai*. Individuals were best divided into four genetic clusters (Ln probability (K) =-7730 ± 5; Figure S1), despite some level of admixture in most colonies. The most north-eastern colony, Girona, formed a separate cluster; however this cluster disappeared once close relatives (TrioML >0.5) were removed from the analysis. Individuals whose haplotypes belonged to the mtDNA North-Central-Eastern clade tended to be assigned to different clusters from individuals from the mtDNA Western clade, with the exception of individuals from the most north-western colony (Ourense). However, most individuals whose haplotypes belonged to the mtDNA Southern clade showed high levels of admixture between clusters, and only an East to West geographic gradient was evident at the nuclear microsatellite level (Figure 2).

Species Distribution Modelling across temporal scales

All SDMs had high predictive ability, did not overfit presence data (full model: AUC=0.89 $\text{AUC}_{\text{crossvalidation}}$=0.80 ±0.04; climatic model: AUC=0.87, $\text{AUC}_{\text{crossvalidation}}$=0.79 ±0.03) and had significantly higher predictive ability than the null models (mean AUC=0.64 ±0.004 [95% Confidence Intervals], range: 0.57-0.67). The best fit model in terms of AIC scores had a regularization value of 1. The main eco-geographical variable contributing to both the climatic and full models was slope. Other important variables contributing to the climatic model were annual rainfall (BIO12), temperature seasonality (BIO4), rainfall seasonality (BIO15) and average temperature of the dry quarter (BIO9), while the habitat variable percent tree cover and the land cover type conifer woodlands were important in the full model (Figures S2-3). Both models show high concordance on predictions for areas occupied by the 16 colonies, though the full model offers a finer resolution, which results in more fragmented habitat suitability in the north-west. All colonies, except for two western colonies (Nabão and Ourense), are currently located in areas predicted to have a high relative occurrence probability for *M. escalerai*, though both are still within 5 km distance of suitable areas (Figure 3a-b; Figure S4).

Paleo-SDMs predicted a substantial decrease in the extent of suitable conditions for *M. escalerai* in Iberia during the LGM compared with present conditions (percent of area above suitability threshold for present: 34%, for LGM: 8.4%). Suitable climatic conditions during the LGM were restricted to isolated areas in the central-west, south and east of Iberia and in south-eastern France, while the Central Plateaus, Western Pyrenees and the north and west coasts were climatically unsuitable. As a result, in the Western Group, only the most central colony Cáceres and the northern colony Entrimio were located in climatically suitable areas (Figure 3c-d). Model predictions were affected by variables outside their training range around the Pyrenees, north-west Iberian coast and northern France (Figure S6). The extent of suitable conditions was also low during the LIG (17%), but suitable areas were restricted to the north Atlantic coast, western Iberia (Portugal) and the southern tip near the Strait of Gibraltar (Figure S5).

Future SDMs predicted a reduction in range suitability for *M. escalerai* in Iberia by 2070 (to 18.1%) with most of the south and interior of Iberia predicted to become climatically unsuitable. However, the northern Atlantic coast, Pyrenees and north-western France are predicted to gain suitable areas (Figure 3e-f). This will result in the majority of colonies and the entire southern lineage being located in climatically unsuitable areas by 2070. However, these predictions should be considered with caution because temperature variables were outside their training range across most of the Peninsula (Figure S7).

ABC inference of demographic/evolutionary history

Model-based inference pointed to the western group being the source population of *M. escalerai*, and to the presence of two separate refugia in the Iberian Peninsula during the LGM, one in the West and one in the North-Central-East. The Southern population diverged from the Western population after the end of the LGM, and later was admixed with gene flow from the North-Central-East population (Scenario 1.1, posterior probability=0.93; type 1 error=0.03, type 2=0.02; Figure 4a; Table S6).

The Western Group analysis identified Cáceres as the representative source population of the Western *M. escalerai* group, from which all other colonies split after the LGM, beginning with the most south-western colony (Amarela) and ending with the adjacent central colony (Nabão) (Scenario 2.1, posterior probability=0.99; type1 error=0.02, type2 error=0.02; Figure 4b; Table S7). Similarly, Castellón was the representative source population in the best supported model for the Eastern Group and all other Eastern colonies split directly from this population post-LGM, with the oldest split being between Castellón and Girona (Scenario 3.1, posterior probability=0.83; type 1=0.05, type 2=0.03; Figure 4b; Table S8). In both analyses, population split dates were estimated to have occurred between the early and mid-Holocene.

Demographic history modelling indicates that the Western group's effective population size has increased more than 10-fold after the end of the LGM, while the Eastern population size remained stable, though currently both groups have similar estimated effective population sizes (Scenario 4.3, posterior probability=1.0, type 1=0.016, type 2=0.01; Figure S8; Table S9). The timing of the western population expansion corresponds with the estimated time of colonisation of the south-western colonies, and therefore may reflect population expansion to areas south of the LGM refugia.

The same full model scenario (Scenario 1.1) was supported by the microsatellite-only dataset (posterior probability=0.99, type 1=0.05, type 2=0.06). There was no clear support for models ran using the extended mtDNA-only dataset, which included the 16 colonies and all the individual samples. Although Scenario 1.4 (West source, colonised East via South) received relatively high support (posterior probability=0.73), error rates were high (type 1=0.39, type 2=0.32), indicating that the analysis was unable to differentiate between the scenarios.

Discussion

The combination of climate change and topographically originated environmental heterogeneity played an important role in shaping the evolutionary history and current genetic population structure of *M. escalerai* within the Iberian refugium, and it is likely to continue shaping the future distribution and patterns of genetic diversity of this restricted range endemic species.

The biogeographic history of *Myotis escalerai*

It is not clear what event caused the divergence of *M. escalerai* from its Moroccan cryptic sister species *M. spB* around 0.99 million years ago (Salicini *et al*. 2013). However, despite this relatively recent speciation event we found strong support for divergence into distinct clades. Quaternary climatic oscillations appear to have left a signature of geographic population structure in *M. escalerai* which corresponds to patterns of deep lineage divergence in other Iberian taxa whose lineages diverged before the Pleistocene (e.g. the *Vipera latastei/monticola* group, Velo-Anton *et al*. 2012).

Based on the mtDNA dataset, *M. escalerai* across Iberia is divided into three main lineages, the Western clade, which is restricted to the Atlantic climatic region in Portugal, the North-

Central-Eastern clade, and the Southern clade. Paleo-SDMs indicate that this split may be the result of the disjunct distribution of suitable climatic conditions during the LGM when suitable areas were restricted to isolated patches in the west, east, south, and near the Pyrenees and southern France. Model projections and the strong association of the phylogenetic divide with geography lend support to the suggestion that during the Pleistocene several geographically separate refugia were present within the Iberian refugium (Gomez & Lunt 2007; Ferrero *et al*. 2011). The strong genetic differentiation of a large number of Iberian species into a western (Atlantic) and eastern (Mediterranean) lineages is thought to reflect the disjunct LGM distribution of the most favourable climatic conditions in the peninsula (Schmitt 2007) and the harsher climate of the central Iberian plateau that separates them (Gomez & Lunt 2007).

Unlike other bat species for whom Iberia was the principal glacial refugia (e.g. *Plecotus austriacus*; Razgour *et al*. 2013), *M. escalerai* is unique as it has never expanded its range beyond the peninsula, even though it is found across the Pyrenees, and therefore Iberia for this species may represent an area of endemism rather than refugium (Stewart *et al*. 2010). Other Iberian endemics, like *Galemys pyrenaicus*, show similar patterns of divergence into distinct evolutionary lineages, suggesting the existence of complex isolation mechanisms as species experienced whole glacial processes of contraction and dispersal within the peninsula (Igea *et al*. 2013).

ABC model-based inference confirms the presence of separate western and eastern refugia during the LGM, and has identified the source populations of each geographical group as colonies that experienced suitable climatic conditions during the LGM based on SDM projections. Moreover, in line with SDM projections of climatic suitability during the LIG, evolutionary history inference suggests that the Western group was the source population. The concordance between the projected distribution of suitable climatic conditions during the LGM and LIG based on SDMs and evolutionary history inference based on genetic data lends support to the presence of niche conservatisms in climatic tolerance in *M. escalerai*. Niche conservatism may limit the ability of species to adapt to novel environmental conditions within the timeframe required to respond to climate changes, suggesting that instead species will either shift their geographic ranges to track suitable climatic regimes or go extinct (Wiens & Graham 2005). However, Pellissier et al. (2013) show that, at least for arctic-alpine plant species, niche conservatism is more pronounced at cold than warm thermal limits because biotic interactions (e.g. competition) play a more important role when conditions are less severe and species are not at their physiological limits.

Yet, climate and topography alone do not determine a species' occurrence, as is evident from the full SDM, in which habitat variables, and in particular the presence of coniferous woodlands, was a strong determinant of occurrence probability. Predicted distribution of forest tree species in Iberia during the LGM (Benito Garzón *et al*. 2007) and evidence from pollen records (Gomez & Lunt 2006; Lopez de Heredia *et al*. 2007; Rodriguez-Sanchez *et al*. 2010) suggest that most of the studied colonies were located in areas where forests persisted during the LGM. Therefore forest availability is not likely to have been a major limiting factor for *M. escalerai* during colder periods. Although LGM forests were dominated by pines (Rodriguez-Sanchez *et al*. 2010), the main woodland type where *M. escalerai* is currently found based on the full SDM, south and south-western Iberia were less forested and dominated by evergreen oaks (Benito Garzón *et al*. 2007). This may explain the ABC inference that *M. escalerai* persisted during the LGM in Western and Eastern areas, while the south was only colonised around the early-mid-Holocene when the predicted distribution of pines extended to the south-west (Benito Garzón *et al*. 2007).

Current patterns of genetic variability and future losses

Population assignment and geographical separation was less clear at the microsatellite than the mtDNA level. Only a slight signature of a geographical West and East divide was evident, most colonies were assigned to more than one genetic population cluster and many individuals showed some level of admixture. Moreover, colony assignment into geographical groups did not always follow the same pattern as the mtDNA dataset. For example, based on the mtDNA dataset, the north-western colony Ourense belongs to the North-Central-Eastern lineage, despite being geographically close to one of the Western colonies, while the microsatellite dataset groups Ourense with the Western colonies. This inconsistency in population assignment may reflect the effect of recent (post-LGM) gene flow disguising older population splits. Microsatellites with their higher evolutionary rates reflect recent or contemporary genetic patterns, while mtDNA is more informative of events that occurred during earlier periods of the species' history (Wan *et al*. 2004).

Alternatively, more limited population structure at the microsatellite level may be the result of male-biased dispersal and female philopatry, a common pattern in bat species (Burland & Worthington Wilmer 2001). Ruedi and Castella (2003) identified a similar pattern in *Myotis myotis*, attributing the absence of population structure at the microsatellite level versus the strong population structure and limited gene flow between colonies at the mtDNA level to the estimated male bias in the proportion of dispersing individuals (>90%). These disparities highlight the importance of combining bi-parentally inherited nuclear markers and maternally inherited mtDNA markers with different evolutionary rates in phylogeographic studies.

Genetic diversity, based on both the mtDNA and microsatellite datasets, is highest in southern colonies, despite their more recent evolutionary history based on the ABC inference. Although this region contains several unique haplotypes and private alleles, high levels of genetic diversity may also be due to this region acting as a ‘hybrid/contact zone’ between the Western and Eastern refugia, in which genetic diversity was enriched by the admixture of divergent lineages (Hewitt 2011). And indeed southern colonies include haplotypes that group with both the Western and North-Central-Eastern clades. On the other hand, high levels of inbreeding and reduced allelic diversity in the most north-eastern colony (Girona) and the island colony (Illes Balears) may reflect their geographic isolation and limited recent gene flow from other populations. In the north-eastern colony in particular, high coancestry values likely reflect inbreeding in a small isolated population, rather than relatedness due to natal philopatry and the presence of mothers and their pups, because this is the only location where samples were collected from a swarming site and not a maternity colony. While bat summer maternity colonies can include a high proportion of relatives due to strong female natal philopatry, during the autumn, the closely related *Myotis nattereri* tends to migrate away from summer roosts to swarming sites that serve large catchment areas of up to 60 km (Rivers *et al.* 2006).

Under future climate change projections, the relative occurrence probability of *M. escalerai* across most of Iberia is predicted to decrease substantially. Range losses are predicted to be greatest in the south, placing the entire southern lineage in climatically unsuitable areas by 2070. Although low levels of population differentiation between Southern colonies and both Western and North-Central-Eastern colonies indicate the presence of gene flow under current conditions, range fragmentation is likely to increase in the future, resulting in colony isolation. Increased isolation can limit future range shifts and lead to increased inbreeding and loss of genetic diversity (Frankham 1995). Future climate change poses a particular threat to *M. escalerai* because it is restricted to the Iberian Peninsula where changes are predicted to be particularly severe (EEA 2012). Other drivers of environmental change, and in particular anthropogenic habitat loss, may hamper the ability of low dispersal and habitat specialist species, like *M. escalerai*, to shift their ranges in response to climate changes (Warren *et al*. 2001).

Forests are predicted to show a time lag in their response to climate change at their trailing edge. Increased temperatures and frequency of droughts are predicted to reduce seedling recruitment and forest regeneration, but adult trees may be able persist in climatically unsuitable areas due to their longevity and phenotypic plasticity (Jump *et al*. 2009). Because forests provide cooler microclimates that can help buffer the effects of macroclimatic warming (De Frenne *et al*. 2013), *M. escalerai* colonies may be able to persist in climatically unsuitable areas in the short-term owing to their association with forests. Yet in the longer term, modelling studies predict severe range contractions of mountain conifer, Mediterranean and sub-Mediterranean forests in central and southern parts of the Iberian Peninsula (Benito Garzón *et al*. 2008)

What restricts an endemic species

SDMs predict that suitable areas for *M. escalerai* are available outside the Iberian Peninsula, in particular along the Mediterranean coast of France. Although only limited genetic sampling has taken place so far, both Salicini et al. (2013) and Puechmaille et al. (2012) genetically identified all samples beyond the Eastern French Pyrenees as its congeners *Myotis spA* or *Myotis nattereri ss.* Yet more extensive sampling is needed in areas outside the Iberian Peninsula identified by our SDMs as potentially suitable. Individual samples from around the

Pyrenees, including the French Pyrenees, fell within the North-Central-Eastern clade and mostly belonged to the common Eastern haplotype, suggesting that this area was colonised from the Eastern refugia, rather than form a putative ‘northern refugia’ (Stewart & Lister 2001).

The range of *M. escalerai* is at least partly restricted by geographical barriers, the Gibraltar Straits in the south and the Pyrenees mountain range in the north (Salicini *et al*. 2013), though the Pyrenees themselves do not appear to form a barrier (Evin *et al*. 2009; Puechmaille *et al*. 2012). The Iberian Peninsula is home to several other restricted range endemic species, whose limited dispersal abilities prevent them from crossing these geographical barriers, and as a result their entire evolutionary history took place within Iberia (e.g. Igea *et al*. 2013). Although flight offers bats greater vagility, the Pyrenees have formed a geographical barrier for several bat species, restricting both post-glacial range re-colonisation from this refugium (Rebelo *et al*. 2012; Dool *et al*. 2013) and current patterns of gene flow (Razgour *et al*. 2014). Similarly, the Gibraltar Straits delimit the range of several bat species despite their relative narrow breadth (Garcia-Mudarra *et al*. 2009).

However, because *M. escalerai* is found across the Pyrenees, including the French side of the Eastern Pyrenees (Evin *et al*. 2009; Puechmaille *et al*. 2012), ecological barriers as a result of biotic interactions, rather than geographical barriers, may have played a more important role. Interspecific competition with its cryptic congeners *M. spA* and *M. nattereri s.s.* that may occupy similar ecological niches across the rest of Europe (Salicini *et al*. 2013) could have limited the spread of *M. escalerai* beyond the Pyrenees. It is possible that a delay in northward population expansion post-LGM due to the longer persistence of ice cover in the Pyrenees meant that advancing competing congeners from the Italian and Balkan refugia restricted the space available for *M. escalerai* north of the Pyrenees, as has been postulated for some Iberian forest tree lineages (Rodriguez-Sanchez *et al*. 2010). This suggests that future range gains predicted around the north coast of Iberia, where *M. escalerai* is sympatric with *M. spA*, and in western France, north of the Pyrenees, where *M. nattereri s.s.* is present, may not help offset extensive range losses in the south and centre of Iberia because competitive exclusion may limit northern population expansion. However, the presence of some altitudinal segregation in sympatric localities (Agirre-Mendi & Ibáñez 2012) could indicate different ecological optima for each of these two species, which may allow them to coexist in areas of range overlap.

Conclusions

A concentration of high genetic diversity and deeply differentiated evolutionary lineages in Iberia has been found in other European bat species with limited long-distance dispersal abilities (Ibáñez *et al*. 2006; Dool *et al*. 2013; Razgour *et al*. 2013), highlighting the evolutionary importance of this peninsula for European bats. Here we resolve the spatial genetic history of a species for which Iberia is not only a glacial refugium but also its range limit, and therefore its future survival prospects are closely tied to climatic processes occurring within the peninsula. We show that past climatic oscillations resulted in the divergence of *M. escalerai* into separate Western and North-Central-Eastern populations, supporting the 'refugia within refugia' hypothesis. In accordance with other studies of Iberian reptiles and mammals, our ABC model-based inference and paleo-SDMs indicates that the Western population is the older, source population. Although contemporary gene flow may mask historic lineage splits, a signature of geographical population structure is still maintained. The role of ecological barriers due to interspecific competition in restricting *M.*

escalerai to the Iberian Peninsula, even when climatic conditions are suitable elsewhere, suggest that this species may struggle to shift its range north of the Pyrenees in the future when most of the peninsula is predicted to become climatically unsuitable.

M. escalerai was identified morphologically a century ago (Cabrera 1904), but because its species status was only recently genetically confirmed (Ibáñez *et al*. 2006), its global conservation status is yet to be formally assessed, though within Portugal it is listed as vulnerable (ICNF 2013). Our findings suggest that conservation management for this species should increase landscape connectivity across Iberia in order to facilitate north-western range shifts in response to future climate change, especially from the southern lineage that is particularly threatened by future changes.

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Data Accessibility

– DNA sequences GenBank accessions numbers: KT365234–KT365283 and KT633874 (Supplementary Table S1-S2).

– Data archived in Dryad (doi:10.5061/dryad.v1v47): aligned mtDNA sequences for all individuals, nexus input file for MrBayes phylogenetic analysis, MrBayes tree file underlying the phylogeny, Structure input files, Structure results and individual assignments, and MaxEnt location records.

Author Contribution

CI, IS and JJ designed the study, collected or organised the sample collection, generated the molecular data and contributed to the manuscript. ER helped with obtaining the microsatellite dataset and information at ISPRA Conservation Genetics Laboratory and contributed to the manuscript. OR wrote the manuscript and performed the genetic analysis (mtDNA and microsatellite), species distribution modelling and ABC evolutionary history analysis.

Figure Captions

Figure 1 – *Myotis escalerai* population structure based on the mtDNA (Cytochrome b) dataset (colonies and individual samples, N=359; Tables S1-S2). A) Map of the location of the colonies (larger circles and names) and individual samples included in the study, colour coded based on phylogenetic clades. B) Bayesian phylogenetic tree showing posterior probability values >0.8. Haplotypes are named based on their respective sampling locations and clades are marked with their respective colours (yellow – West, blue – South, red – North-Central-East). C) Median-joining haplotype network, colour-coded based on phylogenetic clades. Circle sizes correspond to number of samples. Numbers indicate haplotypes separated by >1 mutation. Haplotype CasGiIB is marked with A, and LROurSeg with B.

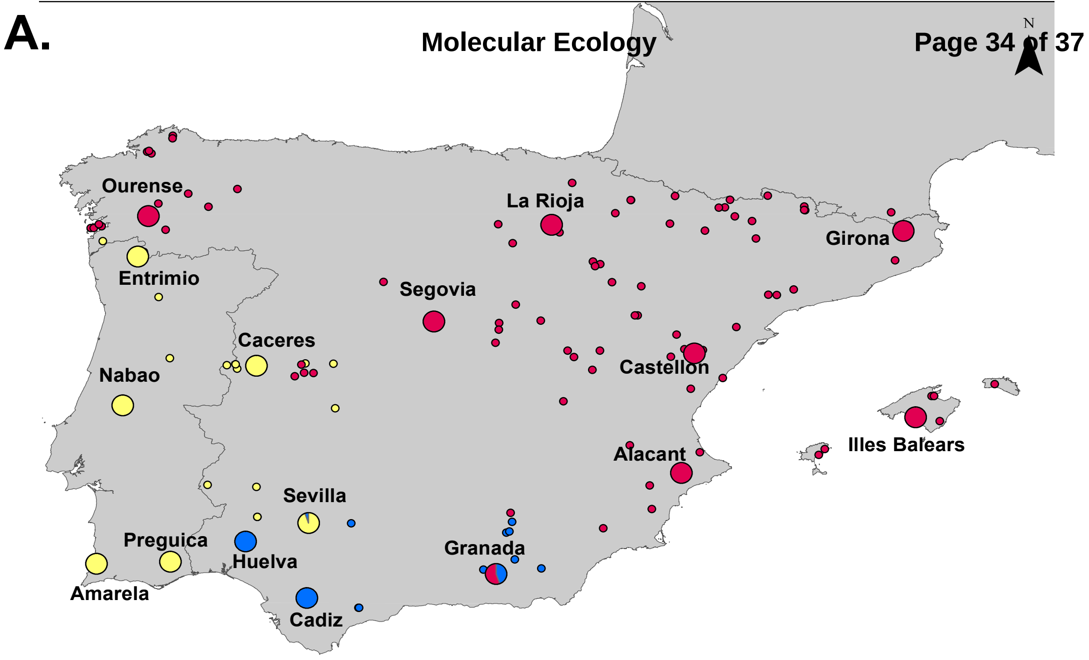
Figure 2 – *Myotis escalerai* population structure based on the microsatellite dataset. A) STRUCTURE analysis including all samples (K=4); and B) STRUCTURE analysis after close relatives (TrioML>0.5) were removed (K=3), showing cluster membership plots and frequency of each cluster in the studied colonies.

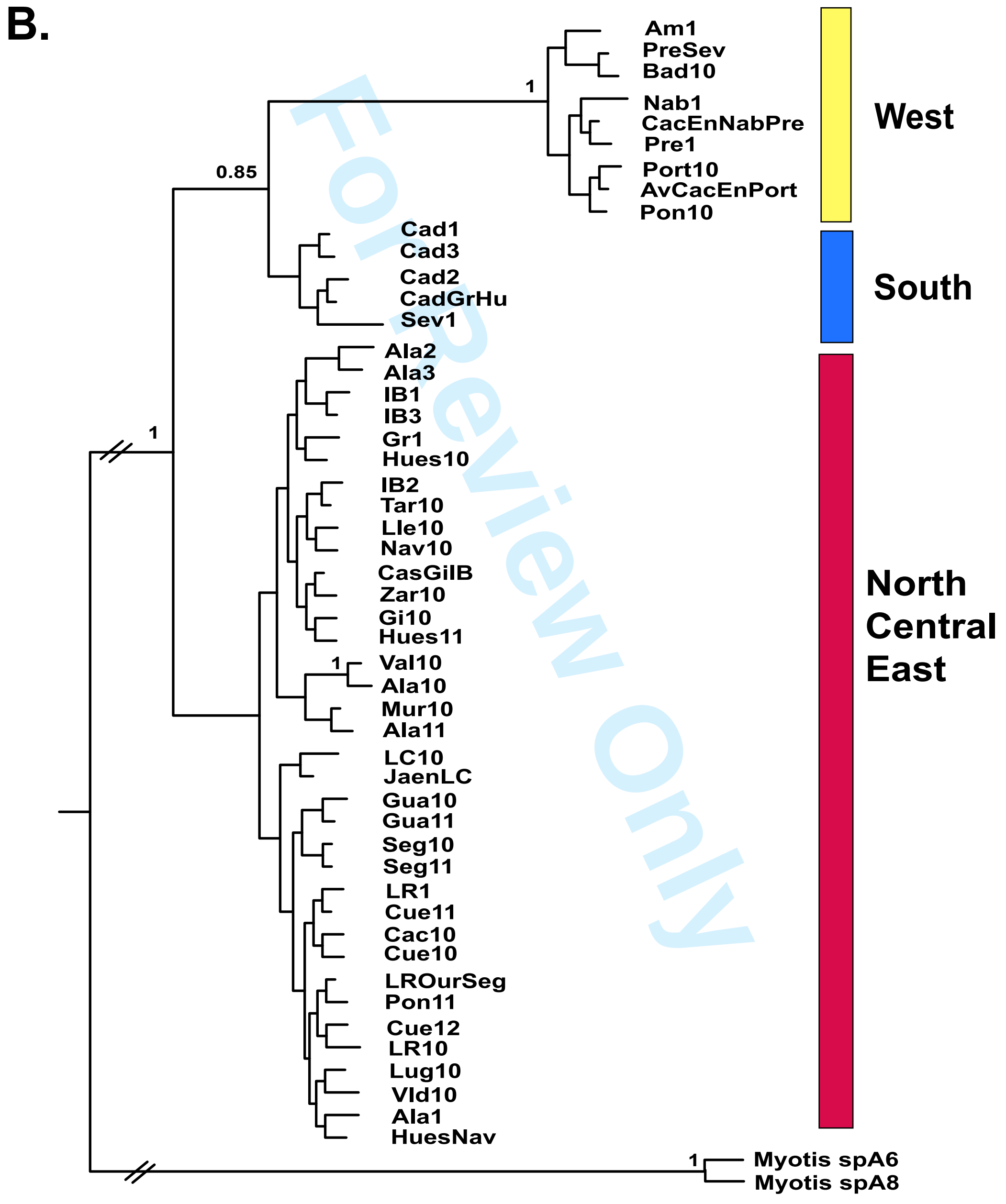
Figure 3 – Species distribution models for *Myotis escalerai* across temporal scales: A-B) present climate model, C-D) Last Glacial Maximum (LGM ~21,000 ybp), and E-F) future (2070, +8.5rcp scenario). Models are presented as a scale of relative occurrence probability from low in yellow to high in dark blue (A,C,E), or as binary maps of potentially suitable areas in black (B,D,F). White circle denote the location of the studied colonies.

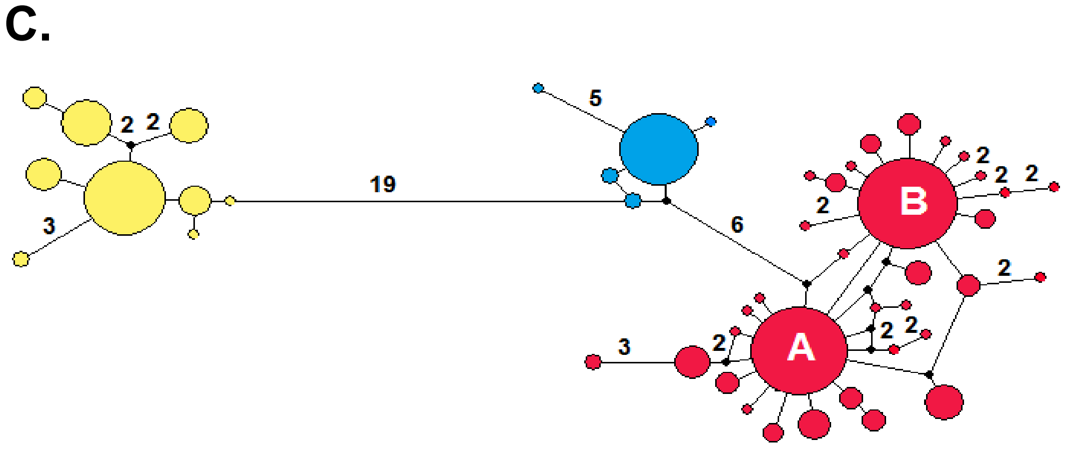
Figure 4 – Results of the Approximate Bayesian Computation analysis of the evolutionary history of *Myotis escalerai*, showing the selected scenarios for A) the full model, and B) the Western and Eastern Group analyses. White circles denote the location of colonies. Arrows represent the direction of colonisation from the source population, with median estimated divergence dates.

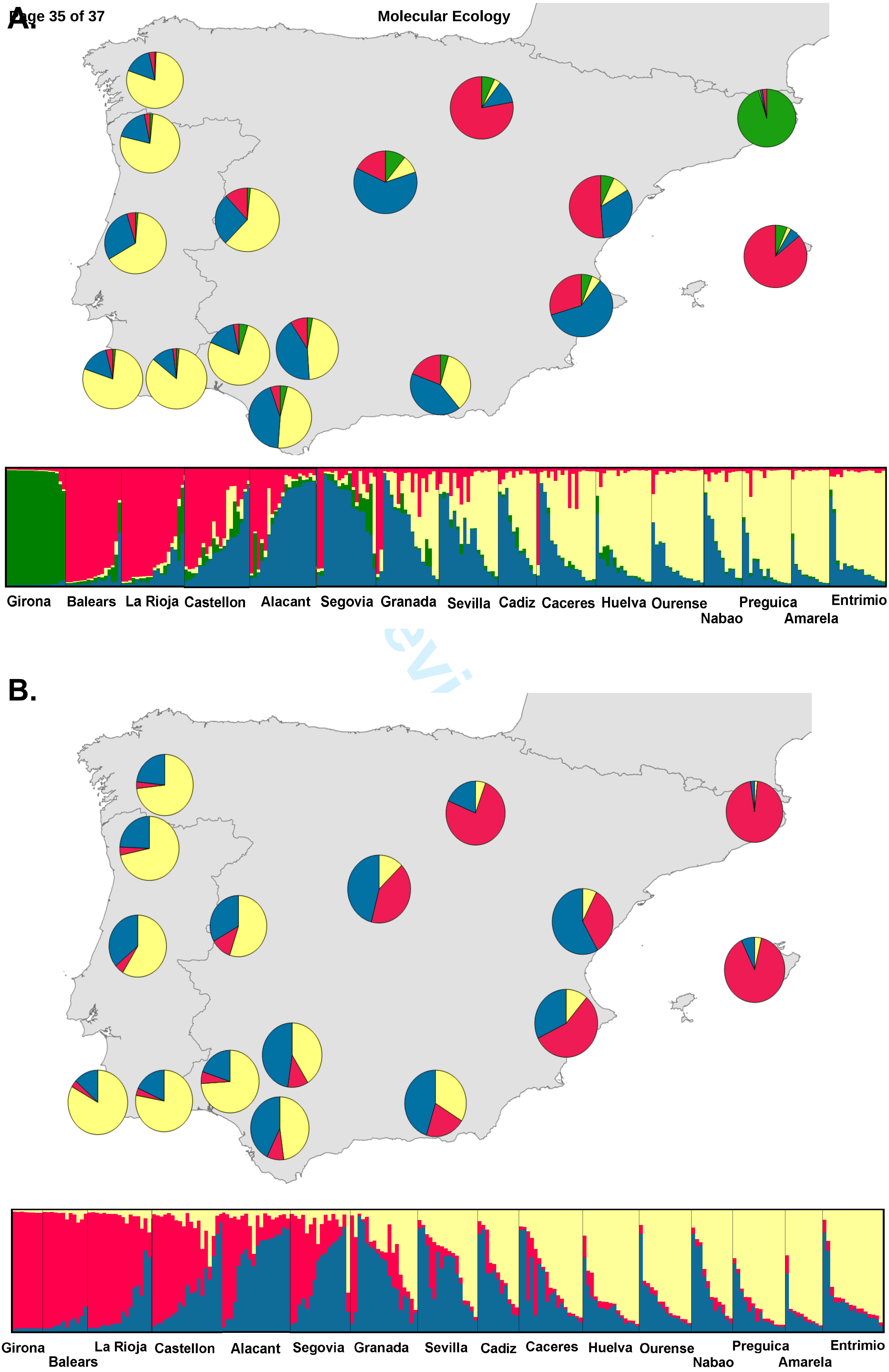
Table 1 – Genetic diversity of *Myotis escalerai* colonies based the microsatellite (first six columns) and the mitochondrial DNA (last four columns) datasets, with sample sizes presented in brackets. Mean allelic richness and gene diversity (± standard deviation) were adjusted based on sample size.

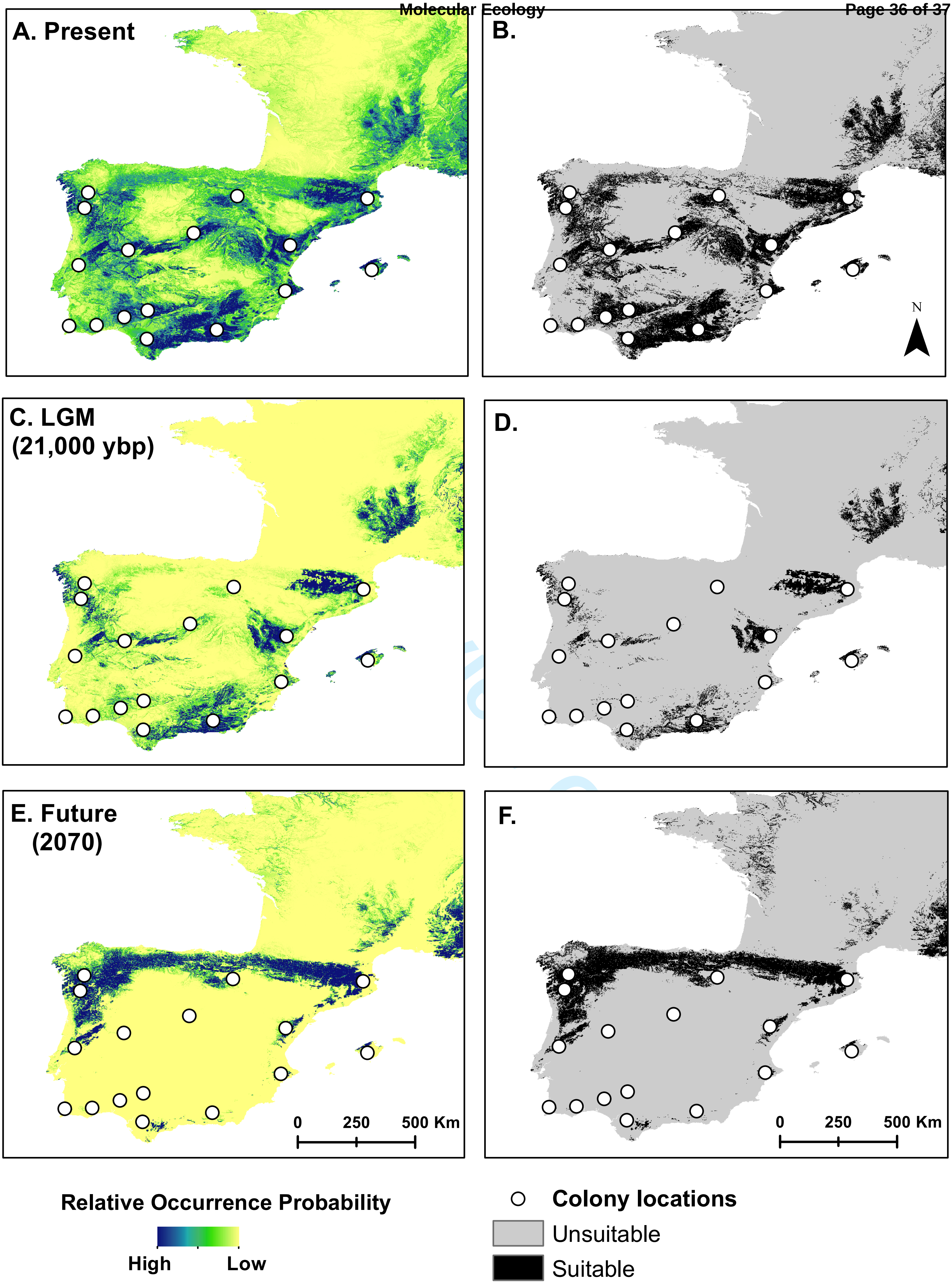
	Mean number of alleles	Shannon Index	Gene diversity	Allelic richness	Number of private alleles	Hetero-zygosity (He)	Number of haplo-types	Number of polymorphic sites	Haplotypic diversity	Nucleotide diversity (Pi)
Alacant (19)	7.44 ±1.0	1.593	0.76 ±0.1	6.0 ±2.2	0	0.739	3	8	0.608	0.0044
Amarela (11)	5.00 ±0.5	1.261	0.67 ±0.1	4.9 ±1.5	0	0.636	1	0	0	0
Cáceres (17)	7.33 ±1.1	1.609	0.75 ±0.2	6.3 ±2.6	3	0.728	1	0	0	0
Cádiz (11)	6.33 ±0.7	1.579	0.77 ±0.2	6.2 ±2.1	0	0.735	4	3	0.691	0.0014
Castellón (19)	7.22 ±1.0	1.586	0.74 ±0.2	6.1 ±2.3	0	0.719	1	0	0	0
Entrimio (16)	6.33 ±0.7	1.433	0.69 ±0.2	5.6 ±1.8	0	0.672	1	0	0	0
Girona (17)	3.44 ±0.7	0.792	0.44 ±0.3	3.1 ±1.6	0	0.431	1	0	0	0
Granada (18)	8.11 ±1.2	1.711	0.77 ±0.2	6.8 ±2.9	4	0.748	2	10	0.526	0.0070
Huelva (16)	6.00 ±0.7	1.440	0.70 ±0.2	5.4 ±2.0	0	0.681	1	0	0	0
I. Balears (16)	5.44 ±0.9	1.192	0.60 ± 0.2	4.7 ±2.2	2	0.581	4	3	0.792	0.0019
La Rioja (18)	6.00 ±0.9	1.396	0.70 ±0.2	5.2 ±2.0	1	0.681	2	1	0.118	0.0002
Nabão (11)	5.78 ±0.7	1.474	0.74 ±0.2	5.6 ±2.0	0	0.702	2	3	0.327	0.0013
Ourense (15)	6.22 ±0.9	1.433	0.69 ±0.2	5.6 ±2.4	0	0.666	1	0	0	0
Preguiça (14)	5.67 ±0.6	1.305	0.65 ±0.2	5.2 ±1.5	0	0.622	3	4	0.538	0.0012
Segovia (17)	7.44 ±1.1	1.568	0.72 ±0.2	6.3 ±2.6	0	0.700	1	0	0	0
Sevilla (17)	7.56 ±1.0	1.600	0.75 ±0.1	6.3 ±2.2	0	0.728	2	25	0.111	0.0037

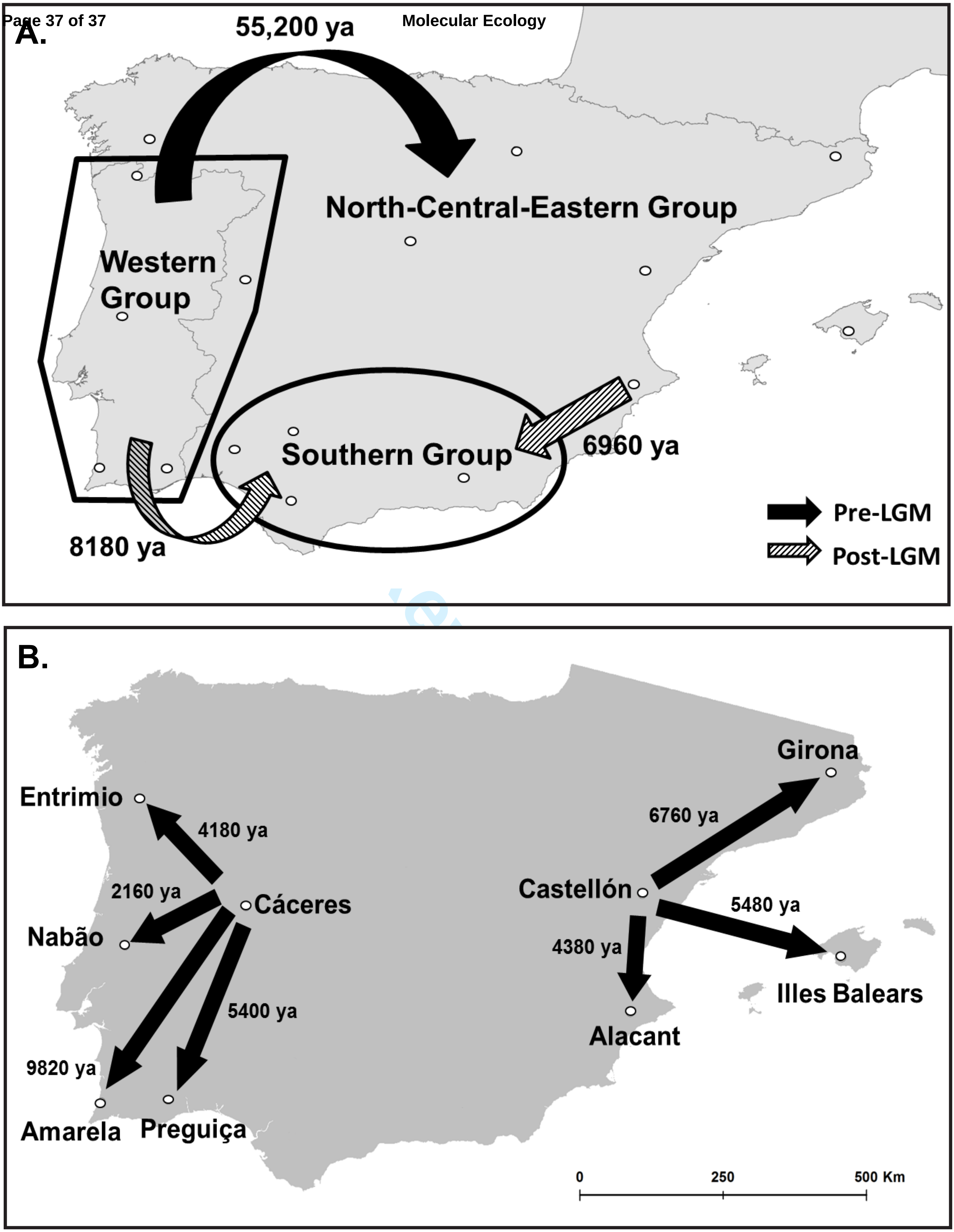

A.
N
Ourense
La Rioja
Girona
Entrimio
Segovia
Caceres
Nabao
Castellon
Illes Balears
Alacant
Sevilla
Preguica
Huelva
Granada
Amarela
Cadiz


B.
Am1
PreSev
Bad10
1
Nab1
CacEnNabPre
Pre1
0.85
Port10
AvCacEnPort
Pon10
West
Cad1
Cad3
Cad2
CadGrHu
Sev1
South
Ala2
Ala3
IB1
IB3
1
Gr1
Hues10
IB2
Tar10
Lle10
Nav10
CasGilB
Zar10
Gi10
Hues11
1
Val10
Ala10
Mur10
Ala11
North
Central
East
LC10
JaenLC
Gua10
Gua11
Seg10
Seg11
LR1
Cue11
Cac10
Cue10
LROurSeg
Pon11
Cue12
LR10
Lug10
Vld10
Ala1
HuesNav
1
Myotis spA6
Myotis spA8


C.
2
2
3
19
5
6
2
2
2
2
B
2
A
3
2
2
2


A.
Girona
Balears
La Rioja
Castellon
Alacant
Segovia
Granada
Sevilla
Cadiz
Caceres
Huelva
Ourense
Preguica
Entrimio
Nabao
Amarela
B.
Girona
Balears
La Rioja
Castellon
Alacant
Segovia
Granada
Sevilla
Cadiz
Caceres
Huelva
Ourense
Preguica
Entrimio
Nabao
Amarela


A. Present
B.
N
C. LGM
(21,000 ybp)
D.
E. Future
(2070)
F.
0 250 500 Km
0 250 500 Km
Relative Occurrence Probability
High
Low
○ Colony locations
Unsuitable
Suitable


A.
55,200 ya
North-Central-Eastern Group
Western Group
Southern Group
6960 ya
8180 ya
Pre-LGM
Post-LGM
B.
Entrimio
4180 ya
2160 ya
Cáceres
Nabão
5400 ya
9820 ya
Amarela
Preguiça
Girona
6760 ya
Castellón
5480 ya
4380 ya
Illes Balears
Alacant
0
250
500 Km