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New insights on postglacial colonization in western Europe: the phylogeography of the Leisler's bat (*Nyctalus leisleri*)

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Despite recent advances in the understanding of the interplay between a dynamic physical environment and phylogeography in Europe, the origins of contemporary Irish biota remain uncertain. Current thinking is that Ireland was colonized post-glacially from southern European refugia, following the end of the last glacial maximum (LGM), some 20 000 years BP. The Leisler's bat (*Nyctalus leisleri*), one of the few native Irish mammal species, is widely distributed throughout Europe but, with the exception of Ireland, is generally rare and considered vulnerable. We investigate the origins and phylogeographic relationships of Irish populations in relation to those across Europe, including the closely related species *N. azoreum*. We use a combination of approaches, including mitochondrial and nuclear DNA markers, in addition to approximate Bayesian computation and palaeo-climatic species distribution modelling. Molecular analyses revealed two distinct and diverse European mitochondrial DNA lineages, which probably diverged in separate glacial refugia. A western lineage, restricted to Ireland, Britain and the Azores, comprises Irish and British *N. leisleri* and *N. azoreum* specimens; an eastern lineage is distributed throughout mainland Europe. Palaeo-climatic projections indicate suitable habitats during the LGM, including known glacial refugia, in addition to potential novel cryptic refugia along the western fringe of Europe. These results may be applicable to populations of many species.

1. Introduction

The origins of the terrestrial fauna and flora of western Europe in general and Ireland in particular are the subject of prolonged debate [1–4]. Similar to the rest of northern Europe, most of Ireland was covered by ice during the last glacial maximum (LGM) some 20 000 years BP; thus, all endemic Irish species are thought to be descendants from colonists arriving post-glacially from refugia in southern Europe. Ireland is species poor compared with Britain and continental Europe. This is attributed to the 'steepchase' effect [5], as the retreating ice cut off Ireland from Britain around 16 000 years BP and continental Europe around 7800 years BP [6]. However, recent studies (see [7]) examining geophysical models simulating the land mass response to glaciohydro-isostatic adjustment [8] provide no evidence for land-bridges between Ireland and Britain during the Holocene, reopening the debate as to the origin of the terrestrial Irish fauna. Biogeographic studies of species such as the Kerry slug (*Geomalacus maculosus*) and the strawberry tree (*Arbutus unedo*) [9] suggest a possible link between Ireland and southwestern Europe. However, phylogeographic studies of Irish flora and fauna have demonstrated that Irish populations are genetically distinct and diverse in relation to their European counterparts [10]. The current consensus is that there are still too few comprehensive phylogeographical studies involving Irish populations to finally resolve the origins of the fauna and flora of western Europe. Here, we use a combination of analytical approaches, including phylogeographic analysis of mitochondrial and nuclear DNA, in addition to palaeo-climatic species distribution modelling (SDM), to investigate the impact of the Pleistocene LGM on

the past and present-day distribution of the Leisler's bat *Nyctalus leisleri* (Kuhl, 1817).

The Leisler's bat is distributed across most of Europe, western Asia as far as the Urals, northwest Africa, the Canary Islands and Madeira [11,12]. Despite widespread distribution, it is rare almost everywhere except Ireland, where it is the most common species after the *Pipistrellus* spp. [13]. The Leisler's bat is the only representative of the *Nyctalus* genus in Ireland [14], but in the rest of its range, it occurs sympatrically with the common noctule *N. noctula* (Schreber, 1774). A closely related species, *N. azoreum*, is endemic to the Azores. The Irish *N. leisleri* bat population is considered the largest in Europe [15] and of international importance.

Morphological and behavioural differences occur between *N. azoreum* and continental European *N. leisleri* [16–19]. Similarly, behavioural differences have been reported between the Leisler's bat in Ireland and elsewhere. Continental *N. leisleri* are forest dwelling, roosting mainly in treeholes, whereas in Ireland, maternity colonies are more commonly found in buildings [20,21]. The *N. leisleri* in Ireland have also been reported to have a lower peak-frequency echolocation call than their counterparts elsewhere [14,22], although such comparisons are difficult owing to disparate recording methods. Continental *N. leisleri* are migratory, with individuals being recorded moving distances over 1500 km between summer and winter roost sites [23–25]. However, in Ireland, it seems *N. leisleri* remain within their summer range to hibernate [26] (E. S. M. Boston and A. Hopkirk 2004, personal observations).

Genetic divergence and phylogeographic history of *Nyctalus* in Europe have been examined by Salgueiro *et al.* [27] using mitochondrial DNA (mtDNA) genes. Their main interest was to establish genetic relationships between *N. azoreum* and *N. leisleri*. They found that *N. azoreum* was characterized by a number of unique mtDNA haplotypes restricted to the Azores. These findings, in addition to a number of morphological, ecological and behavioural differences, were used to argue for full species status for *N. azoreum* [27,28]. The low levels of genetic divergence between *N. azoreum* and *N. leisleri* suggested that the former species originated recently from a European population of *N. leisleri*, and that the colonization of the Azores is most to have likely occurred at the end of the Pleistocene. No *N. leisleri* samples from Ireland or Britain were included in this study and, thus, the phylogeography of *N. leisleri* in western Europe remains far from complete, and the origins of this species in Ireland and Britain remain uncertain. Further, it is not known whether contemporary gene flow occurs between *N. leisleri* populations from Ireland, Britain and mainland Europe. Despite their obvious dispersal potential as a flying mammal, Castella *et al.* [29] demonstrated that in *Myotis myotis*, for instance, large bodies of water can act as effective barriers to gene flow. Thus, any contemporary exchange of genes between island and mainland populations may be restricted to passive (e.g. wind-blown), rather than to active, dispersal.

Here, we aim to determine the origin(s) and relationship of Irish populations with those across the rest of Europe, including the closely related *N. azoreum*. We test the hypotheses that (i) *N. leisleri* in Ireland colonized from a single southern European glacial refugia, via Britain, and (ii) there is limited gene flow among recent populations of *N. leisleri* in continental Europe, Britain and Ireland. Throughout, we examine the systematic and phylogeographic status of *N. leisleri* in continental Europe and offshore islands, and, in particular, the proposed endemic species *N. azoreum*.

2. Material and methods

(a) Sampling

Over two consecutive years, 283 *N. leisleri* were captured leaving maternity colonies in 12 locations throughout Ireland ($n = 138$) and at two sites in northeastern France ($n = 145$). Samples were taken non-destructively, using the sterile wing biopsy technique, as described by Worthington-Wilmer & Barrett [30]. Two 3 mm diameter wing biopsies were taken and placed in 99% molecular grade ethanol. All bats were released immediately after sampling. Biopsy tissue samples from 37 specimens were donated from research colleagues and museums in France, Portugal, Switzerland, Germany, Hungary and Greece, along with 18 faecal samples from Britain (figure 1 and table 1; electronic supplementary material, table A in appendices).

(b) DNA extraction, and mitochondrial DNA sequencing and microsatellite screening

Genomic DNA was extracted from tissue biopsies using the DNeasy tissue kit (Qiagen), whereas the 18 faecal samples were extracted using QIAamp DNA stool mini kit (Qiagen) following the modifications from Puechmaille *et al.* [31], which demonstrates that good-quality DNA can be obtained from bat droppings. A 324 base pair (bp) region of the hypervariable mtDNA control region (D-loop) was amplified for a subset of samples from Ireland ($n = 67$; an average five per site) and France ($n = 15$), and all other European samples, by PCR using the universal primers L16517 [32] and 130 sH651 [29]. PCR amplifications were carried out in 50 μ l reaction volumes as follows: 1–3 μ l of template DNA (approx. 5–25 ng), 2.5 mM MgCl₂, 10 pM of each primer, 200 μ M dNTPs, 1 U of *Taq* DNA polymerase (Invitrogen) with 1 \times of the corresponding PCR buffer. Thermal profiles consisted of an initial denaturation step at 95°C for 3 min, followed by 35 cycles consisting of 45 s at 94°C, 45 s at 50°C, 1 min at 72°C followed by a final extension step of 7 min at 72°C. Amplified products were purified using Roche high purification kits and subsequently sent for commercial sequencing (Macrogen) using the forward primer only. Resulting sequences were imported into BioEDIT [33], and aligned using CLUSTALW [34].

In order to combine our novel mtDNA dataset with existing lineages based on previous studies by Salgueiro *et al.* [27,28], 46 European *N. leisleri* were sequenced for the mtDNA control region, resulting in 13 haplotypes (accession nos. DQ887597–DQ887608 and AY756613–AY756615). In addition, sequences representing 16 haplotypes resulting from the screening of 165 specimens of the closely related *N. azoreum*, and one haplotype from *N. l. verrucosus*, representing two specimens from Madeira Island (accession nos. DQ887596 and AY756598–AY756612), were included in the analysis.

To account for possible biases of phylogeographic inferences resulting from the analysis of a single mtDNA gene [35], 138 Irish tissue samples and 145 tissue samples from two sites in northeastern France were typed at seven autosomal microsatellite loci (*Nleis1*, *Nleis2*, *Nleis3*, *Nleis5*, *Nleis6*, *Nleis8*, *Nleis10*) as described by Boston *et al.* [36]. Genotypic typing was carried out using GENE PROFILER v. 4.05 software (Scanalytics, Fairfax, VA). Control samples of known genotypes were used on each run, and the majority of the typing double checked by an independent researcher.

(c) Mitochondrial DNA analysis

Sample diversity statistics were calculated per site in ARLEQUIN v. 3.0 [37]. To evaluate the overall level of population genetic structuring on a large geographical scale, a global exact test for genetic differentiation was carried out among samples grouped by major regions (i.e. Ireland and Britain, mainland Europe and the Azores) using ARLEQUIN v. 3.0, with 20 000 Markov chain steps. Patterns of genetic

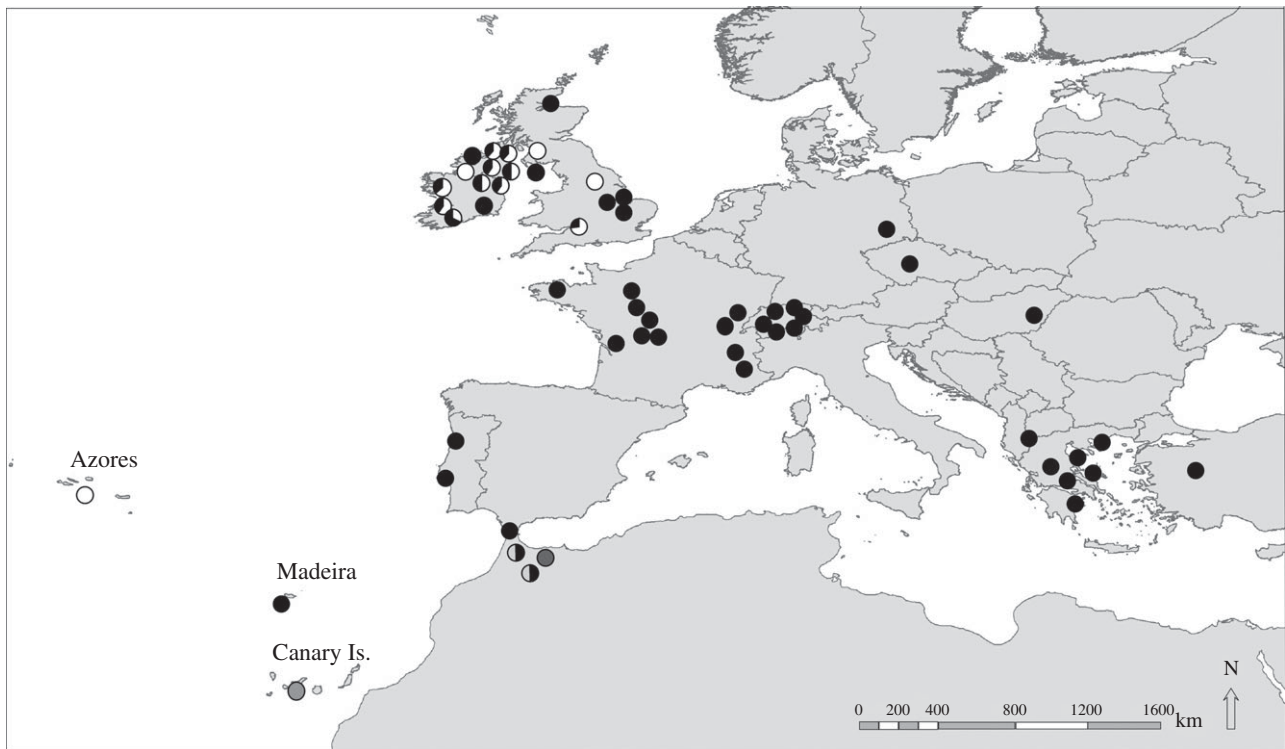


Figure 1. Sampling locations of *N. leisleri*, *N. I. verrucosus* and *N. azureum* used in phylogeographic analysis. Black, white and grey shades in pies represent the proportion of haplotypes belonging to distinct lineages: western (white); eastern (black); Moroccan (Mar1, light grey); Moroccan 2 (Mar2; dark grey); and Canary Islands (Can; medium grey).

Table 1. ISO country code (map ID), number of samples screened for microsatellites (n^n), number of sequences (n^{mt}) and descriptive statistics, including number of haplotypes (nh), number of polymorphic sites, mean number of pairwise differences and haplotype ($h \pm$ s.d.) and nucleotide ($\pi \pm$ s.d.) diversities with standard deviation for each sampling location.

map ID	location	n^n	n^{mt}	nh	poly. sites	mean pairwise	$h \pm$ s.d.	$\pi \pm$ s.d.
IRL	Ireland	138	75	12	18	5.129	0.771 ± 0.036	0.016 ± 0.009
IOM	Isle of Man		7	4	11	3.524	0.81 ± 0.130	0.011 ± 0.007
GBR	Britain		21	6	12	4.485	0.776 ± 0.059	0.014 ± 0.008
	total British Isles		103	15	21	5.106	0.801 ± 0.023	0.016 ± 0.009
FRA	France	145	27	13	15	2.769	0.880 ± 0.044	0.009 ± 0.005
PRT	Portugal		35	6	8	0.947	0.603 ± 0.068	0.003 ± 0.002
ESP	Spain		1	1	0	—	—	—
CHE	Switzerland		7	6	7	2.800	1.000 ± 0.094	0.009 ± 0.006
DEU	Germany		2	2	4	4.000	1.000 ± 0.500	0.013 ± 0.014
HUN	Hungary		2	2	3	3.000	1.000 ± 0.500	0.009 ± 0.010
TUR	Turkey		1	1	0	—	—	—
CZE	Czech Rep		1	1	0	—	—	—
MNE	Montenegro		1	1	0	—	—	—
GRC	Greece		7	5	9	2.571	0.952 ± 0.096	0.008 ± 0.005
	total Mainland Europe		84	29	29	2.227	0.871 ± 0.024	0.007 ± 0.004
MAR	Morocco		4	3	17	10.00	0.833 ± 0.222	0.031 ± 0.021
Can. Is.	Canary Islands		1	1	0	—	—	—
Mad. Is.	Madeira		2	1	0	—	—	—
Az. Is.	Azores		165	16	17	2.762	0.837 ± 0.019	0.009 ± 0.005

subdivision both within and among these regions were evaluated using analysis of molecular variance (AMOVA) as implemented in ARLEQUIN. The statistical significance of the AMOVA was tested

by 10 000 random permutations. To investigate the phylogeographic origin(s) of *N. leisleri* in Ireland in relation to Britain and mainland Europe, and to *N. azureum* and *N. I. verrucosus*,

a haplotypic network was constructed using the median-joining method [38], implemented in NETWORK v. 4.5.0.2 (Fluxus Technology, www.fluxus-engineering.com).

To test for signals of expansion in the recent demographic history of *N. leisleri* and *N. azoreum*, mismatch distribution analysis, using ARLEQUIN, was carried out under a model of sudden (demographic) expansion. The significance of these observed deviations from expectation (i.e. evidence for population growth) was evaluated using the *F*-statistics proposed by Fu & Li [39]. The peak of the mismatch distribution provides an estimate of τ (tau), the starting time of the expansion in units of $1/(2\mu)$ generations. From τ , it is possible to estimate the time (*t*) since the most recent expansion using the equation $\tau = 2\mu t$ [40], where μ is the mutation rate per locus (i.e. the product of the mutation rate per site and the sequence length). We used a range of mutation rates for the control region proposed by Petit *et al.* [41] for the congeneric *N. noctula*, with a generation time of 2 years, a lower rate of 6.3%, a higher rate of 25.2%, with an average of 20% Myr⁻¹. The latter value was used in several other studies, including Salgueiro *et al.* [27] and Chen *et al.* [42]. The approximate Bayesian computation (ABC) approach, implemented in the DIYABC software [43], was also used to test for alternative scenarios for the colonization of Britain and Ireland by *N. leisleri* following the LGM using both microsatellite and mtDNA data (details of this analysis are provided in the electronic supplementary material).

(d) Microsatellite analysis

The Bayesian approach implemented in STRUCTURE v. 2.3.3 was used to examine the extent of concurrent genetic partitioning between Irish and French populations [44]. STRUCTURE was run using an admixture model with correlate allelic frequencies. A burn-in of 5×10^3 was followed by 10^6 iterations over 20 replicated runs for each *K* (1–20). STRUCTURE HARVESTER v. 0.6.1 [45] was used to combine the results files from STRUCTURE and determine the optimal value of *K* using the estimated log-likelihood of *K* according to Pritchard *et al.* [44]. In order to estimate the level of divergence between Irish and French nursery colonies, pairwise *F*_{ST} estimates were calculated using DIVERSITY v. 1.7.0 [46].

(e) Species distribution modelling

To ascertain broadly suitable climatic conditions for *N. leisleri* for both the present day and during the LGM, SDM in conjunction with palaeo-climatic reconstruction was conducted using the maximum entropy approach as implemented in MAXENT v. 3.3.2 [47]. Data for Leisler's bat occurrence, filtered to include only records found within the known distribution range of the species [48], were obtained from the Global Biodiversity Information Facility (GBIF). In total, occurrence data were obtained from 1679 point localities throughout the range of the species. Data were not filtered by season, and thus included some sites used in summer or for hibernation only. Both current (1950–2000) and LGM (Community Climate System Model) global 19 Bioclim variable layers (2.5 arc-min resolution) were downloaded from WordClim—Global Climate Data (www.worldclim.org) and from the Paleoclimate Modelling Intercomparison Project Phase II (<https://pmip2.lsce.ipsl.fr>), respectively. Model performance was evaluated using a randomly selected sample comprising 25% of the occurrence data points. The presence threshold was based on the sensitivity–specificity sum maximization approach [49].

3. Results

(a) Genetic diversity

Levels of mtDNA haplotypic diversity for *N. leisleri* from mainland Europe (0.871 ± 0.024) and the British Isles ($0.801 \pm$

0.023), and those from *N. azoreum* (0.837 ± 0.019), were very similar (table 1). However, nucleotide diversity was almost twofold higher in *N. leisleri* populations from Ireland and Britain than *N. azoreum* (0.016 ± 0.009 versus 0.009 ± 0.005 , respectively). All seven microsatellite loci screened were found to be polymorphic with 3–26 alleles per locus.

(b) Mitochondrial sequence analysis

A 324 bp stretch of the D-loop was sequenced for 147 individual bats. These, in addition to 28 equivalent haplotypic sequences downloaded from GenBank, representing 212 bats, resulted in a total of 359 individual sequences available for analysis. A total of 60 haplotypes were identified in our dataset, with 48% of these ($n = 29$) reported for the first time; these data were submitted to GenBank (see data accessibility information at end of paper). These haplotypes were defined by 64 variable sites, of which 48 were transitions, seven transversions and nine indels. The 22 bp indel found in *N. azoreum* [27] was absent in all *N. leisleri* sequences examined in this study.

Overall, only eight haplotypes (13%) were found in two or more locations, whereas four (*Po1*, *A7*, *Ir1* and *Po2*) were relatively common (i.e. found in three or more locations). One (*Po1*) was widespread throughout Europe, being found in 22% of the samples, and it was the only haplotype to occur in continental Europe, Ireland and Britain. Overall, 87% of haplotypes were restricted to single geographical locations. Surprisingly, *N. azoreum* haplotypes, presumed to be species-specific, were also found in *N. leisleri* from Ireland and Britain. Thus, the *A7* haplotype previously known only in *N. azoreum* from the Azores [28], also occurred in *N. leisleri* from Ireland (7 of 75 individuals), the Isle of Man (three of seven individuals) and Britain (6 out of 21 individuals). Eleven haplotypes (18% of all haplotypes), of which 10 were from Ireland (*Ir1*, *Ir3*, *Ir4*, *Ir5*, *Ir6*, *Ir7*, *Ir8*, *Ir9*, *Ir10* and *Ir11*) and one from Britain (*En2*), were restricted to these islands. The remaining 33 haplotypes (55%) were found in Europe (including Madeira and the Canary Islands) and northern Africa only, whereas 15 additional haplotypes (29%) were restricted to the Azores.

(c) Phylogeographic analysis

The resulting median-joining haplotypic network (figure 2) revealed two very distinct mtDNA haplotypic lineages. One of the lineages comprised haplotypes restricted to the Azores, Ireland and Britain, whereas the second lineage comprised haplotypes which were widespread throughout the rest of Europe, including Ireland and Britain (figure 1). These two major lineages, henceforth referred to as 'western' and 'eastern' lineages, respectively, differ by 2% sequence divergence (i.e. an average of nine nucleotide differences between both lineages), and are characterized by four fixed mutational changes, with a minimum divergence of 1.8% (six nucleotide differences). Haplotype divergence within the eastern lineage was 0.7% (average number of nucleotide differences within lineage = 2.3), whereas for the western lineage, this value was 0.8% (average number of nucleotide differences within lineage = 2.7).

Most *N. leisleri* haplotypes found in Ireland and Britain (*Ir1*, *Ir3*, *Ir4*, *Ir5*, *Ir7* and *Ir11*) were closely related to haplotypes associated with *N. azoreum*, with one haplotype (*A7*) occurring both in *N. azoreum* and in the *N. leisleri* from Ireland and Britain. Within the network, *Po1* was placed in the centre of a typical star-shaped topology of the European lineage, and had the highest frequency and widest geographical

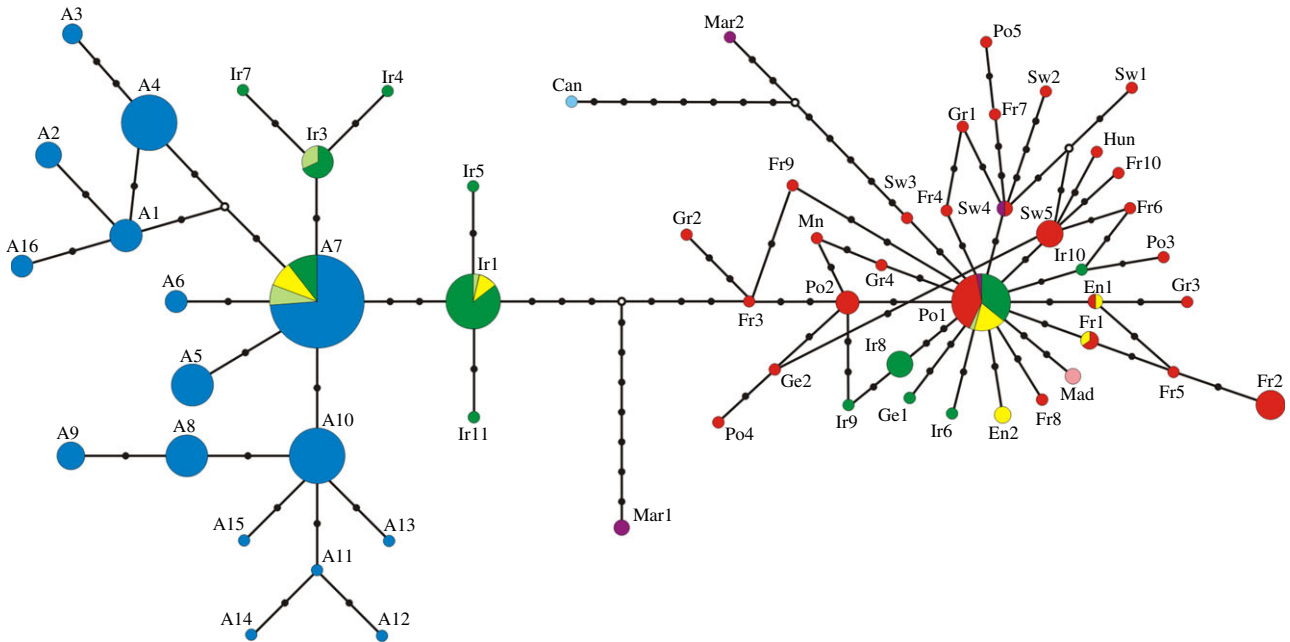


Figure 2. Median-joining network of *N. leisleri*, and *N. azureum* mtDNA haplotypes. Pie sizes are proportional to the number of individuals showing a particular haplotype. Black full circles represent mutational steps; white circles represent inferred haplotypes (not observed in dataset); different colours represent broad geographical haplotype locations (blue, Azores; green, Ireland; pale green, Isle of Man; yellow, England; red, continental Europe; purple, Morocco; light blue, Canary Islands; pink, Madeira) Reticulations for unresolved links between haplotypes are shown.

distribution, which is indicative of its ancestral status in relation to other haplotypes. Four haplotypes unique to Ireland (*Ir6*, *Ir8*, *Ir9* and *Ir10*), and an additional haplotype unique to Britain (*En2*), belonged to the eastern mtDNA lineage, and thus were closely related to the *Po1* haplotype. Two further haplotypes of the eastern lineage were shared between France and Britain, *En1* and *Fr1*. The single haplotype (*Mad*) representing the subspecies *N. l. verrocosus* from Madeira also belonged to the eastern *N. leisleri* lineage. The most divergent haplotypes were found in Morocco (*Mar1* and *Mar2*), and the Canary Islands (*Can1*). Interestingly, *Mar1* and *Mar2* represented two highly divergent haplotypes with 11 fixed differences and 3% sequence divergence between them.

(d) Population structuring

The global exact test for genetic differentiation of mtDNA haplotypes among major regions was highly significant ($p < 0.001$), confirming a considerable degree of genetic structuring among regions. Pairwise F_{ST} comparisons revealed that the majority of this differentiation was observed between regions (i.e. Ireland and Britain, continental Europe and the Azores).

AMOVA between Ireland and Britain, continental Europe and the Azores shows that 45.9% of the observed genetic variation was explained by differences between regions, whereas 38.72% of variation occurred within regions. These findings are supported by the analysis of nuclear microsatellite data between Ireland and France ($F_{ST} = 0.023$, 95% CI 0.015–0.031). The highest probability of the data (i.e. higher estimated log-likelihood) was observed at $K = 2$, clearly separating Irish and French samples (data not shown).

(e) Demographic history

Mismatch distribution demonstrated that both the western ($\tau = 2.71$) and eastern ($\tau = 2.42$) lineages were consistent with a model of sudden demographic expansion. Fu's test was found

to be significant for both western ($F_s = -6.256$, $p = < 0.05$) and eastern ($F_s = -27.386$, $p = < 0.01$) lineages. Assuming an average mutation rate of $20\% \text{ Myr}^{-1}$ (i.e. $\mu = 2 \times 10^{-7} \times 324 \text{ bp} = 6.48 \times 10^{-5}$), the times elapsed from the most recent expansion, using this estimate, were approximately 21 000 (95% CI 10 500–32 000) and approximately 18 600 (95% CI 3000–32 000) years for the western and eastern lineages, respectively. The lower-bound estimates (i.e. $6.3\% \text{ Myr}^{-1}$) for the expansion times for the eastern and western lineages were approximately 69 600 years BP (95% CI 35 000–107 000) and approximately 62 000 years BP (95% CI 10 200–107 500), respectively. The upper-bound estimate ($25.2\% \text{ Myr}^{-1}$) was approximately 16 500 (95% CI 8300–25 500) for the western lineage and approximately 15 000 (95% CI 2400–25 600) for the eastern lineage. Results from the ABC analyses were not entirely consistent. Scenario 1 (i.e. Britain and Ireland diverged from a common ancestor) and scenario 2 (i.e. western and eastern lineages diverged from the common ancestor, whereas Britain and Ireland diverged more recently from the western lineage) had low probability, whereas both scenario 3 (i.e. the Britain and Ireland group diverged more recently from the western lineage) and scenario 4 (i.e. the Britain and Ireland group is the result of an admixture event between the western and eastern lineages) showed the highest probability. Close examination of type I and II errors (data not shown), however, suggests that the current data are insufficient to reliably discriminate among the competing hypotheses.

(f) Species distribution modelling

The predicted geographical distribution for the present day and LGM based on SDM are shown in figure 3. Despite recent criticism [50], MAXENT is thought to be a robust and useful approach for predicting relative occurrence probability [51,52]. Overall, the present-day modelled distribution *N. leisleri* (figure 3a) is in accordance with the known distribution of the species [48]. Discrepancies are most likely to reflect poor data on species

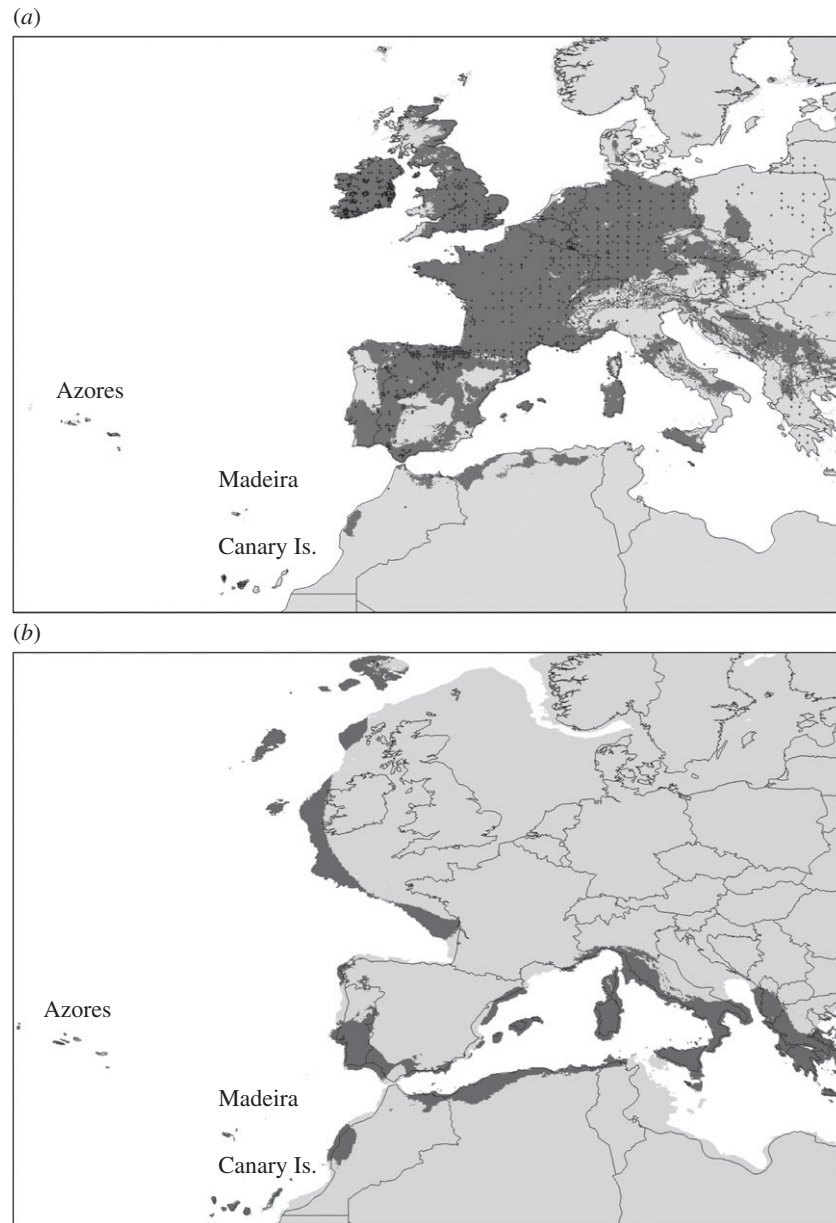


Figure 3. Species distribution model projections of (a) present-day distributions of *N. leisleri* and (b) the distribution during the LGM. Black crosses in (a) indicate *N. leisleri* occurrence data, obtained from the Global Biodiversity Information Facility (GBIF). Dark grey areas in (b) show suitable habitats within the ecological niche of *N. leisleri*, whereas the light grey areas indicate unsuitable habitat.

occurrence/distribution in eastern Europe. Among the 19 Bioclim variable layers, the best predictors for habitat suitability (i.e. accounting for 92.8% of the total contribution to the MAXENT model) were bio19 (precipitation of coldest quarter—21.4%), bio3 (isothermality—17.8%), bio7 (temperature annual range—15.6%), bio6 (minimum temperature of coldest month—15.6%), bio8 (mean temperature of wettest quarter—12.7%), bio4 (temperature seasonality—6.6%) and bio10 (mean temperature of warmest quarter—3.1%).

Projection of the present-day modelled distribution of *N. leisleri* onto reconstructed palaeo-climatic conditions at the LGM (figure 3b) indicates the presence of a number of locations with suitable habitats where the species could have persisted. In addition to well-known glacial refugia (i.e. the south of the Iberian Peninsula, Italy, south of the Alps and the Balkans), other putative regions included parts of northern Spain, the Balearics, northern Africa, Madeira, the Canary Islands and the Azores. Potential terrestrial glacial refuge areas were also identified in now submersed land south of the Bay of Biscay

in France, towards the west/southwest of Ireland, as well as sites probably suitable for hibernation only and as far north as western Scotland and the Faroe Islands.

4. Discussion

(a) Taxonomy and phylogeography

The present results reveal new insights into the taxonomy and evolutionary relationship of the Leisler's bat (*N. leisleri*) and the Azorean bat (*N. azoreum*). Once considered only a subspecies of *N. leisleri* [16], *N. azoreum* has more recently proposed as a good species [17,28]. Analysis of the new molecular data generated in this study, in combination with previous data by Salgueiro *et al.* [27,28], unambiguously demonstrates that the majority of Irish and British *N. leisleri* mtDNA individuals (67%) are represented by haplotypes from the western lineage (i.e. more closely related to *N. azoreum* haplotypes than to any other haplotypes from *N. leisleri* from continental Europe). Thus, from an

mtDNA perspective, *N. azoreum* does not represent a monophyletic group, and hence does not meet the phylogenetic species criterion.

We demonstrate that both the western and eastern mtDNA haplotypic groups represent distinct evolutionary lineages, which probably diverged in allopatry in separate glacial refugia. Based on the results of the network analyses, Ireland and Britain are likely to comprise a zone of secondary contact and admixture between these two lineages. While this is not supported by the ABC analysis, this approach, as applied to our dataset (i.e. combination of samples and molecular data available for analyses), did not discriminate among competing colonization scenarios. The presence of unique derived haplotypes representing both lineages in Ireland and Britain only suggests that this secondary contact is of some antiquity and no longer occurs. Thus, excluding the common eastern lineage *Po1*, the putative ancestral haplotype, and two further haplotypes that occur in Britain (*En1* and *Fr1*), none of the other eastern haplotypes found in Ireland and Britain (*Ir6*, *Ir8*, *Ir9*, *Ir10* and *En2*) are found in mainland Europe. While this could be partially attributed to small sample size, the occurrence of geographically restricted haplotypes is also compatible with new mutations occurring since the time of expansion, which is estimated at between 10 516–32 114 years BP for the western lineage and between 3070–32 268 years BP for the eastern lineage using the average mutation rate of 20% Myr⁻¹, coinciding with the LGM, which peaked some 20 000 years BP. Furthermore, *N. leisleri* is characterized by long migrations in mainland Europe. Thus, even considering small sample sizes per location, our sample size ($n = 186$) over all of Europe is sufficient to argue against sample size bias. Colonization of the Azores archipelago by early ancestors of *N. azoreum*, in agreement with Salgueiro *et al.* [27], is most likely to have occurred from a single source of colonists, given the widespread distribution of the haplotype *A7* throughout Ireland, Britain and the Azores. An additional colonization event took place subsequently in the British Isles, involving ancestors of the eastern European lineage (probably represented by the *Po1* haplotype).

The importance of the Balkans, Italy and the Iberian Peninsula as glacial refugia for northern temperate species during the LGM has been well documented [53,54], and is supported here by SDM of the past climatic conditions for *N. leisleri*. Fossil remains of *N. leisleri* from the middle and late Pleistocene have been found in southern Spain [55], whereas fossil remains of *N. noctula* and *M. myotis* have been found in the Balkans [56,57]. Thus, given the current geographical distribution of the eastern lineage, with no strong signal in the current dataset, *N. leisleri* may have inhabited any one of these refugia during the LGM. The SDM results also suggest the possibility of refugia in northern Africa, northern parts of Italy, Spain and the Balearics. The current distribution of the western lineage, however, is hard to explain by invoking any of these three accepted glacial refugia, given the apparent absence of haplotypes from this lineage in continental Europe. The Iberian Peninsula, being the most westerly of these refugia, would be the most probable location. In support of this, the ‘Lusitanian flora and fauna’ in Ireland proposed by a number of authors [9] links some 15–20 extant species between southwest Ireland and Iberia. In this study, however, no haplotypes of the western lineage were found in Iberia, despite a good sample size ($n = 36$) and geographical coverage of the area, arguing against this region as the refuge for this lineage.

One possible explanation for the absence of this lineage in western Europe is replacement by the expanding eastern lineage. A hypothesis was proposed by Martínková *et al.* [10] investigating the phylogeography of the Irish stoat (*Mustela erminea hibernicus*). These authors suggested that a cold tolerant lineage that initially colonized Britain and Ireland was replaced in Britain by an expanding lineage from Europe, which was assumed to have a fitness advantage under warmer environmental conditions. Piertney *et al.* [58] and Searle *et al.* [59] have used the same mtDNA replacement hypothesis to explain the Celtic fringe distribution seen in many British mammals, including the pygmy shrew (*Sorex minutus*), bank vole (*Clethrionomys glareolus*), field vole (*Microtus agrestis*) and water vole (*Arvicola terrestris*), which is strikingly similar to that described for the Celtic people [60]. In this study, however, the coexistence of both western and eastern lineages of *N. leisleri* in Ireland and Britain, and the fact that according to microsatellite analysis they constitute a single population, argues against mtDNA replacement to explain the absence of the western lineage in continental Europe.

Alternatively, there are several potential histories involving cryptic glacial refugia for the western lineage of *N. leisleri* during the LGM which are independently supported by palaeo-climatic SDM. It is possible that the Irish and British populations originated from populations that diverged in an Azorean refugium where suitable habitat was available according to the SDM. While active dispersal from the Azores to Ireland and Britain is unlikely given the over-water distances involved, it is possible that passive dispersal may have occurred as a result of the prevailing winds in the North Atlantic.

Furthermore, suitable habitats along the coastal regions of western Europe in areas now submerged were identified by SDM methods. The influence of ice loading on the land masses [61] would have meant that ice-free, dry land extended several hundred kilometres into the Atlantic Ocean and Celtic Sea. Exposed land along the coast of western Europe including southern France, the Bay of Biscay and land to the southwest of Ireland, according to our model, would have provided suitable habitat for *N. leisleri* throughout the LGM. In support of this hypothesis, both southern France and the Bay of Biscay have been proposed as potential refugia by a recent study modelling the past geographical distribution of another European bat species, *Plecotus austriacus* [62]. The existence of refugia in northwestern Europe is also supported by the presence of several tree species [63] in the region during the LGM. Furthermore, Mitchell [64] suggests that some tree species colonized Ireland as early as 9500 years BP, supporting the existence of a nearby, forest glacial refugium. Another clue comes from the study of the sibling species *N. noctula* by Petit *et al.* [41]. In their analysis of the mtDNA Dloop of *N. noctula* populations in Europe, they reported on the occurrence of highly differentiated haplotypes (i.e. long-branch haplotypes) among specimens collected in both the UK and Germany. Although their sample size was small, the presence of these haplotypes suggested the existence of highly divergent lineages among the mtDNA Dloop of *N. noctula*. These lineages restricted to western Europe lend weight to the hypothesis of novel cryptic western European refugia for bats. A northwesterly refugia, particularly along the Atlantic coast, could also help to explain why many Irish flora and fauna are genetically distinct and diverse from British and continental European counterparts, including the stoat [10], the natterjack toad [65] and the brown trout [66,67].

(b) Genetic diversity and contemporary gene flow

Regional differentiation in mtDNA indicates limited gene flow between populations of *N. leisleri* across the major geographical areas of Ireland and Britain, mainland Europe and the Azores. This is supported by 42% of the observed genetic variation being explained by differences between these major geographical regions. Results from the nuclear microsatellites amplified for samples from sites in Ireland and France support this, as does the apparent lack of large scale migratory movement observed among populations in Ireland [26], and the timing and number of records of *N. leisleri* recorded on islands in the eastern North Atlantic [68]. Furthermore, this is supported by the reported morphological and behavioural differences in *N. azoreum* [18,19]. We suggest that *N. azoreum* and *N. leisleri* populations from the Azores and continental Europe comprise at least two distinct evolutionary significant units that display distinct genetic diversity, and possibly adaptive features, whereas Britain and Ireland represent a zone of admixture. Owing to a lack of contemporary gene flow, these three areas should be considered separate management units.

(c) Concluding remarks

We demonstrate that populations of *N. leisleri* in Ireland and Britain probably recolonized following the LGM from two distinct glacial refugia. One lineage is consistent with the hypothesis of colonization from a southern European refugium, with no strong evidence to support colonization across a land bridge with Britain. The second lineage is inconsistent with a southern European origin, and thus we cannot refute a hypothesis for the existence of cryptic glacial refugia in western Europe. There is limited contemporary gene flow between recent populations of *N. leisleri* in continental Europe, Britain and Ireland. This indicates that after the

LGM, dispersal of lineages of this species was conservative, underscoring the role of a cryptic refugium in the western isles, and explaining the current distribution of lineages. The post-glacial history revealed here with regard to *N. leisleri* may be applicable to other species that are dependent on deciduous woodland and were able to colonize Britain and Ireland rapidly after the LGM. We also conclude that there is no genetic support for species status for the endemic *N. azoreum*, but that the genetic variation apparent in its populations may play a vital role in securing its future against the challenge of rapid climatic change, and thus is worthy of independent conservation consideration.

Ethics statement. All bats were caught and sampled under licence of the Northern Ireland Environment Agency, the National Parks and Wildlife Service and the Republique Francaise (Prefecture de la Côte-d'Or and Prefecture de la Nièvre).

Data accessibility. DNA sequences for each new haplotype identified in this study are available on GenBank (accession nos. KP462690–KP462718). Microsatellite data (GENEPOP format) are available for download on Dryad (doi:10.5061/dryad.6g6r6).

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