Next-generation phylogeography resolves post-glacial colonization patterns in a widespread carnivore, the red fox (*Vulpes vulpes*), in Europe.

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Abstract

Carnivores tend to exhibit a lack of (or less pronounced) genetic structure at continental scales in both a geographic and temporal sense using various mitochondrial DNA markers on modern and/or ancient specimens. This tends to confound the identification of refugial areas and post-glacial colonization patterns in this group. In this study we used Genotyping-by-Sequencing (GBS) to reconstruct the phylogeographic history of a widespread carnivore, the red fox (*Vulpes vulpes*), in Europe by investigating broad-scale patterns of genomic variation, differentiation and admixture amongst contemporary populations. Using 15,003 single nucleotide polymorphisms (SNPs) from 524 individuals allowed us to identify the importance of refugial regions for the red fox in terms of endemism (e.g. Iberia) and sources of post-glacial re-expansion (e.g. Carpathians and Balkans) across northern regions of the continent. In addition, we tested multiple post-glacial re-colonization scenarios of previously glaciated regions during the Last Glacial Maximum using an Approximate Bayesian Computation (ABC) approach. We identified the role of ancient and temporary land-bridges in the colonization of Scandinavia and the British Isles, with a natural colonization of Ireland deemed more likely than an ancient human-mediated introduction as has previously been proposed. Using genome-wide data has allowed us to tease apart broad-scale patterns of structure and diversity in a widespread carnivore in Europe that was not always evident from using more limited marker sets.

Introduction

Over the last 30 years, phylogeographic studies have highlighted the roles of major past climatic and geophysical events in shaping contemporary genetic structure and diversity in a multitude of species (1–3). During the Last Glacial Maximum (LGM; ~27-19 thousand years ago (kyrs BP) (4)) many terrestrial plant and animal species retreated, and were often restricted, to refugial areas (2,5). In Europe, phylogeographic studies of the most widely studied group, the terrestrial mammals, have shown distinct mitochondrial DNA (mtDNA) lineages in small mammals (6) and ungulates (7) that are consistent with contraction and re-expansion from refugial regions (2).

Carnivores appear to be an exception to this general pattern however, with either a lack of, or less pronounced phylogeographic structure shown across continental scales (8-11). One such carnivore, the red fox (*Vulpes vulpes*), is well-represented in the fossil record in Europe (12) and has numerous records during the LGM in recognized refugial areas such as the Mediterranean peninsulas, and further north in areas in or adjacent to the Carpathian mountains, and the Dordogne region in France (5,12). Previous studies using various mtDNA markers on modern and/or ancient specimens have revealed a general lack of genetic structure on a continental-wide scale, in both a geographic and temporal sense (13–15). The lack of phylogeographic structuring in the red fox and other carnivores has been previously attributed to these species persisting outside the traditional refugial areas during the LGM, and effectively existing as a large interbreeding population on a continental scale (13,14). However, despite the abundant carnivore fossil data, there is a distinct lack of fossils from central Europe or further north during the LGM (12,16).

Alternatively, long-range gene flow between isolated refugia could explain the lack of phylogeographic structure in these species (8). More recent studies (17,18) identified mtDNA haplotypes that were unique to particular regions (e.g. Iberia) that potentially indicate long-term separation from other European populations.

The concerns about the use of short mtDNA sequences is that they may not fully capture the signals of retraction and re-expansion in species with high dispersal capabilities (19). One solution is the utilisation of microsatellite markers in conjunction with mtDNA data (18). However, several carnivore species (e.g. badgers *Meles meles* and otters *Lutra lutra*) show a similar lack of resolution in terms of broad-scale genetic structure across continental Europe using microsatellites (10,11). For the red fox, several distinct groups in Europe were identified using microsatellite markers from Bayesian clustering analysis, with distinction between foxes in Ireland, Britain, Spain, Italy and Scandinavia being apparent (18). The rapid mutation rate of microsatellites leaves it unclear whether divisions reflect ancient isolation or more recent population structure, owing to recently limited gene flow. The advent of next-generation sequencing technologies holds great promise for phylogeographic studies, allowing for thousands of single nucleotide polymorphisms (SNPs) to be genotyped in non-model organisms and providing a representation of the organism's entire genome (20,21). The use of reduced-representation techniques (e.g. genotyping-by-sequencing, GBS) has already demonstrated their potential in resolving phylogeographic patterns in non-model organisms that are not fully captured with data with a limited number of genetic markers (22,23).

Using GBS data from over 500 individuals, the purpose of the present study was to reconstruct the phylogeographic history of the red fox in Europe by investigating broad-scale genomic variation, differentiation, and admixture amongst contemporary populations. From there, we simulated different phylogeographic scenarios within an Approximate Bayesian Computation (ABC; (24)) framework to distinguish between multiple post-glacial re-colonization scenarios of previously glaciated regions that were not fully resolved in previous studies using mtDNA, Y chromosomal, and microsatellite markers.

Materials and Methods

Laboratory methods

Red fox tissue samples were obtained from freshly culled (not directly related to this study), roadkill, frozen, ethanol- (70-95%) and DMSO-preserved material. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Ltd.) according to manufacturer's protocols (with the additional treatment of RNase). A total of 30–100 ng/µl of high molecular weight DNA was sent to the Genomic Diversity Facility in Cornell University (USA) where GBS was used for constructing reduced representation libraries (25) using the restriction enzyme EcoT22I (ATGCAT). Further details of the library preparation are provided in the Supplementary Material.

Bioinformatics

The raw Illumina sequence data from 568 individuals were processed through the GBS analysis pipeline implemented in TASSEL v5.2.31 (26). Due to concerns about the performances of *de novo* approaches to identify SNPs in reduced representation genomic techniques (particularly for demographic analyses (27)) the reference genome of the domestic dog (CanFam3.1; *Canis lupus familiaris*) was used to align the sequence tags on individual chromosomes using the Burrows-Wheeler alignment tool (28).

A total of 15,003 SNPs in 524 individuals (Table S1) remained after bioinformatic filtering in TASSEL and PLINK v1.07 (29), the removal of SNPs in linkage disequilibrium and deviating from Hardy-Weinberg equilibrium (within populations), and outliers putatively under selection (see Supplementary Material for further details of all filtering steps). Two datasets were created for subsequent

analyses; one containing all 524 individuals (*524dataset*; Table S1), and the other containing 494 individuals (*494dataset*; Tables S1 and S2), consisting of 29 'populations' with seven or more individuals for population-level analyses (Fig. 1A-C).

Individual-based analyses

Individual-based clustering analysis on the *524dataset* was conducted in fastSTRUCTURE v1.0 (30). fastSTRUCTURE was performed using the simple prior with *K* values of 1–30 over five independent runs. The number of clusters (*K*) was obtained by using the 'chooseK.py' function on each of these independent runs. Visualization of individual assignments to clusters per population was initially performed using the 'distruct.py' function, with the final figure produced using a custom R script.

Population-based analyses

For the *494datset*, two measures of genomic diversity, allelic richness (AR) and expected heterozygosity (H_E), were calculated in GENODIVE (31) and HP-RARE (32). AR and H_E values were mapped with interpolation using ArcGIS 10.2.1. Geostatistical Analyst. One population from Siberia (Russia) was excluded from this analysis because it was geographically distant from all the other populations. Interpolation was carried out using an Inverse Distance Weight model (IDW, power=1, based on 12 neighbours; (33)).

Discriminant Analysis of Principal Components (DAPC) as implemented in the R package *adegenet* v2.0.1 (34) was performed on the *494dataset*. DAPC does not

make assumptions about Hardy-Weinberg equilibrium or linkage disequilibrium and provides a graphical representation of the divergence among pre-defined populations/groups. To infer the evolutionary relationships and admixture among red fox populations, we used the maximum-likelihood method implemented in TREEMIX v1.12 (35). This software estimates a population tree based on SNP allele frequency data and allows for admixture to be incorporated into the tree building process. The length of a branch is therefore proportional to the amount of genetic drift that occurred along the branch.

Phylogeographic reconstruction

Approximate Bayesian Computation was implemented in DIYABC v2.1.0 (36,37) to further investigate the dynamics of the re-colonization process of red foxes in Europe. We followed the approach of Kotlík et al. (38) where all SNPs were used, and using a tree-based classification method known as 'random forest' in ABC allowing demographic scenarios to be distinguished based on 1,000s to 10,000s of simulations for each scenario (38–40). We randomly chose a subset of the individuals in each grouping to save on computational time (Table S1).

Following on from outstanding issues in relation to unresolved colonization scenarios (18) we first investigated the colonization history of red foxes in the British Isles. Two scenarios were incorporated between which the data and analysis presented by Statham et al. (18) could not distinguish between. The first was that Ireland was colonized naturally overland from Britain after the end of the LGM (i.e. before Ireland became an island between approximately 19 and 15 kyrs BP; Fig. S1). In the second scenario, Ireland's red foxes were transported to the island from

Britain after the earliest evidence of human presence on the island at 12.7 kyrs BP (41) right up to the present day (Fig. S1). For the second ABC-based analysis, we investigated the colonization history of Scandinavia. It was proposed that Scandinavia was colonized from multiple sources based on mtDNA and Y chromosome data but there was no attempt to date the progression or order of these events (42). In the first of these, 'Scandinavia' was the result of admixture in the east and subsequent colonization between 'Central Europe' and 'Russia' populations after the region became ice-free after the Younger Dryas Glaciation (12.9–11.7 kyrs BP) and disappearance of land-bridges connecting it to central Europe (43; Fig. S2). In the second scenario, the first colonization wave occurred from central Europe over land-bridges (14 to 9 kyrs BP), with later admixture occurring from Russia (9 kyrs BP to present). In the third scenario, the first colonization wave occurred from Russia (9 kyrs BP to present), with later admixture from Central Europe from an eastern route (9 kyrs BP to present but restricted to after the first Russian colonization wave; Fig. S2). To check the reliability of the observed summary statistics for both ABC-based analyses, a Principal Component Analysis (PCA) was performed on the summary statistics from the simulated datasets and compared against the summary statistics from the observed dataset in order to evaluate how the latter is surrounded by the simulated datasets (Fig. S3). A more detailed description of the ABC-based analyses is provided in the Supplementary Material.

Results

Individual-based analyses

fastSTRUCTURE identified *K* = 7 as the lower limit of clusters in each of the five independent runs of *K* = 1–30 (Fig. 1C), with the upper limit of *K* fluctuating from 10-13 between runs. Focusing on *K* = 7, distinct clusters were identified in each of Ireland ('Ireland') and Great Britain ('Britain'). Iberian populations formed a distinct cluster ('Iberia'). Populations in France, Switzerland, Belgium, Germany, Poland, Slovenia, Croatia, Serbia and the Ukraine formed a distinct cluster ('Central Europe', named for simplicity because of the approximate location of the cluster relative to the other clusters). Localities with small numbers of individuals in Lithuania, Estonia, Belarus and western Russia also belonged to this cluster. These populations in eastern Europe showed evidence of admixture with individuals from Scandinavia (Figs. 1A and 1C), who formed another distinct cluster ('Scandinavia'). Individuals from European Russia were admixed between this Scandinavian cluster and individuals from Siberia (another distinct cluster; 'Siberia'). Finally, individuals from central Italy formed a distinct cluster ('Italy'), with individuals in northern Italy showing admixture with the central European group (Figs. 1A and 1C). For *K* = 8 revealed an additional cluster with the European Russian populations and for *K* = 9, French, Belgian and Swiss populations formed another cluster (with admixture from Central Europe; Fig. S4). Further admixture was identified within populations in Central Europe at *K* = 10–13 (data not shown).

Population-based analyses

Direct estimates of genomic diversity (Table S2) and the IDW interpolation of allelic richness (AR) and expected heterozygosity (H_E) in 28 fox populations showed that

diversity is highest in Central and Eastern Europe and decreases westwards and northwards (Fig. 2). Genomic diversity was notably lower in the British Isles, with the Irish populations showing the lowest levels of diversity (Fig. 2). The DAPC revealed distinct groupings of Iberian samples, Irish samples, British samples, Siberian and Scandinavia/Russian samples in general agreement with the individual-based analysis in fastSTRUCTURE (Fig. 1B). Populations in western, central and eastern Europe were grouped closely together, but the populations in France, Belgium and Switzerland were more separated from the main European group on the first axis, aligned with the individual-based Bayesian analyses at K = 9 (Fig. S4). Although the population in central Italy formed its own genomic cluster in the individual-based analysis (Fig. 1C), it grouped more closely with the central European group than the French, Belgian and Swiss populations in this analysis (Fig. 1B).

The topology of trees generated by TREEMIX were consistent across the independent runs. The Central European populations are grouped together as shown in fastSTRUCTURE. As demonstrated in the DAPC analysis, France, Belgium and Switzerland are grouped together, with the British and Irish populations stemming from these (Fig. S5). The long branch lengths of the Irish and British populations are reflective of their isolation and subsequent drift as island populations. The Italian and Iberian populations are closely grouped, and the Scandinavian and Russian populations are grouped similarly to the DAPC analysis. In terms of admixture, having zero migration edges explained 97.4% of the model's variation, with this rising to 99.2% when 10 migration edges were included. Patterns of admixture were consistent with results from fastSTRUCTURE and DAPC, with

populations close to each other geographically showing more admixture (Figs. 1 and S5).

For the ABC-based analysis, first focusing on the colonization history of the British Isles, scenario 1 (Fig. S1) was chosen as the most likely ($p = 0.748$). The ancestral population for Ireland and Britain was estimated to have split from the European mainland at 17.1 kyrs BP (95% CI: 15.32–18.86 kyrs BP), with the Irish population originating at 15.64 kyrs BP (95% CI: 15.2–17.88 kyrs BP; Fig. 3; Table S3). For Scandinavia, scenario 2 (Fig. S2) was chosen as the most likely ($p = 0.452$). The initial split of Scandinavia from the Central European population was estimated at 13.06 kyrs BP (95% CI: 10.02–13.98 kyrs BP) and then subsequent admixture with foxes from Russia was estimated at 3.46 kyrs BP (95% CI: 0.092–8.32 kyrs BP; Fig. 3; Table S3).

Discussion

In this study, we provided a genome-wide assessment of population structure and diversity in the red fox in Europe. By incorporating over 15,000 SNPs and over 500 individuals, we were able to advance previous work by investigating broad-scale patterns of structure and variation to identify putative glacial refugia and post-glacial re-colonization patterns in this widespread species.

Phylogeographic structure of the red fox in Europe

Individual- and population-based analyses revealed congruent patterns of genomic structuring at the broad scale of Europe (and Siberia), with certain important nuances being revealed by different approaches. (Fig. 1A, 1B and S4). Earlier studies had proposed that red foxes may have existed as a single, large panmictic population during the LGM based on a lack of distinct structure at mtDNA markers using modern and/or ancient DNA (13,14). If this was the case, we might have expected to find a more continuous population (excluding the islands potentially) and this is not evidenced here with this greatly expanded dataset in terms of genetic markers, individuals and spatial coverage. In addition, a continuous population over the whole continent at the LGM is not generally congruent with the fossil data and the lack of fossil records beyond the more accepted refugial regions (5,12). Our study demonstrates that the observed patterns of genomic variation in contemporary red fox in Europe were mainly shaped by distinct refugial populations, with subsequent post-glacial admixture and isolation when this species had expanded into what is now its current distribution range in Europe (12).

Within continental Europe, most of the central European (defined here as those outside of the Mediterranean peninsulas and Scandinavia) and Balkan populations formed a single genomic cluster at *K* = 7 (Figs. 1A and 1C). This and the elevated values of genomic diversity (Fig. 2) are likely reflective of a more widespread and connected populations occupying the Balkans and Carpathian regions during the LGM (as is known from the fossil records;(5)) and a subsequent expansion into the rest of central Europe in the post-glacial period. A similar scenario has been proposed for other large mammals (10,44). At *K* = 7, French, Belgian and Swiss individuals were grouped with other central European populations but population-level analyses (DAPC and TREEMIX) showed that these populations were distinct from other populations in close proximity (and they formed their own cluster at *K* = 9 in the individual-based Bayesian analysis; Fig. S4). Fossil records of the red fox are known from the Dordogne region in France during the LGM (5) so these populations may stem from a previously isolated refugial population in the area, and now show post-glacial admixture with populations stemming from eastern/Balkan and Iberian refugia (Figs. 1B and 1C). The Iberian populations form a distinct cluster/group (Figs. 1B and 1C) and this is in line with previous findings using fewer molecular markers. Statham et al. (18) identified mtDNA haplotypes that were endemic to the region, while microsatellites identified Spanish individuals as being distinct from those in other European populations. A similar pattern was found previously in badgers, with Iberian populations having many unique mtDNA haplotypes not found elsewhere on the continent (10). The Pyrenees Mountains have remained a formidable barrier for post-glacial re-colonization, and there appears to be little contribution to subsequent northwards expansion when the ice-sheets receded for many terrestrial species (45). Even though the maximum

dispersal capabilities of the red fox are up to 1,000 km in Europe (46), this mirrors the pattern of mountains acting as significant barriers for the species in North America (47). This is in contrast to red foxes in the other Mediterranean peninsula, Apennine (Italy). Although red foxes from central Italy are identified as a distinct cluster in fastSTRUCTURE, admixture was identified with neighbouring populations north of the Alps in central Europe and the Balkans (Figs. 1B and 1C).

Glaciated regions during the LGM such as the British Isles and Scandinavia present differing problems in terms of how contemporary populations of terrestrial species colonized these areas in post-glacial periods. The island of Ireland has long presented a biogeographical quandary in terms of how and when terrestrial species colonized it (48). It has existed as an island for approximately 15,000 years (almost twice as long as Britain; 49) and humans have been proposed as the primary mechanism of transport for its mammalian fauna in ancient and modern times (10,48). An estimate of 10.2 kyrs BP was estimated for a split between Irish and British red foxes using mtDNA data, but with a 95% CI range that incorporated the possibility of natural colonization before Ireland became an island (18). Both Irish and British populations are distinct from mainland European populations (Figs. 1B and 1C) and have patterns of diversity and structure consistent with colonization and subsequent isolation (Table S2; Figs. 1B and 2). Using an ABC-based approach, a scenario in which Ireland was colonized before humans were known to be present (approximately 15 kyrs BP) was deemed the most likely (Fig. 3; Table S3). Although this conflicts with the current fossil evidence where the oldest known specimen in Ireland is from the Bronze Age (approximately 3.8 kyrs BP; (12)), this is congruent with previous studies demonstrating high haplotype diversity and the identification of

many unique haplotypes at mitochondrial markers on the island (14,18). Another carnivore, the stoat (*Mustela erminea*) was proposed to be an early colonizer of Ireland over a post-glacial land bridge from Britain inferred from molecular dating and fossil data (50) and several potential prey species (e.g. mountain hare *Lepus timidus* and arctic lemming *Dicrostonyx torquatus*) were also present in the early post-glacial period. While humans were an important factor in determining later faunal assemblages on Ireland (10,48), the early post-glacial period clearly warrants further investigation on the island (41,48).

Although glacial refugia in Scandinavia during the LGM have been proposed for several species including mammals (51,52), the red fox does not appear in the fossil records in southern Scandinavia until after ~9,000 yrs BP (12). Based on evidence from mtDNA, microsatellites and Y chromosome data, multiple colonization events have been proposed from the south and east (42,53) but the progression of these events remain untested prior to this study. Here, we examined three different hypotheses for the colonization of Scandinavia (Fig. S2). The most likely scenario for the colonization of Scandinavia is that first colonization wave occurred from central Europe over land-bridges approximately 13 kyr BP (95% CI: 10–14 kyrs BP), with later admixture occurring from Russia in the east at approximately 3.5 kyr BP (95% CI: 0.09–8 kyrs BP; Fig. 3). Southern Scandinavia was ice-free during the Younger Dryas (12.9–11.7 kyrs BP; (54)) and early colonization over temporary land-bridges (which existed up until approx. 9 kyrs BP; (43)) has now been proposed for several mammals (10,43). When the ice retreated from northern Scandinavia after the Younger Dryas, a lack of geographic barriers led to later dispersal into the region from the east (42,53), a pattern that is evident in other carnivores also (19,55).

Using genome-wide data allowed us to tease apart broad-scale patterns of structure and diversity in a widespread carnivore in Europe that was not evident from more limited marker sets. The use of genomic data allowed us to identify the importance of refugial regions in terms of both endemism (e.g. Iberia) and sources of post-glacial re-expansion across the continent (e.g. the Carpathians and Balkans). In conjunction with ABC-based analyses, we identified the potential role of ancient and temporary land-bridges in the colonization of Scandinavia and the British Isles. Given the genomic resources now available (56), the application of ancient genomics on the extensive fossil material available for this species (12) should fall into line with other charismatic carnivores (57) to fully understand re-colonization and temporal patterns that have not been captured in previous studies of ancient red fox specimens (13,14).

Competing interests

We declare we have no competing interests.

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Author contributions

JMW, ADM, RK, JM, CP and ER conceived and acquired funding for the study. ADM, JMW, RK, MJS, BNS, CP and ER designed the study. MJS, ACF, KN, EÅ, JL, MB, CF, PS, DGT, MS, AG, MI, MP, AK, IMO, AS, BP, KF, VL, SWRH, DC, ER, BNS and RK contributed samples and data towards the study. IR performed the laboratory work. ADM, AR-G and IC generated the final SNP panel. ADM, IC, SSB, LR and JMW designed and performed the data analyses. ADM, JMW, IC, JS and SSB wrote the manuscript, with all authors contributing to edits and discussion.

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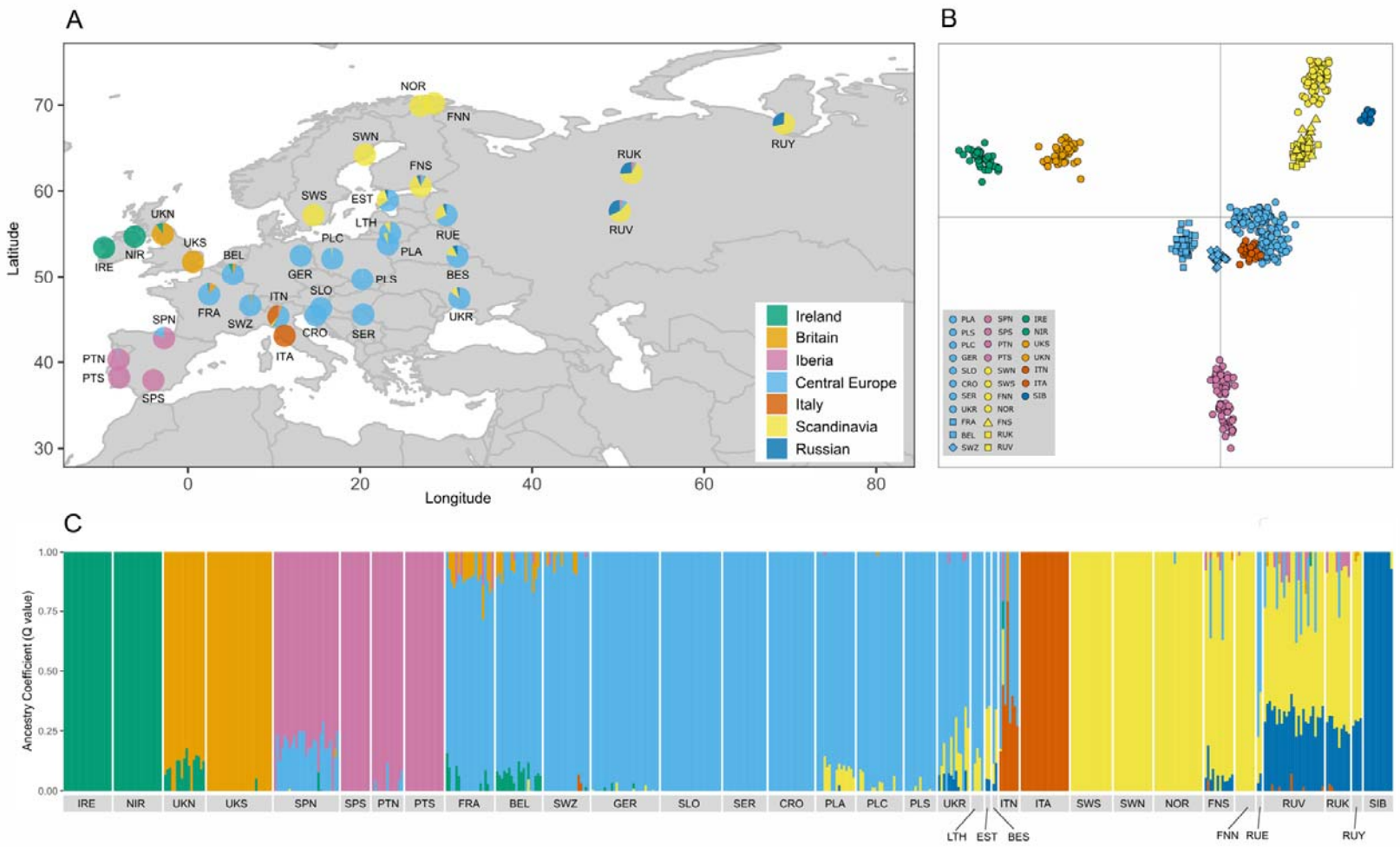

Figure 1. Approximate locations of the studied populations (A) and the genomic clusters to which they have been assigned based on Discriminant Analysis of Principal Components (DAPC; B) and Bayesian analysis in *fastSTRUCTURE* at $K = 7$ (C). The proportion of admixture in each population (A) is based on the ancestry coefficients determined in *fastSTRUCTURE* (C).

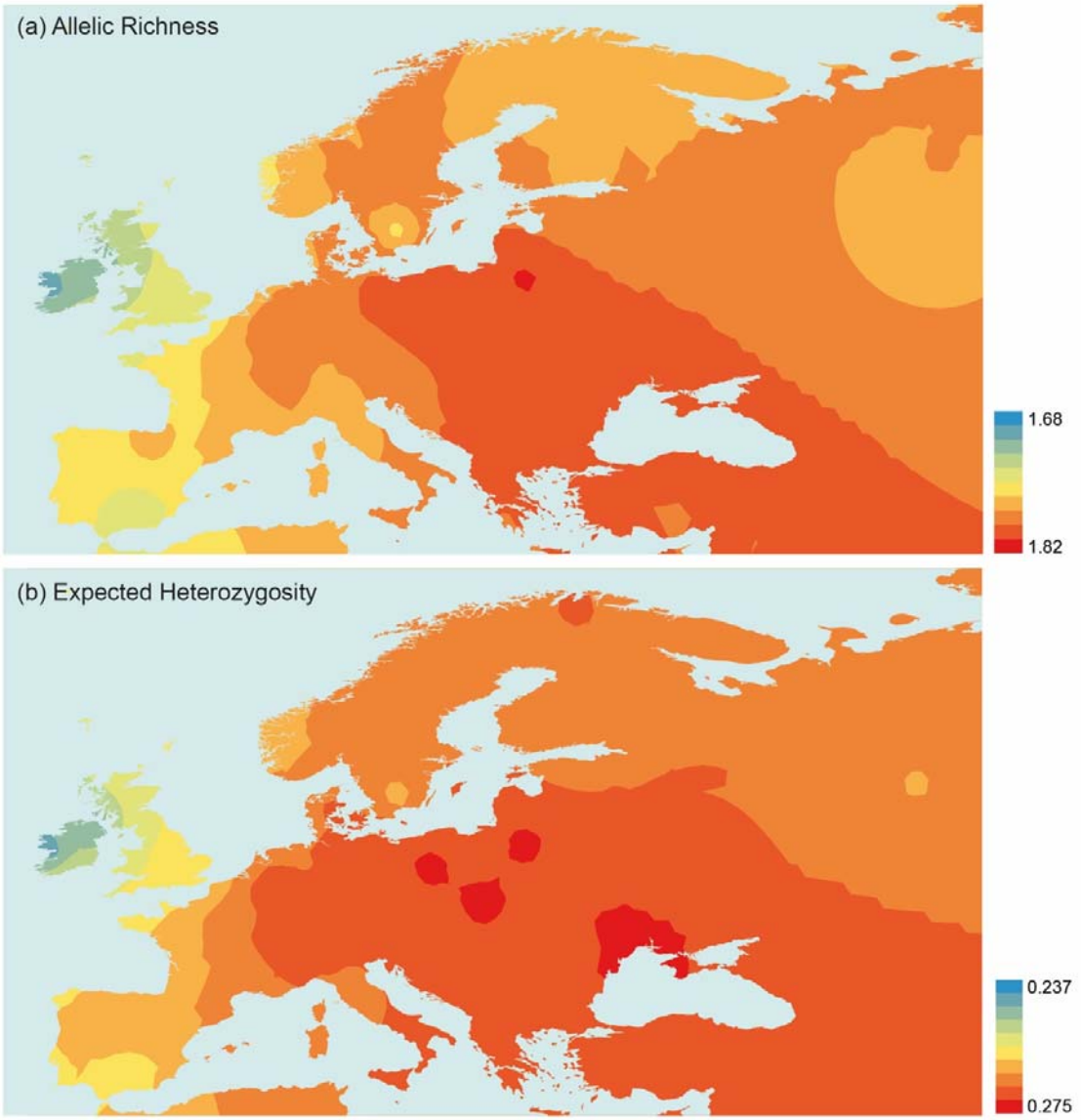

Figure 2. Interpolation of allelic richness and expected heterozygosity in 28 red fox populations (Siberia was excluded in this analysis) in Europe. The interpolated values of both indices are presented in the maps in different colours on a low (blue) to high (red) scale according to the legends.

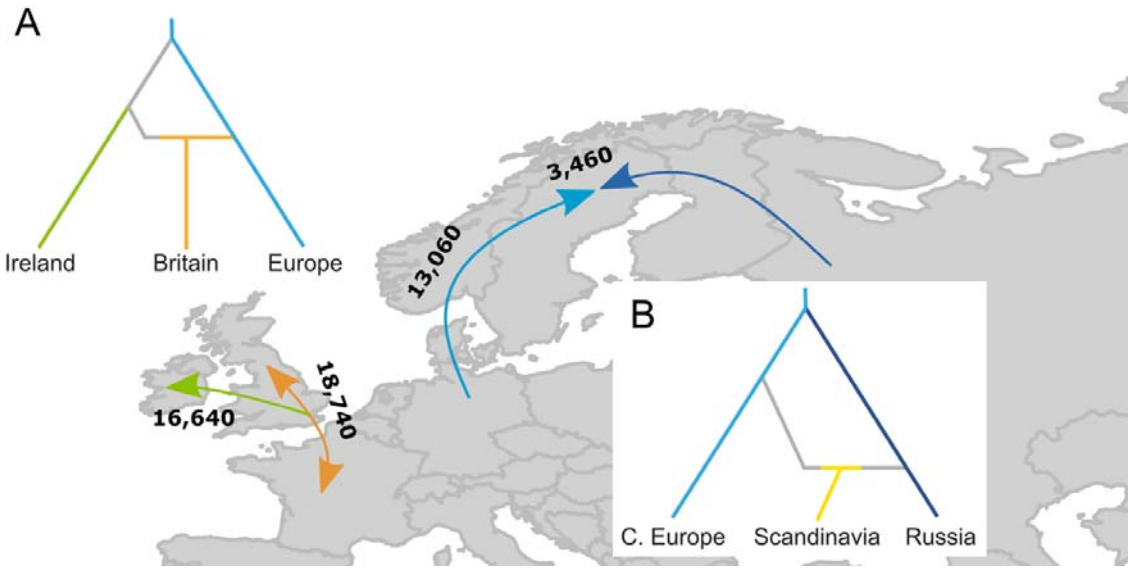

Figure 3. Graphical representation of the most likely post-glacial colonization scenarios for the British Isles (A) and Scandinavia (B) inferred from Approximate Bayesian Computation. Arrows on the map represent the median timing of these events in years before present.