Title: Genetic analyses of European red foxes reveals multiple distinct peripheral populations and central continental admixture

Keywords: mitochondrial DNA, nuclear DNA, Holocene, Pleistocene, phylogeography, Spain, Ireland, Britain, Scandinavia, *Vulpes vulpes*

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Abstract

Temperate terrestrial species in Europe were hypothesized to have been restricted to southern peninsular refugia (Iberia, Italy, Balkans) during the height of the last glacial period. However, recent analyses of fossil evidence indicate that some temperate species existed outside these areas during the last glacial maximum (LGM). Red foxes (*Vulpes vulpes*) in particular, could have been distributed across the southern half of the continent, potentially forming one continuous population. To investigate these hypotheses, we used 21 nuclear microsatellite loci and two fragments (768 bp) of mitochondrial DNA to characterize the population structure among a continent-wide sample of 288 European red foxes. We tested whether European red foxes clustered into discrete populations corresponding to the hypothetical peninsular refugia. Additionally, we sought to determine if distinct northern populations were formed after post-glacial recolonization. Our results indicated that only the foxes of Iberia appeared to have remained distinct over a considerable period of time (32–104 kya). Spanish red foxes formed their own genotypic cluster; all mtDNA haplotypes were endemic and closely related, and together both the mitochondrial and nuclear datasets indicated this population contributed little to postglacial recolonization of Northern Europe. In contrast, red foxes from Italy and the Balkans contributed significantly to, or were part of, a wider, admixed population stretching across mid-latitude Europe. In Northern Europe, we identified a Scandinavian population that had an ancestral relationship with red foxes to the south, and a more recent relationship with those to the east, in Russia. We also resolved two distinct populations on the islands of Ireland and Britain that had been separated from one another, and from those on the continent, since the late Pleistocene/mid Holocene (~4–24 kya).

1. Introduction

The climatic oscillations of the Pleistocene caused range expansions and contractions, extinctions and the evolution of novel lineages (Hewitt 2000; Lister 2004; Stewart 2010; Morales-Barbero et al 2017). During the last glacial maximum (LGM, 26 thousand years ago, kya [Peltier and Fairbanks 2006]), ranges of many temperate terrestrial species in Europe were pushed southward, where they became isolated in (primarily peninsular) refugia (Hewitt 2004). Geographically distinct lineages have been observed in many European species and are attributed to this vicariant event, as well as the uneven range expansion following climatic warming. Although individual species responded differently to potential barriers depending on their particular physiology and dispersal abilities (Taberlet et al. 1998; Stewart 2010), one of three models has been typically invoked to describe common patterns observed across temperate species: the grasshopper (*Chorthippus paralleus*), where northern populations stem from the Balkans; the hedgehog (*Erinaceus europeus* and *E. concolor*), where populations expanded from Iberia, Italy and the Balkans; and the European brown bear (*Ursus arctos*), where populations expanded from Iberia and the Balkans (Hewitt 1999).

A review of faunal assemblages from archaeological sites has called these models into question (Sommers and Nadachowski 2006). An examination of fossil records dated to the LGM not only revealed the presence of temperate fauna in putative southern peninsular refugia of Iberia, Italy, and the Balkans, but also in a number of mid-latitude European sites from Southwestern France through Austria, Hungary, Czech Republic, Slovakia, Slovenia, to Moldova, in the east. This pattern suggests that temperate species could have retained a more continuous distribution than typically assumed throughout much of southern Europe, potentially facilitating genetic exchange and therefore countering population differentiation. Nevertheless, subsequent phylogeographic analyses indicate major subdivision attributable to contraction into refugia during the last glaciation, even for large vagile species such as the red deer (*Cervus elaphus*; Skog et al. 2009). Thus, it remains unclear what impact the last glacial cycle had on the generation or maintenance of distinct lineages across Europe.

Red foxes (*Vulpes vulpes*) are currently distributed across Europe, from the south of Spain to the most northerly point of Norway (Macdonald and Reynolds 2004). During the last glacial period red foxes exhibited a more southerly distribution (Sommers and Benecke 2005); in particular, sub-fossil remains indicate the presence of red foxes no farther north than England

and Poland just prior to the LGM. For a period of >7,000 years (23–16 kya), red foxes were apparently pushed further south. Sub-fossil remains from this time indicate that red foxes were present in the southern peninsulas of Iberia, Italy, and Balkans, as well in a number of mid-latitude locations stretching from France in the west, through to Moldova in the east (Sommers and Nadachowski 2006). Thus, red foxes apparently maintained a large continuous population across the southern half of the European continent at the height of the last glaciation (Sommers and Nadachowski 2006). By 16 kya, red foxes had expanded as far north as southeastern Germany (Sommers and Benecke 2005). By the mid Holocene (8.2–4.2 kya; Walker et al 2012), red foxes had apparently expanded into most of their current range (Sommers and Benecke 2005). Given the species' history of responding to changing climate, and its ability to cope with a range of environmental conditions (Macdonald and Reynolds 2004), the extent to which populations were isolated and subdivided during the LGM is unknown. Such demographic changes, however, often leave genetic signatures in modern populations.

Increasingly extensive sampling and more highly resolving genetic analyses have provided a shifting understanding of European red fox phylogeography and of how current populations are structured. Such studies have either had widespread sampling but were based primarily on mitochondrial DNA (mtDNA), or used multiple nuclear loci but with a more geographically restricted sampling. An early study using mitochondrial cytochrome *b* sequence data and allozymes indicated low contemporary gene flow between populations across the Mediterranean Basin (Frati et al. 1998). A subsequent analysis used short segments of cytochrome *b* and D-loop from both modern and ancient DNA samples and found a lack of spatial structure and change in population size over the last 40,000 years (Teacher et al. 2011). Edwards et al (2012) followed with a geographically and numerically larger sampling, with particular emphasis on representation from Britain and Ireland. Analyzing portions of cytochrome *b* and D-loop, these authors identified clear differentiation between continental red foxes and those from the islands of Britain and Ireland along with their closest continental neighbour, the Netherlands. Recently a small number of studies have used nuclear microsatellites to investigate regional population substructure within Europe, in Poland (Mullins et al. 2014), Britain (Atterby et al. 2015), and Scandinavia (Norén et al 2015). However, no study has used high-resolution nuclear markers to investigate the continent-scale population genetics of a large number of European red foxes.

We used a panel of 21 nuclear microsatellites and mitochondrial DNA sequences to assess the population substructure, phylogeography, and the timing of vicariant events within a continent-wide sample of European red foxes. The use of multiple loci allowed an independent assessment of the population structure relative to that identified with maternally-inherited mtDNA. Specifically, we sought to determine whether (a) red foxes across southern Europe constituted a single continuous population, or if (b) multiple discrete populations were evident. Given that much of northern Europe was uninhabitable by the red fox during the period around the LGM and that current populations in those areas stem from postglacial colonization, we also tested the predictions of (c) little or no differentiation among northern populations and their southern sources, versus (d) geographically discrete populations consistent with colonization from different sources populations or subsequent isolation. Our analyses also allowed us to assess the validity of current subspecies designations within the red fox.

2. **Methods**

2.1. Samples

All samples used in this analysis were collected and DNA extracted as described in previous studies (Edwards et al. 2012; O'Mahoney et al. 2012; Statham et al. 2014). In total, 288 DNA samples were collected from across Europe: Ireland, Britain, Spain, Italy, Serbia, France, Netherlands, Germany, Denmark, Poland, Estonia, Norway, Sweden, and Russia (Figure 1; Appendix). These samples comprised tissue (n = 232) and faeces (n = 56). The faecal samples were from Ireland (n = 52) and the Kola Peninsula, Russia (n = 4), and were previously genetically identified to species (O'Mahoney et al. 2012; Statham et al. 2014).

2.2. PCR amplification and Microsatellite Genotyping

We amplified 21 microsatellite loci (*AHT133, AHTh171, C01.424, C04.140, C08.618, CPH11, CPH18, CPH2, CXX-468, CXX-602, FH2001, FH2004, FH2010, FH2054, FH2080, FH2289, FH2328, FH2380, FH2457, FH2848, REN54P11*) in three multiplexes for populations of ≥5 individuals (Figure 1). The primers, PCR chemistry, and cycling conditions were described by Moore et al. (2010). We genotyped the faecal DNA samples ≥3 times each and assigned a consensus genotype based on the results.

2.3. Microsatellite Analyses

We used the program Micro-checker v 2.3.3 (Van Oosterhout et al. 2004) to screen the microsatellite dataset for null alleles. To estimate the random allelic dropout of the faecal samples, we obtained consensus types from the 3 replicates for each sample, identified heterozygous loci from these consensus genotypes, calculated the cumulative proportion of them that had homozygous replicates, and raised this proportion to the third power as the estimate of allelic dropout rate in the consensus genotypes (Bonin et al. 2004). We tested for deviations from Hardy-Weinberg equilibrium using Arlequin 3.5 (Excoffier and Lischer 2010), and from gametic equilibrium using Genepop (*http://genepop.curtin.edu.au/*). We calculated the observed (*H*o) and expected (*H*e) heterozygosities and average number of alleles per locus (*A*) in Microsatellite Tool Kit (Park 2001). We calculated allelic richness (*Ar*), and inbreeding coefficient (F_{IS}) in FSTAT v 2.9.3.2 (Goudet 1995), and the rarefied number of private alleles (*Pr*) in HP-Rare v1.1 (Kalinowski 2005). We assessed how nuclear genetic variation was partitioned across the species

range using a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) in Arlequin. We calculated pairwise F_{ST} among sampling sites using Arlequin. Excluding geographic locations with sample sizes <10 (Estonia and Denmark), we evaluated the relationship between Euclidean geographic distance and genetic distance ($F_{ST}/1- F_{ST}$) using Mantel tests in Arlequin. We created a matrix of genetic distance (Nei's D_A; Takezaki and Nei 1996) with 999 bootstrap replicates and used these values to create a neighbor-joining tree using the program Populations 1.2.32 (Langella 1999)

We examined population substructure using the model-based Bayesian clustering method implemented in the program STRUCTURE v.2.3.3 using the admixture model with correlated allele frequencies (Pritchard et al. 2000; Falush et al. 2003). This technique allowed us to evaluate population substructure without the need for *a priori* assignment of individuals to populations. Iterations were run at *K* values of 1-10, with a burn-in of 100,000 followed by a run of 1 million iterations. Simulations were repeated 5 times at each *K* value to assess consistency across runs. We determined the most meaningful *K* values by plotting the Ln P(D) values and determining where the greatest support was found (Pritchard 2009) and using the delta *K* method (Evanno et al. 2005), implemented in Structure Harvester (Earl and vonHoldt 2012).

2.4. mtDNA analyses

We generated all mtDNA sequence data in our previous study (Statham et al. 2014). We conducted analyses of 275 concatenated mitochondrial DNA sequences for individuals that provided partial cytochrome *b* (397 bp), and partial D-loop (371 bp) sequences. The two fragments totaled 768 bp, which was slightly longer than the 697 bp fragment analyzed by Statham et al. (2014). For populations ≥5 we calculated basic diversity statistics in Arlequin. We also examined our data for evidence of previous demographic events using Tajima's *D* (Tajima 1989), calculated in Arlequin, and Strobecks' *S* statistic (Strobeck 1987) in DNAsp v5.10.01 (Librado and Rozas 2009). To investigate the relationship among haplotypes we created a median joining network (Bandelt et al. 1999) with cytochrome *b* mutations conservatively weighted double those of D-loop mutations in the program in Network 4.2.0.1 (*www.fluxus-engineering.com*). We calculated pairwise Φ_{ST} among sampling sites with >5 individuals using Arlequin. Excluding geographic locations with sample sizes <10 (France, Holland and Estonia),

we evaluated the relationship between Euclidean geographic distance and mtDNA genetic distance (Φ_{ST} /1- Φ_{ST}) using Mantel tests in Arlequin.

We investigated the locations of phylogeographic breaks by comparing pairwise Φ_{ST} and geographic distance among sampling sites in the program SAMOVA v1.0 (Dupanloup et al. 2002). The analyses were run for *K* values 2-10, with 100 simulated annealing processes.

2.5. Population splitting times using mtDNA

We tested hypotheses regarding splitting times among European red fox populations using MCMC simulations in the program IMa2 (Hey 2010). We used the model 'isolation without migration' when comparing island populations, and used the model 'isolation with migration' when examining the relationship between populations with potential overland connectivity. We conducted analyses on a segment of concatenated cytochrome *b* and D-loop, truncated to 572 bp to allow inclusion of sequences from Edwards et al. (2012). We used the HKY substitution model. Following several initials trials with the recommended starting parameters (Hey 2011), we ran 30 chains with geometric heating. Burn-in was set at a minimum of 1.5 x 10^6 generations and parameters estimates were calculated based the subsequent 2.5 x10^6 generations of data, sampling every 100 generations, resulting in 25,000 sampled steps. We repeated each analysis once with a different random seed to assess consistency. We converted the resulting mutation-scaled parameters to time values using a mutation rate of 9.36% per million generations (Edwards et al. 2012). Previous studies have based divergence times on a generation time of 1 year, which would be the minimum possible for a monoestrous canid. We assumed a generation time of 2 years, and thus a mutation rate of 4.68% per million years (Goddard et al. 2015).

3. Results

3.1. Microsatellites

All loci tested were polymorphic with a range of 4–22 alleles per locus. We identified two loci (*RF2457*, *FH2088*) as having null alleles in a large number of the populations analyzed using the program Microchecker, and, therefore, excluded these loci from further analyses. We identified ten population locus-pairs as statistically linked after Bonferroni correction. All linked pairs of loci were only identified in individual populations rather than systematically across populations, indicating gametic disequilibrium (e.g. due to population substructure) rather than physical linkage. Therefore, the remaining 19 loci were retained for further analyses. We estimated the allelic dropout rate for fecal samples based on 50 triplicated 19-locus genotypes to be 5.5%.

We identified the greatest average number of alleles per locus in Ireland, the location with the largest sample size and greatest number of sampling sites (Table 1; Appendix). When accounting for sample size, allelic richness was similar across most locations, although all locations had positive F_{IS} values, consistent with substructure.

An analysis of molecular variance (AMOVA) indicated significant overall population structure (F_{ST} = 0.058), with the majority of the variation (>94%) found within populations. Analyses of population pairwise F_{ST} revealed significant differentiation in 55 of 78 pairs of populations after Bonferroni correction for multiple tests (Table 2). We identified the highest pairwise F_{ST} between Italy and Spain (F_{ST} = 0.111), lending support for the differentiation of these two putative refugial populations. Additionally, Italy and Spain were significantly differentiated from the majority of other locations. The northern peripheral locations of Ireland, Britain, Sweden, and Norway, were significantly differentiated from nearly all other populations, supporting the establishment of distinct red fox populations after postglacial colonization. In contrast, when considering pairwise comparisons among more centrally located populations (France, Netherlands, Germany, Denmark, Estonia, Serbia), 14 of 15 pairs were not significantly differentiated, consistent with a continuous population across these areas. We did not detect a significant relationship between genetic and geographic distance (isolation by distance, IBD) in red fox populations throughout Europe (r = 0.03, P = 0.39). However, as greater isolation of island and peripheral peninsular populations could have obscured an isolation by distance relationship among the central sites, we conducted a second analysis using only the central continental sites; i.e. France, Netherlands, Germany, Serbia, and Yamal, Russia, which revealed

a substantial (although statistically non-significant) relationship between geographic and genetic distance (r = 0.71, P = 0.16).

Our analyses of population subdivision conducted in STRUCTURE provided increased support with each successive K value up to K = 8. Values ranging K = 1–6 produced sensible geographic subdivisions (Figure 2). At K = 7 the output was less informative and identified additional admixed individuals within populations across central Europe (data not shown). We identified the most basal subdivision (K = 2) within European red foxes between the island populations of Ireland and Britain versus other populations. This subdivision was also identified as having the greatest ΔK, with a secondary peak at K = 5. At K = 5, where support values began to plateau, the following populations were evident: Ireland, Britain, Spain, Italy, and Norway/Sweden. Yamal (Russia) largely split off to form a separate cluster at K = 6. At K = 3–6, individual animals from throughout central Europe (France through to Estonia and Serbia) appeared admixed, with portions of their ancestry assigned to multiple clusters that otherwise dominated in distinct peripheral locations. In an effort to resolve the subdivision within central Europe we ran separate analyses in STRUCTURE (K = 1–10) excluding peripheral areas. All K values >1 had lower support, indicating a lack of major subdivision among central European red foxes. These results support the presence of distinct southern refugial populations, a large continuous population across Central Europe, and differentiation of relatively recent populations formed after postglacial recolonization. A population tree based on genetic distance (Nei's D_A) was broadly consistent with the structure analyses and indicated a close relationship between populations in Britain and Ireland, as well as among populations in Norway, Sweden and Yamal (Figure 3).

3.2. mtDNA

We obtained mitochondrial sequence data from 288 individuals, resulting in 275 composite cytochrome *b*/D-loop sequences, which in turn provided 72 distinct haplotypes (Figure 4). We assigned haplotypes to four subclades within the Holarctic clade, a clade also dominating in Asia and northwestern North America (Statham et al. 2014). Most locations exhibited high haplotype diversity (0.82–0.92). However, lower diversity was identified in a number of more northerly locations (Table 3). All three southern peninsular populations (Spain, Italy, Serbia) had positive (but non-significant) Tajima's D values consistent with a decreasing population size (Table 3). In

contrast, negative values (indicating an excess of low frequency polymorphisms), consistent with an expansion, were only found in northern populations, with Denmark having the only significant value. We identified a significant signature of admixture in the samples from Serbia (Strobeck's *S*; Table 3). We did not detect a significant relationship between genetic and geographic distance (IBD) in red fox populations throughout Europe ($r = 0.20$, $P = 0.18$), nor when we ran the analysis excluding island and peripheral peninsular populations ($r = 0.18$, $P = 0.30$).

All SAMOVA analyses ranging $K = 2–10$ identified statistically significant subdivision (Table 4). The most basal split ($K = 2$) separated three western populations (Ireland, Britain, Netherlands) from all others. France grouped with the three western populations at higher K values. The greatest increase in Φ_{CT} was found at $K = 6$, which resolved the following geographic groupings: (1) Ireland, Britain, Netherlands, France; (2) Italy, Germany, Estonia; (3) Denmark, Sweden; (4) Serbia, Yamal; (5) Spain; and (6) Norway. This analysis resolved the phylogeographic relationship and postglacial colonization history among European red foxes, specifically, the contribution of the southern peninsulas of Italy and the Balkans (but not Iberia) to northern recolonization, and central continental populations to the colonization of Britain, Ireland, and the Scandinavian Peninsula.

Population splitting times estimated with mtDNA

We estimated that populations in Britain and Continental Europe split 14.2 kya (95% HPD = 4.8–24 kya; Table 5). Ireland became an island prior to Britain, therefore we ran our analyses under two different scenarios. Allowing for an early colonization of Ireland prior to the separation of Britain and continental Europe, we identified a splitting time of 14 kya (95% HPD = 6–22.4 kya). Allowing for a late colonization after both Ireland and Britain were islands, we identified a slightly earlier splitting time of 10.2 kya (95% HPD = 4.2–16.4 kya). Allowing for migration, we estimated that red fox populations in Spain and Central Europe split 120 kya (95% HPD = 34–372 kya). This analysis indicated that the level of migration between Spain and Central Europe included zero. Therefore, we also carried out analyses excluding migration and estimated an overlapping but more recent splitting time of 66 kya (95% HPD = 32–104 kya). All the above results produced unimodal parameter estimates and splitting times that were consistent

across independent runs with different random seeds. Independent runs also had high effective samples sizes (>1000) and trend plots free of systematic changes, indicating good mixing.

We also attempted to generate splitting time estimates between Central European populations and those in Italy and Fennoscandia because these populations were differentiated in other analyses. However, those estimates were inconsistent across runs and produced bimodal peaks for multiple parameter estimates. The inability to estimate splitting times could have been due to insufficient resolving power in the dataset, relatively recent genetic exchange between populations, or perhaps in the case of Scandinavia, multiple colonization events.

4. Discussion

We used genetic analyses to test hypotheses about the impact of historically changing climate, from the last glacial maximum to the Holocene, on the population structure of red foxes across Europe. Despite an apparent broad distribution across the southern half of Europe during the LGM, our results indicate that several discrete populations of red foxes were present. Our work adds to the limited number of species, with similar LGM distributions, that also show evidence of discrete populations (Randi et al. 2004; Skog et al. 2009). Additionally we found evidence that multiple genetically distinct northern populations formed after postglacial recolonization. Below we expand on and provide support for these conclusions.

The red fox population we identified in Spain was among the most highly differentiated within Europe. Bayesian cluster analysis indicated that this population formed a discrete genetic cluster, which was supported by one of the highest average pairwise F_{ST} values across all locations sampled. All mtDNA haplotypes identified in Spain were endemic and closely related, indicating long-term differentiation of Iberian red foxes from those elsewhere. In addition, the presence of a number of well-represented haplotypes, with no sign of sudden radiation, was indicative of a large, long-standing population in this area. Multiple lines of evidence indicate that this population made only a minor contribution to the gene pool of other western European populations. For example, the SAMOVA identified connectivity between central Europe and Italy, and these two regions also shared mtDNA haplotypes, but Spain parsed as distinct and was estimated in the IMa2 analyses to have diverged 32–104 kya. Although this time period substantially precedes the global LGM (~26 kya), it was consistent with separation since the local last glacial maximum in the Pyrenees of ca. 50–70 kya (Jiménez-Sánchez et al. 2013).

The east-west orientation of Pyrenees Mountains at the northern extreme of the Iberian Peninsula poses a substantial barrier to dispersal (Taberlet et al. 1998), which would have been exacerbated during the last glacial period. In addition, the presence of established red fox populations in the southwest of France during the LGM (Sommers and Nadachowski 2006) apparently negated a colonizing front stemming from Iberia. Distinct Iberian lineages within species have been described previously, notably, for the grasshopper (Cooper et al. 1995), the model species for one of the three paradigms of postglacial colonization (Hewitt 2000). Further work focusing on red foxes either side of the Pyrenees will be needed to evaluate the magnitude and directionality of contemporary and historical gene flow between these populations.

One unusual result was that a single endemic haplotype from Ireland grouped with haplotypes found in Spain (Figure 4), while a portion of the gene pool in Irish and Dutch populations assigned to the Spanish structure cluster (Figure 2). This indicates some contribution of Iberian red foxes to northern populations. The finding of a connection between Ireland and Iberia, referred to as a Lusitanian (or Hiberno-Lusitanian) distribution (Edwards and Bradley 2009; Beatty and Provan 2013), has also been observed in a range of other mammal species (e.g. Davison et al. 2001; Mascheretti et al. 2003; O'Meara et al. 2012). This pattern has variously been attributed to anthropogenic introductions associated with historical cultural connections (Mascheretti et al. 2003; O'Meara et al. 2012), population expansion causing a replacement of intervening populations (O'Meara et al. 2012), or a population bottleneck causing a loss of connecting haplotypes from intervening populations (Jordan et al. 2012). Given the history of fox translocation globally (Long 2003; Statham et al. 2012), a potential population size reduction in Britain due to hunting (Atterby et al. 2015), and the greater diversity of haplotypes found in Ireland than in Britain, any one of these scenarios could explain the patterns seen. Additionally, increased sampling in Britain may uncover the same or similar Spanish type haplotypes, thus indicating genetic continuity between British and Irish red foxes.

Genetic analyses of Italian red foxes indicated that they were distinct from, yet with a history of interconnection with, central European populations. Italian red foxes formed a cohesive genetic cluster, with minimal evidence of admixture from other populations. However, both the mitochondrial and nuclear datasets indicated that the Italian population contributed significantly to central European populations. For example, at $K = 5$ in the structure output (where ΔK analyses indicated a peak of support), the cluster encompassing all Italian red foxes was also evident to the east in Serbia, as well as across all of the central European locations sampled. This interconnected relationship was also evident in the shared and closely related mtDNA haplotypes, particularly between Italy and Germany to the north, and the consistent grouping of these locations with SAMOVA. In contrast to Spain and Italy, both mitochondrial and nuclear DNA indicated high genetic connectivity of Serbia (i.e., Balkans) to central Europe.

In addition to our support for distinct populations in two of the southern refugia, we found evidence of major differentiation among populations that arose more recently, following postglacial recolonization of the north. Bayesian cluster analysis indicated that the northwestern island populations of Britain and Ireland formed the primary splinter group found among

European red foxes. The mtDNA dataset was in close agreement and also resolved an ancestral relationship with the neighboring populations of the Netherlands and France. The relationship between Britain, Ireland, and the Netherlands had previously been noted based analyses of a shorter sequence of mtDNA (Edwards et al. 2012); however, the identification of a close relationship with France was novel to this study. The genetic differentiation of Britain and Ireland from populations elsewhere was likely driven by a bottleneck during recolonization, followed by subsequent physical and genetic isolation as sea-level rose. This scenario was supported by the low mtDNA nucleotide diversity found in both island populations.

We also uncovered ancient differentiation between British and Irish populations. Both formed distinct structure clusters and were significantly differentiated from one another (F_{ST} = 0.049, Φ_{ST} = 0.14, from microsatellite and mtDNA respectively). We estimated that Britain split from the wider European population 4.8–24 kya. This period overlaps with that estimated previously for the separation of a combined British and Irish dataset from continental Europe (5.7–14.5 kya; Edwards et al. 2012), and is in keeping with the last overland connection between Britain and continental Europe, via Doggerland, which existed into the Holocene, and finally flooded around 7.8 kya (Montgomery et al. 2014). Ireland has existed as an island for twice as long as Britain (Clark et al. 2012). This early isolation has led to considerable debate regarding whether many Irish terrestrial species colonized on their own or were aided by humans (Montgomery 2014 and citations within). Therefore, we investigated two scenarios: allowing for natural overland colonization of Ireland (when Britain was still connected to continental Europe), or allowing for human translocation (when both Ireland and Britain were islands). The analysis where Britain was still part of a continental population produced an estimate of 6.2–22.4 kya, which encompasses the last overland/ice connection between Ireland and the rest of Europe (~18 kya; Clark et al. 2012). The analysis between island populations returned a slightly more recent splitting time of 4.2–16.4 kya, which is close in age to the earliest Irish red fox subfossil at 3.8 kya (Montgomery et al. 2014). Unfortunately, both analyses produced overlapping splitting time estimates, which also encompassed the earliest evidence of human presence in Ireland (12.7 kya; Dowd and Carden 2016). Thus, our data do not allow us to resolve whether red foxes colonized Ireland naturally or were aided by human intervention. Ultimately, analysis of a greater proportion of the genome will be necessary to determine when (and how) red foxes colonized Ireland.

Red foxes in the Scandinavian Peninsula also comprised a distinct population. The microsatellite dataset indicated a close relationship between foxes from Sweden and Norway, which together had a more distant relationship with populations to the east in Siberia (as represented by samples from the Yamal Peninsula in Russia). The affiliation with Russia and other eastern European locations was supported by shared and closely related mtDNA haplotypes. The mtDNA also indicated an ancestral relationship with populations to the south of the Scandinavian Peninsula, with SAMOVA consistently grouping the populations of Sweden and Denmark. Similarly, mtDNA analysis by Edwards et al. (2012) suggested bidirectional colonization of Scandinavia, while Norén et al (2015) identified differentiation between red foxes in southern Sweden and Finland. Taken together these results suggest that the Scandinavian Peninsula was colonized by red foxes from two directions; from the south across a land bridge from Denmark, and also from the east through Finland and Russia. Once the final land bridge to the south was flooded (9.2–10.3 kya; Björck 1995; Herman et al. 2014), continued gene flow was only possible to the east, which was supported by our microsatellite analyses. Similar southern and eastern colonisation of Scandinavia has been inferred in a range of other species (e.g. Lundqvist 2011; Ruiz-Gonzalez 2013; Herman et al. 2014).

4.1 Comparison of genetic subdivision with recognized subspecies

Based on gross morphological differences, five red fox subspecies have been described in Europe (Macdonald and Reynolds 2004). Our genetic data allows us to assess the validity of these designations, which have never been empirically tested. The nominate subspecies *V. v. vulpes* was described in Scandinavia (Macdonald and Reynolds 2004), which is consistent with the genetic distinctiveness that we observed. Red foxes in Iberia belong to the subspecies *V. v. silacea*, and our genetic evidence broadly supports this designation. We did not sample foxes from two other European named subspecies from the Mediterranean islands of Cyprus (*V. v. induta*), and Sardinia and Corsica (*V. v. ichnusae*). All remaining European red fox populations were considered to belong to a single subspecies, *V. v. crucigera*, initially described in Germany (MacDonald and Reynolds 2004). This subspecies designation includes several distinct populations resolved in our study, including those in Italy, and on the islands of Ireland and Britain, which have been physically and genetically isolated since the late Pleistocene/early Holocene. Despite translocations into Britain during historical times (Long 2003; Atterby et al.

2015), this population has maintained a distinct genetic character. Taken together, these data indicate that both Irish and British red foxes should be considered evolutionarily distinct units within the red fox.

4.2. Conclusions

During the LGM populations in the Iberian and Italian peninsula were distinct and isolated from one another. Genetic evidence suggests that Italian populations contributed to neighboring populations in central Europe and the Balkans. The potential for connection with the Balkans is supported by fossil evidence, which indicates the presence of red fox during the LGM in Slovenia, at the nexus of the Italian and Balkan peninsulas (Sommers and Nadachowski 2006). The admixed nature of the Serbian (i.e., Balkan) population also indicates a degree of genetic exchange with populations to the east. During the LGM, and for a period afterward, Britain was connected via land and ice bridges with continental Europe (Montgomery et al. 2014). Mitochondrial DNA evidence indicates that red fox populations in France and the Netherlands were likely the source populations (or were part of the same population) that colonized Britain and Ireland. After colonization, red foxes in Ireland and Britain became isolated both from one another and from the continent by rising sea levels, thus facilitating the formation of distinct populations. In the meantime, gene flow across much of central Europe was largely unimpeded. Mitochondrial DNA evidence indicates that the central European population colonized northward via Denmark across a land bridge to Sweden, and this connection was subsequently lost due to rising sea level. The Scandinavian Peninsula was also colonized from the east. In relative isolation on the Peninsula, these foxes formed a distinct genetic unit with a degree of ongoing gene flow with populations to the east.

While red foxes were not restricted to glacial refugia during the LGM, we can compare the colonization pattern observed in the European red fox to the three paradigms of postglacial colonization described by Hewitt (1999; 2000). Similar to the grasshopper (Cooper et al. 1995), Iberian red fox populations appear to have made limited impact on northern populations. In contrast to the pattern seen in the grasshopper, Italian, as well as Balkan, red foxes contributed to, or were part of, more northerly populations, more consistent with the pattern described for hedgehogs (Seddon et al. 2001). Thus, European red foxes do not easily fit one of the classic models, indicating that the postglacial colonization pattern observed is distinct.

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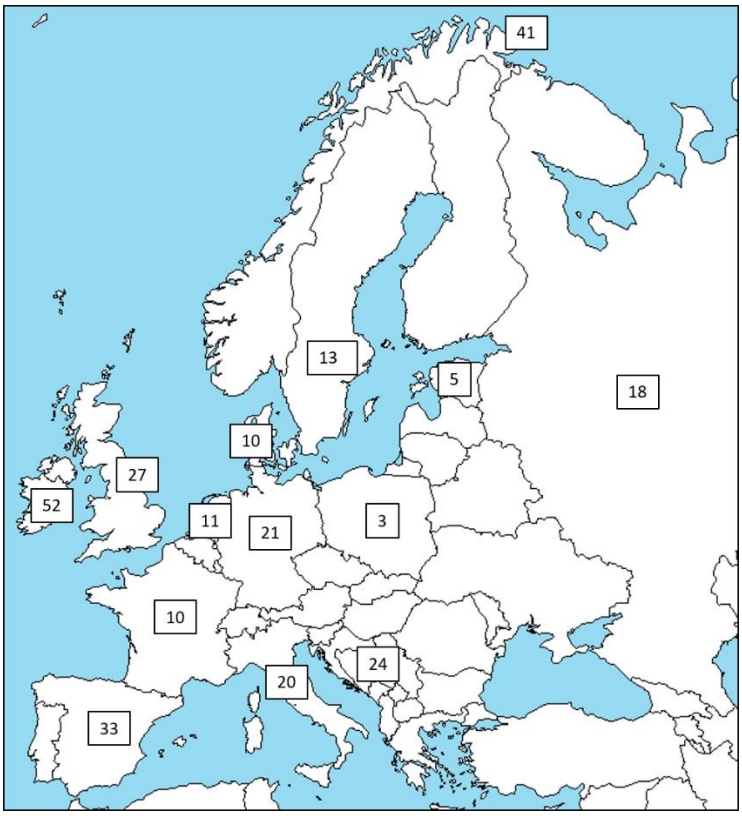
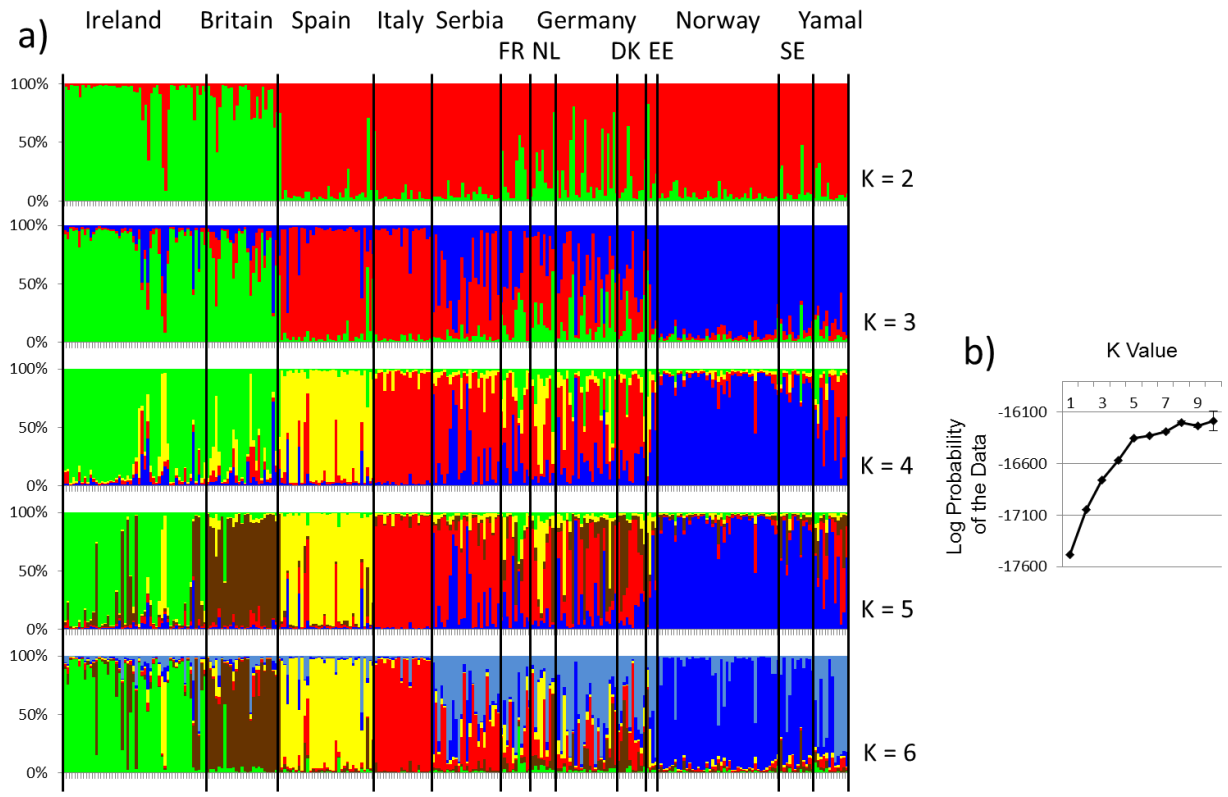


Figure 1. Map of red fox samples. The number indicates the total number of samples from that country. More specific sampling information is provided in the Appendix.



640

641 Figure 2. Bayesian cluster analysis of individual European red foxes generated in the program
642 Structure. a) Vertical bars represent individual foxes and the shading represents the proportional
643 assignment to different clusters. FR = France, NL = Netherlands, DK = Denmark, EE = Estonia,
644 SE = Sweden. b) Support value for each level of *K*, based on five iterations of *K* = 1–10.

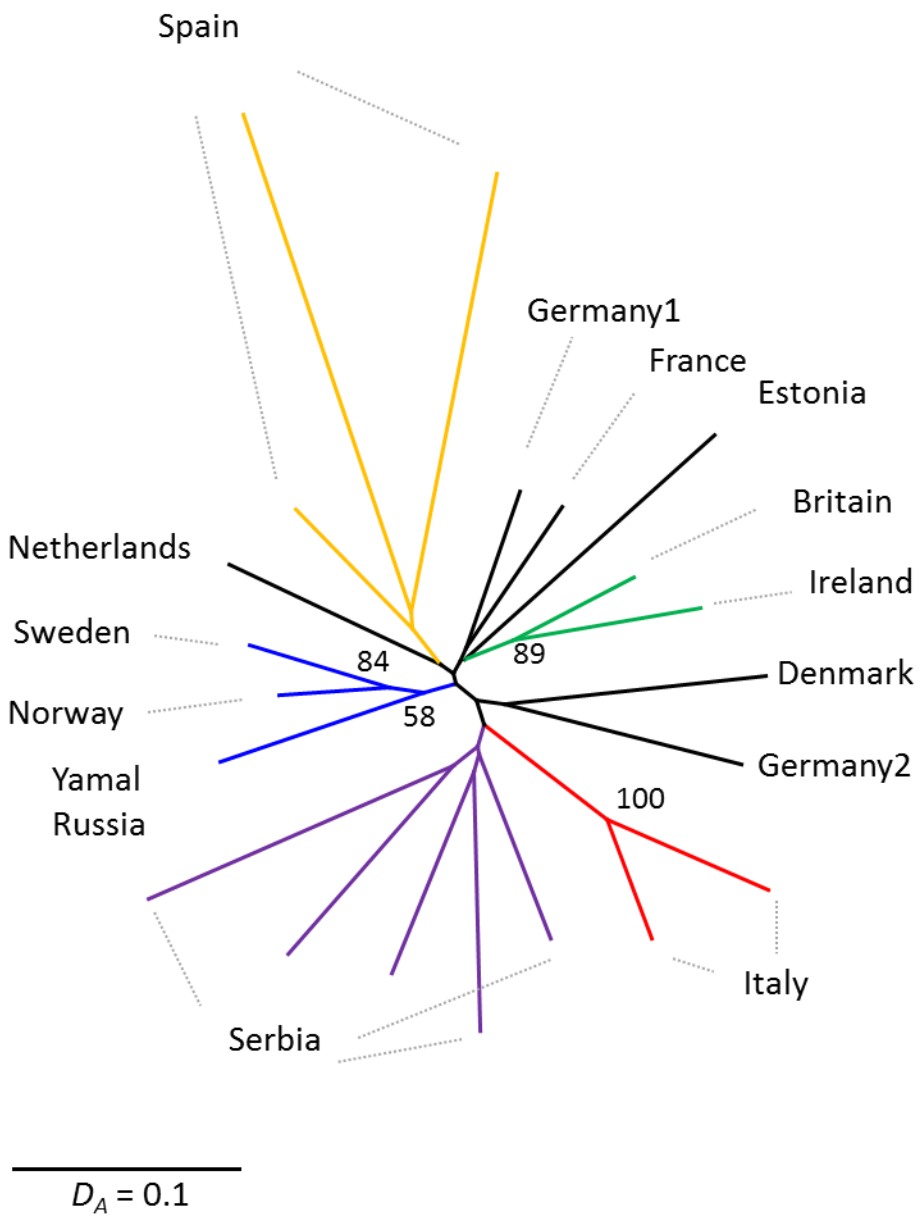


Figure 3. Neighbor joining population tree of European red foxes from 21 sampling sites. Based on Nei's genetic distance (DA; Takezaki and Nei 1996) calculated using 19 microsatellite loci. Values at the nodes indicate bootstrap support.

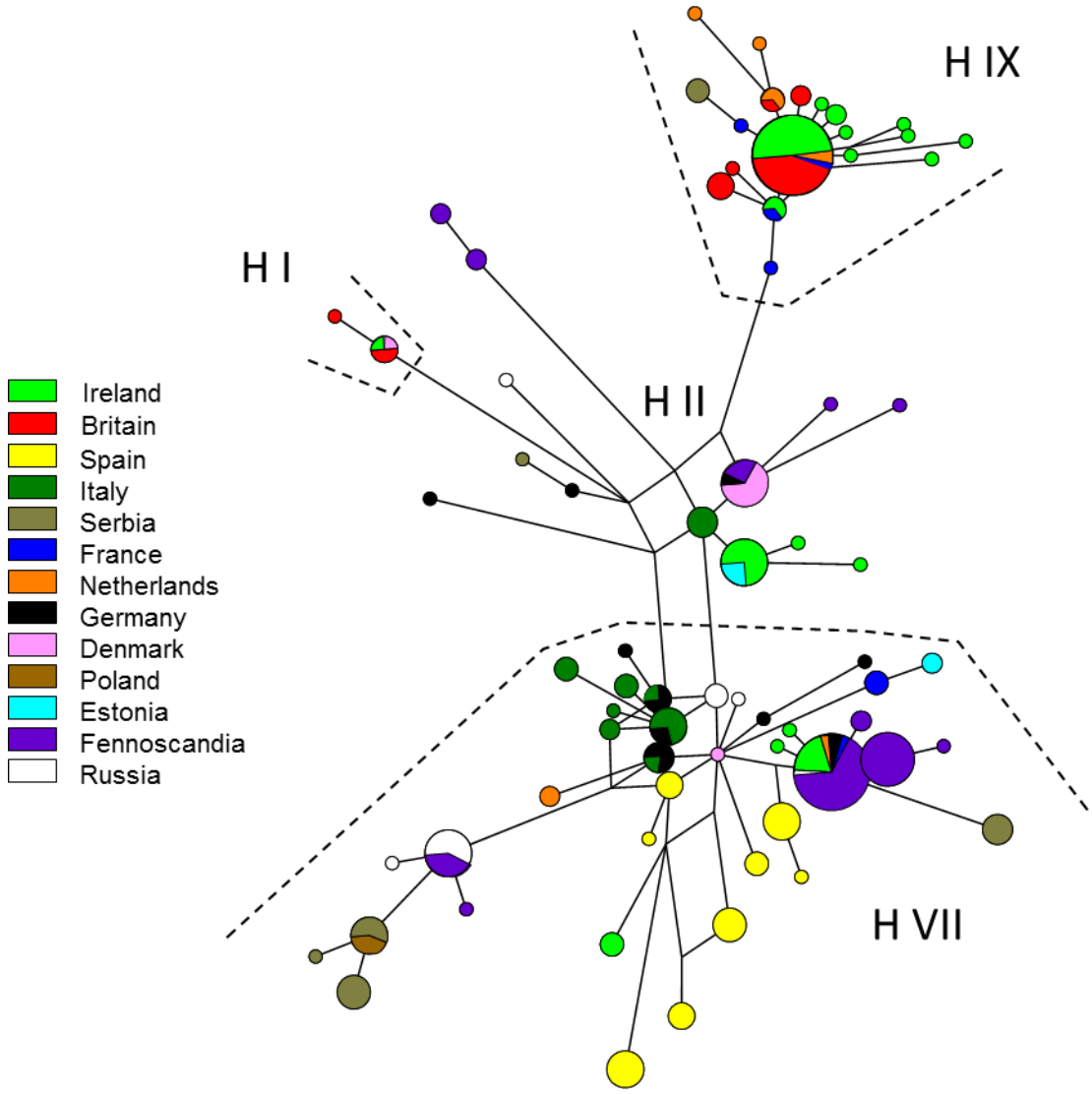


Figure 4. Haplotype network of European red fox mtDNA. Calculated based on 768bp of concatenated cytochrome *b* and D-loop from 275 red foxes with cytochrome *b* mutations weighted double that of D-loop. Russia includes samples from Yamal, as well as two samples from Tver. Fennoscandia includes Sweden and Norway, as well as four samples from the Kola Peninsula, Russia. Nodes are colour coded by population composition, with the size of the node indicating the number of individuals represented (smallest = 1, largest = 37). All haplotypes belong to the Holarctic clade, while division into subclades is indicated with a dashed line and is based on Statham et al. (2014).

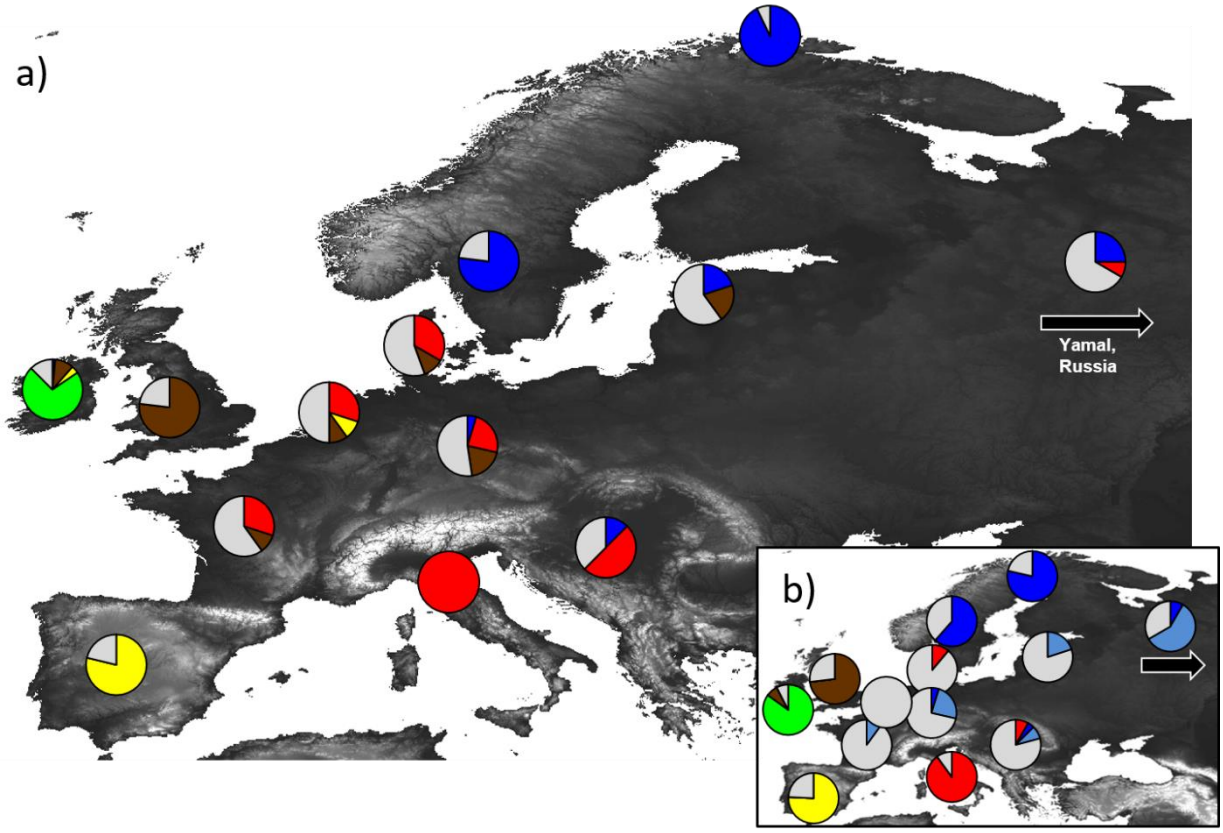


Figure 5. Geographic distribution of genetic groups of red fox foxes within Europe as indicated by the program Structure. The colours used to indicate genetic clusters are the same as those used in Figure 2. Individuals were considered to belong to a cluster if they assigned ≥75%. Admixed individuals (<75% assignment) were colour coded grey. a) Genetic clusters at $K = 5$. b) Genetic clusters at $K = 6$. The background map is shaded by elevation, with lighter shades indicating higher elevation.