

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

3

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5 Ireland, Britain, Scandinavia, *Vulpes vulpes*

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17 **Abstract**

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

39 **1. Introduction**

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

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

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

70 and Poland just prior to the LGM. For a period of >7,000 years (23–16 kya), red foxes were
71 apparently pushed further south. Sub-fossil remains from this time indicate that red foxes were
72 present in the southern peninsulas of Iberia, Italy, and Balkans, as well in a number of mid-
73 latitude locations stretching from France in the west, through to Moldova in the east (Sommers
74 and Nadachowski 2006). Thus, red foxes apparently maintained a large continuous population
75 across the southern half of the European continent at the height of the last glaciation (Sommers
76 and Nadachowski 2006). By 16 kya, red foxes had expanded as far north as southeastern
77 Germany (Sommers and Benecke 2005). By the mid Holocene (8.2–4.2 kya; Walker et al 2012),
78 red foxes had apparently expanded into most of their current range (Sommers and Benecke
79 2005). Given the species' history of responding to changing climate, and its ability to cope with a
80 range of environmental conditions (Macdonald and Reynolds 2004), the extent to which
81 populations were isolated and subdivided during the LGM is unknown. Such demographic
82 changes, however, often leave genetic signatures in modern populations.

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

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

113 2. Methods

114 2.1. Samples

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

122

123 2.2. PCR amplification and Microsatellite Genotyping

124 We amplified 21 microsatellite loci (*AHT133*, *AHT171*, *C01.424*, *C04.140*, *C08.618*, *CPH11*,
125 *CPH18*, *CPH2*, *CXX-468*, *CXX-602*, *FH2001*, *FH2004*, *FH2010*, *FH2054*, *FH2080*, *FH2289*,
126 *FH2328*, *FH2380*, *FH2457*, *FH2848*, *REN54P11*) in three multiplexes for populations of ≥ 5
127 individuals (Figure 1). The primers, PCR chemistry, and cycling conditions were described by
128 Moore et al. (2010). We genotyped the faecal DNA samples ≥ 3 times each and assigned a
129 consensus genotype based on the results.

130

131 2.3. Microsatellite Analyses

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

144 range using a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) in
145 Arlequin. We calculated pairwise F_{ST} among sampling sites using Arlequin. Excluding
146 geographic locations with sample sizes <10 (Estonia and Denmark), we evaluated the
147 relationship between Euclidean geographic distance and genetic distance ($F_{ST}/1 - F_{ST}$) using
148 Mantel tests in Arlequin. We created a matrix of genetic distance (Nei's D_A ; Takezaki and Nei
149 1996) with 999 bootstrap replicates and used these values to create a neighbor-joining tree using
150 the program Populations 1.2.32 (Langella 1999)

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

160

161 2.4. mtDNA analyses

162 We generated all mtDNA sequence data in our previous study (Statham et al. 2014). We
163 conducted analyses of 275 concatenated mitochondrial DNA sequences for individuals that
164 provided partial cytochrome *b* (397 bp), and partial D-loop (371 bp) sequences. The two
165 fragments totaled 768 bp, which was slightly longer than the 697 bp fragment analyzed by
166 Statham et al. (2014). For populations ≥ 5 we calculated basic diversity statistics in Arlequin. We
167 also examined our data for evidence of previous demographic events using Tajima's D (Tajima
168 1989), calculated in Arlequin, and Strobecks' S statistic (Strobeck 1987) in DNAsp v5.10.01
169 (Librado and Rozas 2009). To investigate the relationship among haplotypes we created a
170 median joining network (Bandelt et al. 1999) with cytochrome *b* mutations conservatively
171 weighted double those of D-loop mutations in the program in Network 4.2.0.1 ([www.fluxus-](http://www.fluxus-engineering.com)
172 [engineering.com](http://www.fluxus-engineering.com)). We calculated pairwise Φ_{ST} among sampling sites with >5 individuals using
173 Arlequin. Excluding geographic locations with sample sizes <10 (France, Holland and Estonia),

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

176 We investigated the locations of phylogeographic breaks by comparing pairwise Φ_{ST} and
177 geographic distance among sampling sites in the program SAMOVA v1.0 (Dupanloup et al.
178 2002). The analyses were run for K values 2-10, with 100 simulated annealing processes.

179

180 *2.5. Population splitting times using mtDNA*

181 We tested hypotheses regarding splitting times among European red fox populations using
182 MCMC simulations in the program IMA2 (Hey 2010). We used the model ‘isolation without
183 migration’ when comparing island populations, and used the model ‘isolation with migration’
184 when examining the relationship between populations with potential overland connectivity. We
185 conducted analyses on a segment of concatenated cytochrome *b* and D-loop, truncated to 572 bp
186 to allow inclusion of sequences from Edwards et al. (2012). We used the HKY substitution
187 model. Following several initial trials with the recommended starting parameters (Hey 2011),
188 we ran 30 chains with geometric heating. Burn-in was set at a minimum of 1.5×10^6 generations
189 and parameter estimates were calculated based on the subsequent 2.5×10^6 generations of data,
190 sampling every 100 generations, resulting in 25,000 sampled steps. We repeated each analysis
191 once with a different random seed to assess consistency. We converted the resulting mutation-
192 scaled parameters to time values using a mutation rate of 9.36% per million generations
193 (Edwards et al. 2012). Previous studies have based divergence times on a generation time of 1
194 year, which would be the minimum possible for a monoestrous canid. We assumed a generation
195 time of 2 years, and thus a mutation rate of 4.68% per million years (Goddard et al. 2015).

196 3. Results

197 3.1. Microsatellites

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

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

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

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

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

249 250 3.2. *mtDNA*

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

258 contrast, negative values (indicating an excess of low frequency polymorphisms), consistent with
259 an expansion, were only found in northern populations, with Denmark having the only
260 significant value. We identified a significant signature of admixture in the samples from Serbia
261 (Strobeck's S ; Table 3). We did not detect a significant relationship between genetic and
262 geographic distance (IBD) in red fox populations throughout Europe ($r = 0.20$, $P = 0.18$), nor
263 when we ran the analysis excluding island and peripheral peninsular populations ($r = 0.18$, $P =$
264 0.30).

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

275 276 *Population splitting times estimated with mtDNA*

277 We estimated that populations in Britain and Continental Europe split 14.2 kya (95% HPD =
278 4.8–24 kya; Table 5). Ireland became an island prior to Britain, therefore we ran our analyses
279 under two different scenarios. Allowing for an early colonization of Ireland prior to the
280 separation of Britain and continental Europe, we identified a splitting time of 14 kya (95% HPD
281 = 6–22.4 kya). Allowing for a late colonization after both Ireland and Britain were islands, we
282 identified a slightly earlier splitting time of 10.2 kya (95% HPD = 4.2–16.4 kya). Allowing for
283 migration, we estimated that red fox populations in Spain and Central Europe split 120 kya (95%
284 HPD = 34–372 kya). This analysis indicated that the level of migration between Spain and
285 Central Europe included zero. Therefore, we also carried out analyses excluding migration and
286 estimated an overlapping but more recent splitting time of 66 kya (95% HPD = 32–104 kya). All
287 the above results produced unimodal parameter estimates and splitting times that were consistent

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

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

296 4. Discussion

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

305 The red fox population we identified in Spain was among the most highly differentiated
306 within Europe. Bayesian cluster analysis indicated that this population formed a discrete genetic
307 cluster, which was supported by one of the highest average pairwise F_{ST} values across all
308 locations sampled. All mtDNA haplotypes identified in Spain were endemic and closely related,
309 indicating long-term differentiation of Iberian red foxes from those elsewhere. In addition, the
310 presence of a number of well-represented haplotypes, with no sign of sudden radiation, was
311 indicative of a large, long-standing population in this area. Multiple lines of evidence indicate
312 that this population made only a minor contribution to the gene pool of other western European
313 populations. For example, the SAMOVA identified connectivity between central Europe and
314 Italy, and these two regions also shared mtDNA haplotypes, but Spain parsed as distinct and was
315 estimated in the IMA2 analyses to have diverged 32–104 kya. Although this time period
316 substantially precedes the global LGM (~26 kya), it was consistent with separation since the
317 local last glacial maximum in the Pyrenees of ca. 50–70 kya (Jiménez-Sánchez et al. 2013).

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

327 One unusual result was that a single endemic haplotype from Ireland grouped with
328 haplotypes found in Spain (Figure 4), while a portion of the gene pool in Irish and Dutch
329 populations assigned to the Spanish structure cluster (Figure 2). This indicates some contribution
330 of Iberian red foxes to northern populations. The finding of a connection between Ireland and
331 Iberia, referred to as a Lusitanian (or Hiberno-Lusitanian) distribution (Edwards and Bradley
332 2009; Beatty and Provan 2013), has also been observed in a range of other mammal species (e.g.
333 Davison et al. 2001; Mascheretti et al. 2003; O'Meara et al. 2012). This pattern has variously
334 been attributed to anthropogenic introductions associated with historical cultural connections
335 (Mascheretti et al. 2003; O'Meara et al. 2012), population expansion causing a replacement of
336 intervening populations (O'Meara et al. 2012), or a population bottleneck causing a loss of
337 connecting haplotypes from intervening populations (Jordan et al. 2012). Given the history of
338 fox translocation globally (Long 2003; Statham et al. 2012), a potential population size reduction
339 in Britain due to hunting (Atterby et al. 2015), and the greater diversity of haplotypes found in
340 Ireland than in Britain, any one of these scenarios could explain the patterns seen. Additionally,
341 increased sampling in Britain may uncover the same or similar Spanish type haplotypes, thus
342 indicating genetic continuity between British and Irish red foxes.

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

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

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

366 We also uncovered ancient differentiation between British and Irish populations. Both
367 formed distinct structure clusters and were significantly differentiated from one another ($F_{ST} =$
368 0.049 , $\Phi_{ST} = 0.14$, from microsatellite and mtDNA respectively). We estimated that Britain split
369 from the wider European population 4.8–24 kya. This period overlaps with that estimated
370 previously for the separation of a combined British and Irish dataset from continental Europe
371 (5.7–14.5 kya; Edwards et al. 2012), and is in keeping with the last overland connection between
372 Britain and continental Europe, via Doggerland, which existed into the Holocene, and finally
373 flooded around 7.8 kya (Montgomery et al. 2014). Ireland has existed as an island for twice as
374 long as Britain (Clark et al. 2012). This early isolation has led to considerable debate regarding
375 whether many Irish terrestrial species colonized on their own or were aided by humans
376 (Montgomery 2014 and citations within). Therefore, we investigated two scenarios: allowing for
377 natural overland colonization of Ireland (when Britain was still connected to continental Europe),
378 or allowing for human translocation (when both Ireland and Britain were islands). The analysis
379 where Britain was still part of a continental population produced an estimate of 6.2–22.4 kya,
380 which encompasses the last overland/ice connection between Ireland and the rest of Europe (~18
381 kya; Clark et al. 2012). The analysis between island populations returned a slightly more recent
382 splitting time of 4.2–16.4 kya, which is close in age to the earliest Irish red fox subfossil at 3.8
383 kya (Montgomery et al. 2014). Unfortunately, both analyses produced overlapping splitting time
384 estimates, which also encompassed the earliest evidence of human presence in Ireland (12.7 kya;
385 Dowd and Carden 2016). Thus, our data do not allow us to resolve whether red foxes colonized
386 Ireland naturally or were aided by human intervention. Ultimately, analysis of a greater
387 proportion of the genome will be necessary to determine when (and how) red foxes colonized
388 Ireland.

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

405

406 *4.1 Comparison of genetic subdivision with recognized subspecies*

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

420 2015), this population has maintained a distinct genetic character. Taken together, these data
421 indicate that both Irish and British red foxes should be considered evolutionarily distinct units
422 within the red fox.

423

424 *4.2. Conclusions*

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

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

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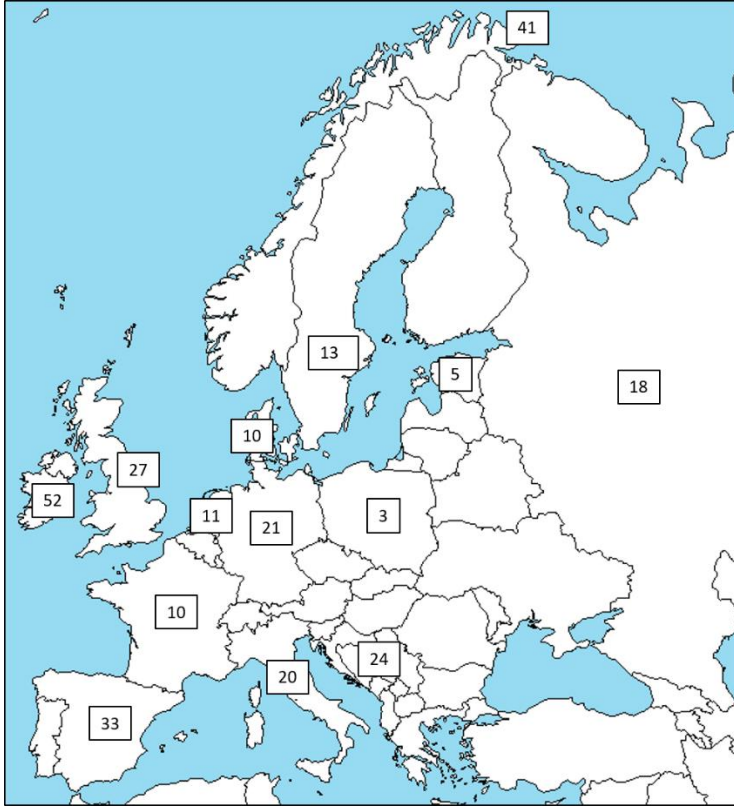
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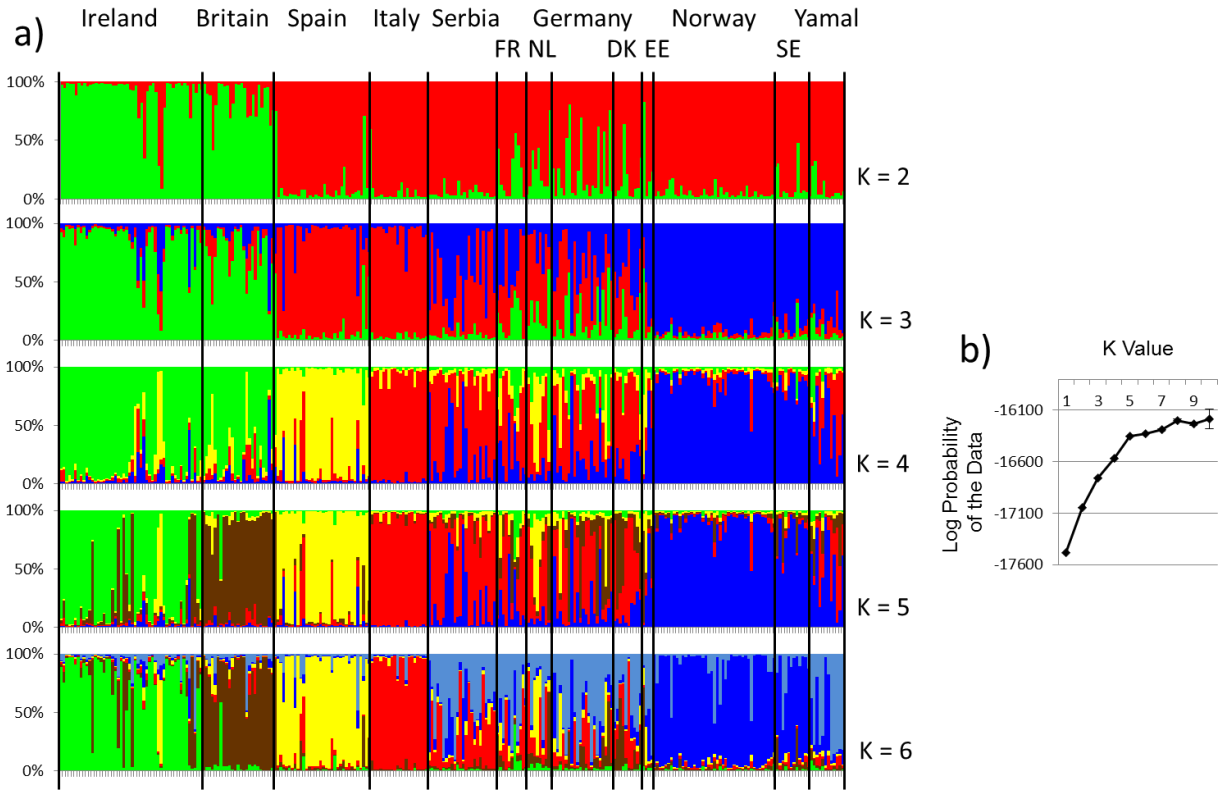
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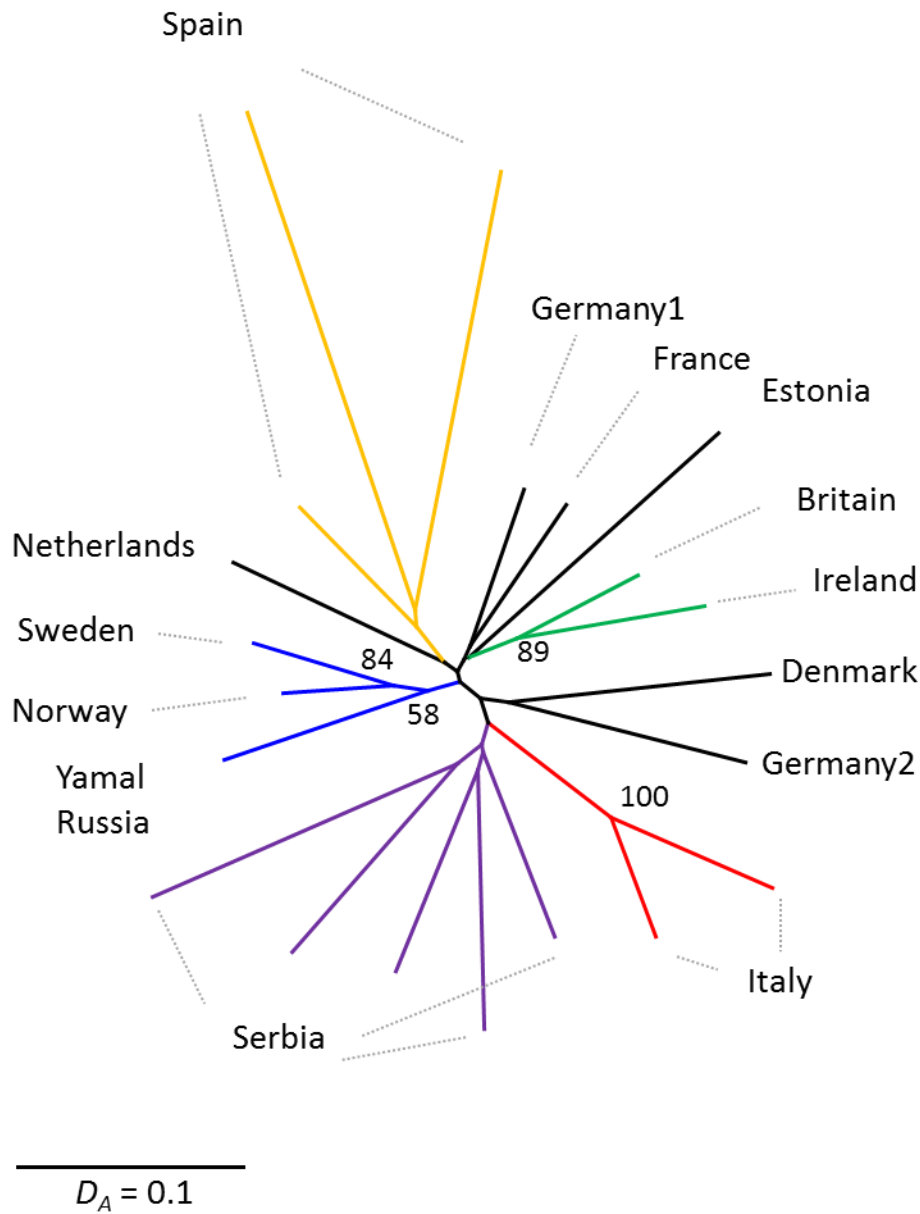
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638 Figure 1. Map of red fox samples. The number indicates the total number of samples from that
639 country. More specific sampling information is provided in the Appendix.



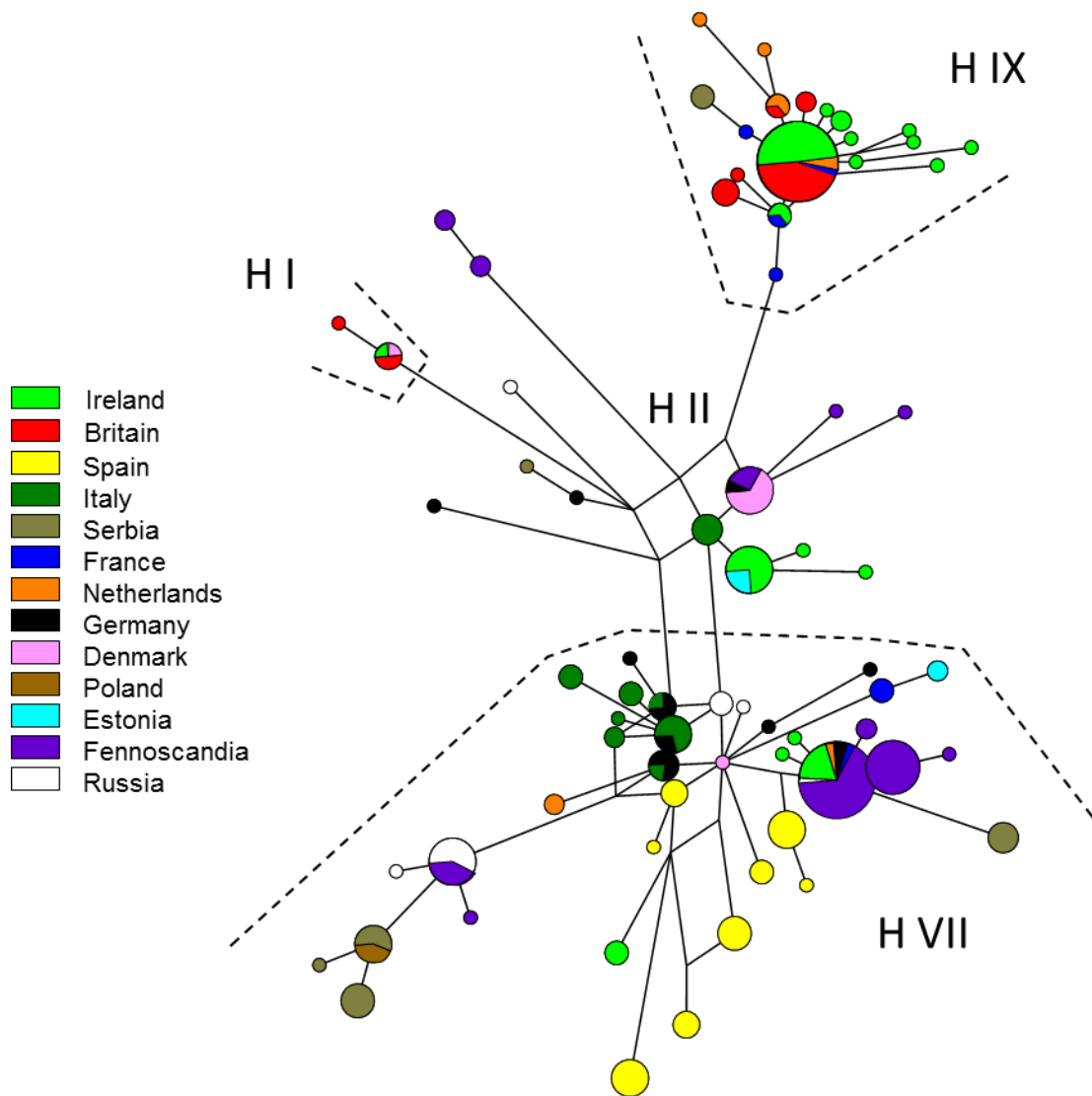
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641 Figure 2. Bayesian cluster analysis of individual European red foxes generated in the program
 642 Structure. a) Vertical bars represent individual foxes and the shading represents the proportional
 643 assignment to different clusters. FR = France,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



645
 646 Figure 3. Neighbor joining population tree of European red foxes from 21 sampling sites. Based
 647 on Nei's genetic distance (D_A ; Takezaki and Nei 1996) calculated using 19 microsatellite loci.
 648 Values at the nodes indicate bootstrap support.
 649

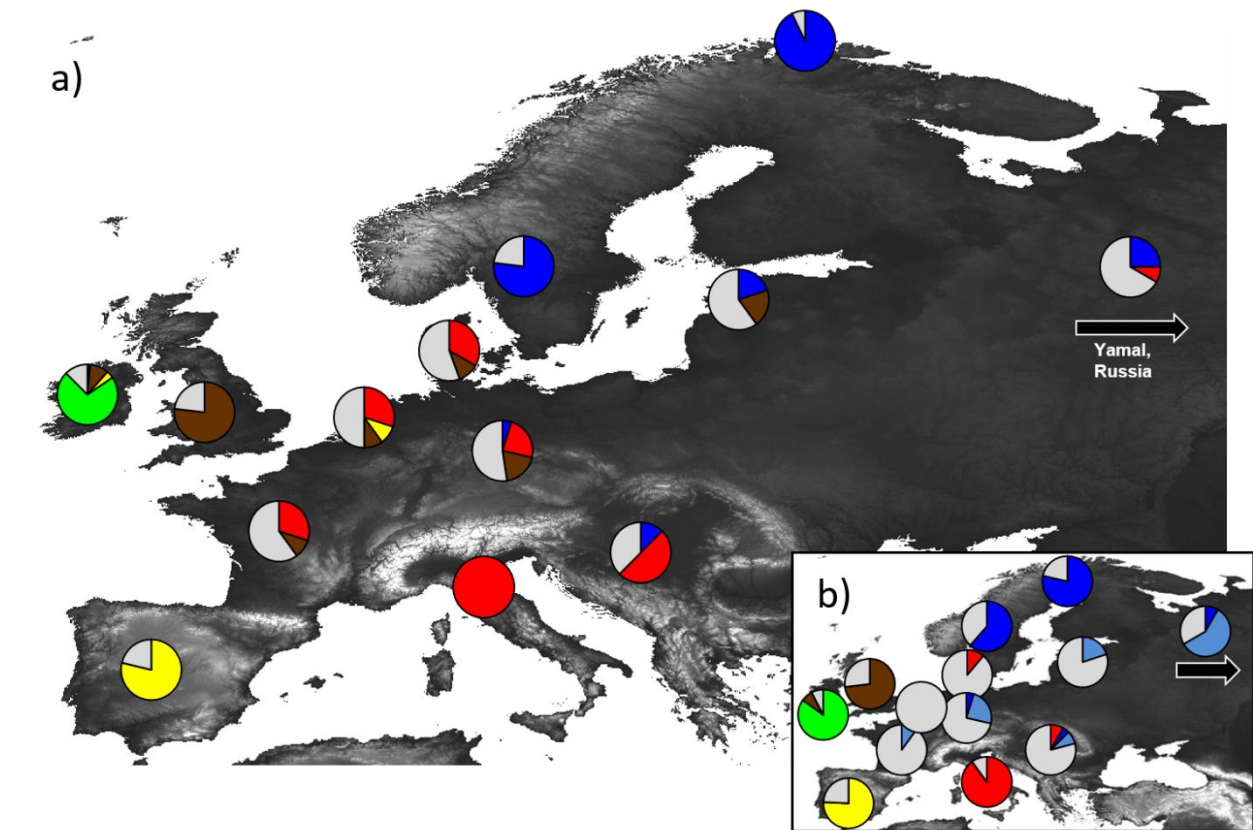
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653 Figure 4. Haplotype network of European red fox mtDNA. Calculated based on 768bp of
654 concatenated cytochrome *b* and D-loop from 275 red foxes with cytochrome *b* mutations
655 weighted double that of D-loop. Russia includes samples from Yamal,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

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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 $\geq 75\%$. 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.