

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

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56

57 **Abstract**

58 Carnivores tend to exhibit a lack of (or less pronounced) genetic structure at
59 continental scales in both a geographic and temporal sense and this can confound the
60 identification of post-glacial colonization patterns in this group. In this study we used
61 genome-wide data (using Genotyping-by-Sequencing (GBS)) to reconstruct the
62 phylogeographic history of a widespread carnivore, the red fox (*Vulpes vulpes*), by
63 investigating broad-scale patterns of genomic variation, differentiation and admixture
64 amongst contemporary populations in Europe. Using 15,003 single nucleotide
65 polymorphisms (SNPs) from 524 individuals allowed us to identify the importance of
66 refugial regions for the red fox in terms of endemism (e.g. Iberia). In addition, we tested
67 multiple post-glacial re-colonization scenarios of previously glaciated regions during
68 the Last Glacial Maximum using an Approximate Bayesian Computation (ABC)
69 approach that were unresolved from previous studies. This allowed us to identify the
70 role of admixture from multiple source population post-Younger Dryas in the case of
71 Scandinavia and ancient land-bridges in the colonization of the British Isles. A natural
72 colonization of Ireland was deemed more likely than an ancient human-mediated
73 introduction as has previously been proposed and potentially points to an increased
74 mammalian fauna on the island in the early post-glacial period. Using genome-wide
75 data has allowed us to tease apart broad-scale patterns of structure and diversity in a
76 widespread carnivore in Europe that was not evident from using more limited marker
77 sets and provides a foundation for next-generation phylogeographic studies in other
78 non-model species.

79

80 **Keywords**

81 Approximate Bayesian Computation; Genotyping-by-Sequencing; Ireland; Mammal;

82 Last Glacial Maximum; Scandinavia; SNP

83

84 **Introduction**

85 Over the last 30 years, phylogeographic studies have highlighted the roles of major
86 past climatic and geophysical events in shaping contemporary genetic structure and
87 diversity in a multitude of species (Avice et al., 1987; Pedreschi et al., 2019; Stojak,
88 Borowik, McDevitt, & Wójcik, 2019; Taberlet, Fumagalli, Wust-Saucy, & Cosson,
89 1998). During the Last Glacial Maximum (LGM; ~27-19 thousand years ago (kyrs BP;
90 Clark et al., 2009)) many terrestrial plant and animal species retreated, and were often
91 restricted, to refugial areas (Sommer & Nadachowski, 2006; Taberlet et al., 1998). In
92 Europe, several overarching patterns have emerged from fossil data and
93 phylogeographic studies, with refugia identified in the three 'classic' Mediterranean
94 (Iberian, Apennine and Balkan) peninsulas (Hewitt, 1999; Sommer & Nadachowski,
95 2006; Taberlet et al., 1998), but also further north in areas in or adjacent to the
96 Carpathians mountains and the Dordogne region in France as examples of 'cryptic
97 refugia' (McDevitt et al., 2012; Provan & Bennett, 2008; Stojak et al., 2016).
98 Phylogeographic studies of the most widely studied group, the terrestrial mammals,
99 have shown distinct mitochondrial DNA (mtDNA) lineages in small mammals (Searle
100 et al., 2009; Stojak et al., 2016; Vega et al., 2020) and ungulates (Carden et al., 2012;
101 Sommer et al., 2008) that are consistent with contraction and re-expansion from these
102 refugial regions (Hewitt, 1999; Taberlet et al., 1998).

103

104 Carnivores appear to be an exception to this general pattern however, with
105 either a lack of, or less pronounced phylogeographic structure shown across
106 continental scales (Frantz et al., 2014; Hofreiter et al., 2004; Korsten et al., 2009;
107 Mucci et al., 2010). One such carnivore, the red fox (*Vulpes vulpes*), is well-
108 represented in the fossil record in Europe (Sommer & Benecke, 2005) and has

109 numerous records during the LGM in recognized refugial areas such as the
110 Mediterranean peninsulas, and further north in areas in or adjacent to the Carpathian
111 mountains, and the Dordogne region in France (Sommer & Benecke, 2005; Sommer
112 & Nadachowski, 2006). Previous studies using mtDNA on modern and/or ancient
113 specimens have revealed a general lack of genetic structure on a continental-wide
114 scale, in both a geographic and temporal sense (Edwards et al., 2012; Kutschera et
115 al., 2013; Teacher et al., 2011). The lack of phylogeographic structuring in the red fox
116 and other carnivores has been previously attributed to these species persisting outside
117 the traditional refugial areas during the LGM, and effectively existing as a large
118 interbreeding population on a continental scale (Edwards et al., 2012; Teacher et al.,
119 2011). However, despite the abundant fossil data for red foxes, there is a distinct lack
120 of fossils from central Europe or further north during the LGM (Sommer & Benecke,
121 2004, 2005).

122

123 More recent studies (Statham et al., 2014, 2018) identified mtDNA haplotypes
124 that were unique to particular regions (e.g. Iberia) that potentially indicate long-term
125 separation from other European populations. The concerns about the use of short
126 mtDNA sequences is that they may not fully capture the signals of post-glacial
127 colonization patterns in species with high dispersal capabilities (Keis et al., 2013;
128 Koblmüller et al., 2016). One solution is the utilisation of microsatellite markers in
129 conjunction with mtDNA data (Statham et al., 2018). However, several carnivore
130 species (e.g. badgers *Meles meles* and otters *Lutra lutra*) show a similar lack of
131 resolution in terms of broad-scale genetic structure across continental Europe using
132 microsatellites (Frantz et al., 2014; Mucci et al., 2010). For the red fox, several distinct
133 groups in Europe were identified using microsatellite markers from Bayesian clustering

134 analysis, with distinction between foxes in Ireland, Britain, Spain, Italy and Scandinavia
135 being apparent (Statham et al., 2018). The rapid mutation rate of microsatellites leaves
136 it unclear whether divisions reflect ancient isolation or more recent population
137 structure, owing to recently limited gene flow. This uncertainty and lack of resolution
138 in phylogeographic structure outside the ‘traditional’ (and well-established) refugia
139 means that inferring post-glacial colonization patterns of previously glaciated regions
140 during the LGM is challenging with more limited genetic marker sets. This is
141 particularly evident in the British Isles and Scandinavia where these regions present
142 differing problems in terms of how and when contemporary populations of terrestrial
143 species reached these areas in post-glacial periods (Carden et al., 2012, 2020;
144 Herman et al., 2014).

145

146 The island of Ireland has long presented a biogeographical quandary in terms
147 of how and when terrestrial species colonized it (Carden et al., 2012, 2020; McDevitt
148 et al., 2011). The area’s latitude means that it was covered almost entirely by the ice
149 sheet during the LGM and it didn’t become an island until approximately 15,000 years
150 ago (almost twice as long as Britain; Edwards & Brooks, 2008). Because of this,
151 humans have been proposed as the primary mechanism of transport for its
152 mammalian fauna in ancient and modern times (Carden et al., 2012; Frantz et al.,
153 2014). An estimate of 10.2 kyrs BP was estimated for a split between Irish and British
154 red foxes using mtDNA data, but with a 95% CI range that incorporated the possibility
155 of natural colonization before Ireland became an island (Statham et al., 2018).
156 Haplotype diversity at different mtDNA markers is high in Irish red foxes (Edwards et
157 al., 2011; Statham et al., 2018) which would appear to contradict a more recent,
158 human-mediated origin that has been inferred from the only fossil dated to the Bronze

159 Age (3.8 kyrs BP; Sommer & Benecke, 2005). Indeed, natural colonization of other
160 species (e.g. stoat *Mustela erminea*) have been proposed over post-glacial land
161 bridges (Martínková, McDonald, & Searle, 2007) and a recent re-evaluation of fossil
162 dates has now placed multiple mammalian species on the island close to the end of
163 the LGM period (Carden et al., 2020). Therefore, a more thorough investigation of the
164 red fox's colonization history on the island has implications beyond this species only.

165

166 Fennoscandia (present-day Sweden, Norway, Finland and Denmark) was
167 covered by a huge ice sheet during the LGM and would have been inhospitable to
168 most species until its complete retreat approximately 10,000 years ago (Patton et al.,
169 2017). However, some parts of southern Sweden and western Norway would have
170 had ice free regions during the later Younger Dryas Glaciation (12.9–11.7 kyrs BP)
171 and several temporary land-bridges connected these countries to Denmark between
172 approximately 13.1 and 9.2 kyrs BP (Herman et al., 2014; Marková et al., 2020).
173 Although glacial refugia in Scandinavia during the LGM have been proposed for
174 several species, including some mammals (Lagerholm et al., 2014; Westergaard et
175 al., 2019), the red fox does not appear in the fossil records in southern Scandinavia
176 until after ~9,000 yrs BP (Sommer & Benecke, 2005). Based on evidence from mtDNA,
177 microsatellites and Y chromosome data, multiple colonization events have been
178 proposed from the south and east for the red fox (Norén et al., 2015; Wallén et al.,
179 2018) but the progression of these events remain untested. Several mammalian
180 species have been proposed to have colonized from the south over temporary land-
181 bridges and persisted through the Younger Dryas before being supplemented with
182 further colonization wave(s) from the east (Herman et al., 2014; Marková et al., 2020).

183 It is unclear however if this is a frequently-used post-glacial colonization route for many
184 of the mammals that are currently present in the region.

185

186 The advent of next-generation sequencing technologies holds great promise
187 for phylogeographic studies, allowing for thousands of single nucleotide
188 polymorphisms (SNPs) to be genotyped in non-model organisms and providing a
189 representation of the organism's entire genome (Garrick et al., 2015; McCormack,
190 Hird, Zellmer, Carstens, & Brumfield, 2013). The use of reduced-representation
191 techniques (e.g. genotyping-by-sequencing, GBS) has already demonstrated their
192 potential in resolving phylogeographic patterns in non-model organisms that are not
193 fully captured with data with a limited number of genetic markers (Emerson et al., 2010;
194 Jeffries et al., 2016; Puckett et al., 2016; Marková et al., 2020). Using GBS data from
195 over 500 individuals, the purpose of the present study was to reconstruct the
196 phylogeographic history of the red fox in Europe by investigating broad-scale patterns
197 of genomic variation, differentiation, and admixture amongst contemporary
198 populations to investigate if contemporary red fox populations are the result of isolation
199 in particular refugia during the LGM (Statham et al., 2018); or if these patterns likely
200 emerged in the post-glacial period following the LGM (Edwards et al., 2012; Teacher,
201 Thomas, & Barnes, 2011). From there, we adopted an Approximate Bayesian
202 Computation (ABC; (Beaumont, Zhang, & Balding, 2002)) framework to distinguish
203 between multiple post-glacial re-colonization scenarios of previously glaciated regions
204 that were not fully resolved in previous studies using mtDNA, Y chromosomal, and
205 microsatellite markers (Statham et al., 2018; Wallén et al., 2018). We first focused on
206 the colonization of Ireland and Britain, and whether the red fox colonized the island of
207 Ireland naturally or was introduced later by humans (Statham et al., 2018). Finally, we

208 investigated the post-glacial colonization patterns of northern Europe, with a focus on
209 the mechanism and timing of the colonization of Scandinavia around and after the
210 Younger Dryas period (Wallén et al., 2018).

211

212 **Materials and Methods**

213

214 *Laboratory methods*

215 Red fox tissue samples were obtained from freshly culled (not directly related to this
216 study), roadkill, frozen, ethanol- (70-95%) and DMSO-preserved material from
217 previous studies. No material was collected for the purpose of the present study.

218 Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Ltd.)
219 according to manufacturer's protocols (with the additional treatment of RNase). A total

220 of 30–100 ng/μl of high molecular weight DNA was sent to the Genomic Diversity
221 Facility in Cornell University (USA) where GBS was used for constructing reduced

222 representation libraries (Elshire et al., 2011) using the restriction enzyme EcoT22I
223 (ATGCAT) in six GBS libraries (each consisting of 95 uniquely barcoded individuals

224 and one negative control). Individual ligations were then pooled and purified from
225 adaptor excess using the QIAquick PCR Purification Kit (Qiagen). For library

226 preparation genomic adaptor-ligated fragments were then PCR amplified in 50 μL
227 volumes with 10 μL of DNA fragment pool, 1 × Taq Master Mix (New England Biolabs

228 Inc.) and 12.5 pmol each of the following Illumina primers: 5'-
229 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG

230 ATCT and 5'-

231 CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCT
232 TCCGATCT (the underlined parts will hybridize to the two Illumina flowcell oligos).

233 Temperature cycling consisted of 72 °C for 5 min, 98 °C for 30 s followed by 18 cycles
234 of 98 °C for 30 s, 65 °C for 10 s, and 72 °C for 30 s, with a final extension step at 72

235 °C for 5 min. The EcoT22I GBS libraries (now containing ID tags and Illumina flowcell
236 adaptors) were purified again using the QIAquick PCR Purification Kit (Qiagen). An

237 aliquot was run on the BioAnalyzer™ 2100 to verify fragment sizes. Library DNA was

238 then quantified on a Nanodrop 2000 (ThermoFisher Scientific) and subsequently
239 sequenced on an Illumina HiSeq 2000 (Cornell University, Life Sciences Core Facility).

240

241 *Bioinformatics*

242 The raw Illumina sequence data from 568 individuals were processed through the GBS
243 analysis pipeline implemented in TASSEL v5.2.31 (Glaubitz et al., 2014). Due to
244 concerns about the performances of *de novo* approaches to identify SNPs in reduced
245 representation genomic techniques (particularly for demographic analyses; Shafer et
246 al., 2017) the reference genome of the domestic dog (CanFam3.1; *Canis lupus*
247 *familiaris*) was used to align the sequence tags on individual chromosomes using the
248 BWA-backtrack method in the Burrows-Wheeler alignment tool (Li & Durbin, 2009).

249

250 A total of unique 9,249,177 tags were found, with 6,267,895 tags (67.8%)
251 uniquely aligned to the dog genome. The rest of the tags were disregarded from further
252 analyses. SNPs were initially filtered by removing those with a minor allele frequency
253 (MAF) of <0.01 and missing data per site of >0.9. This resulted in 144,745 SNPs with
254 a mean individual depth of 15.853 (\pm SD = 4.629). After the removal of indels, SNPs
255 with an observed heterozygosity >0.5 (to filter out potential paralogs), removing SNPs
256 and individuals with call-rates of <0.8, SNPs with a MAF of <0.05, and SNPs located
257 on the X chromosome, a total of 19,795 bi-allelic SNPs and 524 individuals were
258 retained after filtering in TASSEL v5.2.31 (Glaubitz et al., 2014) and PLINK v1.07
259 (Purcell et al., 2007). Because of the potential for linkage disequilibrium (LD) to bias
260 the results of population genetic analyses, we pruned 3,571 SNPs with LD of $r^2 > 0.2$
261 (Schweizer et al., 2016) in a window of 50 SNPs (sliding window with five SNPs
262 overlapping at a time) in PLINK (Purcell et al., 2007). To filter out SNPs deviating from

263 Hardy-Weinberg Equilibrium (HWE), individuals were grouped into 29 populations,
264 consisting of seven or more individuals (see below). A least-squares F_{IS} estimator
265 (based on an AMOVA) was implemented in GenoDive v2.0b23 (Meirmans & Van
266 Tienderen, 2004) using 999 permutations. A total of 869 SNPs which deviated from
267 HWE ($p < 0.01$) in 15 or more populations ($\geq 51.7\%$) were removed from subsequent
268 analyses (Van Wyngaarden et al., 2017).

269

270 Given that the focus of the study was to investigate demographic and
271 phylogeographic patterns, loci putatively under selection were identified using
272 Principal Component Analysis (PCA) and Mahalanobis distance implemented in the R
273 package *pcadapt* v3.0 (Luu, Bazin, & Blum, 2017). A false discovery rate (FDR) of α
274 = 0.05 was applied, with q -values smaller than α considered as candidate SNPs under
275 selection. A total of 352 SNPs were removed according to these criteria. Two datasets
276 were created for subsequent analyses; one containing all 524 individuals (*524dataset*;
277 Table S1), and the other containing 494 individuals (*494dataset*; Tables 1 and S1),
278 consisting of 29 ‘populations’ with seven or more individuals for population-level
279 analyses (Fig. 1A-C).

280

281 *Individual-based analyses*

282 Individual-based clustering analysis on the *524dataset* was conducted in
283 fastSTRUCTURE v1.0 (Raj, Stephens, & Pritchard, 2014). fastSTRUCTURE was
284 performed using the simple prior with K values of 1–30 over five independent runs.
285 The number of clusters (K) was obtained by using the ‘chooseK.py’ function on each
286 of these independent runs. Visualization of individual assignments to clusters per
287 population was initially performed using the ‘distruct.py’ function, with the final figure

288 produced using a custom R script. In our second individual-based analysis on the
289 *524dataset*, we incorporated the capture or sampling location of each individual, using
290 the spatially-explicit software *TESS3* (Caye, Deist, Martins, Michel, & François, 2016)
291 The default values of the program were implemented and each run was replicated five
292 times. The optimal value of K corresponded to the minimum of the cross-entropy
293 criterion, across values of $K = 1-30$.

294

295

296 *Population-based analyses*

297 For the *494dataset*, two measures of genomic diversity, allelic richness (AR) and
298 expected heterozygosity (H_E), were calculated in GENODIVE (Meirmans & Van
299 Tienderen, 2004) and HP-RARE (Kalinowski, 2005). AR and H_E values were mapped
300 with interpolation using ArcGIS 10.2.1. Geostatistical Analyst. One population from
301 Siberia (Russia) was excluded from this analysis because it was geographically distant
302 from all the other populations. Interpolation was carried out using an Inverse Distance
303 Weight model (IDW, power=1, based on 12 neighbours; Stojak et al., 2016).
304 Discriminant Analysis of Principal Components (DAPC) as implemented in the R
305 package *adegenet* v2.0.1 (Jombart & Ahmed, 2011) was performed on the
306 *494dataset*. DAPC does not make assumptions about Hardy-Weinberg equilibrium or
307 linkage disequilibrium and provides a graphical representation of the divergence
308 among pre-defined populations/groups.

309

310 *Phylogeographic reconstruction*

311 Approximate Bayesian Computation was implemented in DIYABC Random Forest
312 v1.0 (Collin et al., 2021) to further investigate the dynamics of the re-colonization

313 process of red foxes in Europe. As the method is computationally intensive and has
314 generally required 100,000 to 1,000,000 simulations to distinguish between
315 demographic scenarios being tested, several studies have performed their ABC
316 analyses on subsets of their SNPs randomly selected from the full dataset (usually
317 ~1,000 SNPs; e.g. Huang et al., 2017; Jeffries et al., 2016). However, recent
318 applications using a tree-based classification method known as 'random forest' in ABC
319 allow demographic scenarios to be distinguished based on 1,000s to 10,000s of
320 simulations for each scenario (Fraimout et al., 2017; Kotlík, Marková, Konczal, Babik,
321 & Searle, 2018; Pudlo et al., 2015; Marková et al., 2020). We followed the approach
322 of Kotlík et al., (2018) where all SNPs were used, but we chose a subset of the
323 individuals in each grouping to save on computational time (Table S1).

324

325 Based on recent simulation studies, ABC-based methods have received
326 criticism for their ability to capture the true demographic models under consideration
327 (Cabrera & Palsboll, 2017; Shafer, Gattepaille, Stewart, & Wolf, 2015). Following
328 recommendations by Cabrera & Palsboll (2017), scenarios were kept as simple and
329 different from each other as possible in order to distinguish between the major
330 demographic events under consideration. In addition, the number of comparable
331 scenarios was always kept low (Cabrera & Palsboll, 2017). Following on from
332 outstanding issues in regards to unresolved colonization scenarios (Statham et al.,
333 2018), we first investigated the colonization history of red foxes in the British Isles. We
334 grouped individuals into three 'populations' of interest to ascertain the most likely
335 scenario for the timing and source of existing populations in Ireland and Great Britain
336 based on the analyses performed in fastSTRUCTURE, DAPC (See Results) and
337 previous studies. These were 'Ireland' (IRE + NIR populations), 'Britain' (UKS + UKN)

338 and 'Europe' (FRA + BEL + SWZ). Three scenarios were incorporated that the data
339 and analysis presented by Statham et al. (2018) could not distinguish between. The
340 first is that Ireland was colonized naturally overland from an unsampled ancestral
341 population (N4) after the LGM (i.e. before Ireland became an island between
342 approximately 19,000 and 15,000 yrs BP; t_2 ; Fig. S1). In this scenario, Britain
343 originated from this ancestral population also but mixes with the European population
344 before it became isolated from the mainland after the flooding of Doggerland around
345 8 kyrs BP (19–8 kyrs BP; t_a). Europe diverged from the unsampled ancestral
346 population at 19–15 kyrs BP with $t_1 > t_2$. In the second scenario, Ireland's red foxes
347 were founded directly from Britain naturally before it became an island (t_2). In the third
348 scenario, Ireland's foxes were transported by humans from Britain after the earliest
349 evidence of human presence on the island at 12,700 yrs BP (Dowd & Carden, 2016)
350 right up to the present day (t_3 ; Fig. S1). Effective populations sizes were allowed to
351 range between 10 and 500,000 individuals for Irish and British populations and
352 between 10 and 1,000,000 for Europe and the unsampled ancestral population.
353 Generation time was assumed to be 2 years (Statham et al., 2018).

354

355 For the second ABC-based analysis, we investigated the colonization history of
356 Scandinavia. Wallen et al. (Wallén et al., 2018) proposed that Scandinavia was
357 colonized from multiple sources based on mtDNA and Y chromosome data but did not
358 attempt to date the progression of these events. We attempted to distinguish between
359 five scenarios for the timing and progression of these colonization events. In the first
360 of these, 'Scandinavia' (FNS + FNN + SWN + SWS + NOR) was the result of admixture
361 in the east and subsequent colonization (9,000 yrs BP to present; t_b) between 'Central
362 Europe' (PLA + PLC + PLS + GER) and 'Russia' (RUK + RUV) populations after the

363 region became ice-free after the Younger Dryas Glaciation and disappearance of land-
364 bridges connecting it to central Europe (Herman et al., 2014). The Central Europe and
365 Russian populations split at t_1 (27,000–19,000 yrs BP). In the second scenario, the
366 first colonization wave occurred from central Europe over land-bridges (14,000 to
367 9,000 yrs BP; t_a), with later admixture occurring from Russia (9,000 yrs BP to present;
368 t_b). In the third scenario, the first colonization wave occurred from Russia (9,000 yrs
369 BP to present; t_b), with later admixture from Central Europe from an eastern route
370 (9,000 yrs BP to present but restricted to after the first Russian colonization wave; t_b
371 $>t_c$). In the fourth scenario, Russia was the result of admixture between Scandinavia
372 and Central Europe at t_b . Finally, the fifth scenario had all three populations diverging
373 at t_1 . Effective populations sizes were allowed to range between 10 and 1,000,000
374 individuals.

375

376 Ten thousand simulated datasets per scenario were used to produce posterior
377 distributions. Each scenario was considered to be equally probable at the outset. To
378 check the reliability of the observed summary statistics, a Principal Component
379 Analysis (PCA) was performed on the summary statistics from the simulated datasets
380 and compared against the summary statistics from the observed dataset in order to
381 evaluate how the latter is surrounded by the simulated datasets (Collin et al., 2021).
382 All simulated datasets were used in each Random Forest training set. We used five
383 noise variables and ran 1,000 Random Forest trees to select the most likely scenario
384 and estimate parameters (Collin et al., 2021).

385

386 **Results**

387 *Individual-based analyses*

388 fastSTRUCTURE identified $K = 7$ as the lower limit of clusters in each of the five
389 independent runs of $K = 1-30$ (Fig. 1C), with the upper limit of K fluctuating from 10-
390 13 between runs. Focusing first on $K = 7$, distinct clusters were identified in each of
391 Ireland ('Ireland') and Great Britain ('Britain'). Iberian populations formed a distinct
392 cluster ('Iberia'), a known glacial refugium. Populations in France, Switzerland,
393 Belgium, Germany, Poland, Slovenia, Croatia, Serbia and the Ukraine formed a
394 distinct cluster ('Central Europe', named for simplicity because of the approximate
395 location of the cluster relative to the other clusters). Several of these populations are
396 in proximity to the Balkan and Carpathian glacial refugia. Localities with small numbers
397 of individuals in Lithuania, Estonia, Belarus and western Russia also belonged to this
398 cluster. These populations in eastern Europe showed evidence of mixed ancestry with
399 individuals from Scandinavia (Figs. 1A and 1C), who formed another distinct cluster
400 ('Scandinavia'). Individuals from European Russia had mixed ancestry between this
401 Scandinavian cluster and individuals from Siberia (another distinct cluster; 'Siberia').
402 Finally, individuals from central Italy formed a distinct cluster ('Italy'), another well-
403 defined glacial refugium. Individuals in northern Italy showing mixed ancestry with the
404 central European cluster (Figs. 1A and 1C). For the lower values of K , the islands of
405 Ireland and Britain were separated from all other samples at $K = 2$. Scandinavia and
406 Russia samples were further separated at $K = 3$, Iberia samples at $K = 4$, Siberia at K
407 $= 5$ and Ireland and Britain each becoming distinct clusters at $K = 6$ (Fig. 2). For $K =$
408 8 , an additional cluster with the European Russian populations was identified. For $K =$
409 9 , another cluster consisting of French, Belgian and Swiss populations was found (with
410 mixed ancestry from Central Europe; Fig. 2). These populations are in close proximity

411 to the known glacial refugium located in the Dordogne region. Further admixture was
412 identified within populations in Central Europe at $K = 10\text{--}13$ (data not shown). The
413 spatially-explicit software *TESS3* (Caye et al., 2016) failed to resolve the genomic
414 structure within the system, with the minimum of the cross-entropy criterion decreasing
415 up to $K = 30$ (Fig. S4). Further test runs increasing K up to a maximum of 40 failed to
416 resolve the genomic structure (data not shown).

417

418 *Population-based analyses*

419 Direct estimates of genomic diversity (Table 1) and the IDW interpolation of allelic
420 richness (AR) and expected heterozygosity (H_E) in 28 fox populations showed that
421 diversity is highest in Central and Eastern Europe and decreases westwards and
422 northwards (Fig. 2). Genomic diversity was notably lower in the British Isles, with the
423 Irish populations showing the lowest levels of diversity (Fig. 2). The DAPC revealed
424 distinct groupings of Iberian samples, Irish samples, British samples, Siberian and
425 Scandinavia/Russian samples in general agreement with the individual-based
426 analysis in fastSTRUCTURE (Fig. 1B). Populations in western, central and eastern
427 Europe were grouped closely together, but the populations in France, Belgium and
428 Switzerland were more separated from the main European group on the first axis,
429 aligned with the individual-based Bayesian analyses at $K = 9$ (Fig. 2). Although the
430 population in central Italy formed its own genomic cluster in the individual-based
431 analysis (Fig. 1C), it grouped more closely with the central European group than the
432 French, Belgian and Swiss populations in this analysis (Fig. 1B).

433

434 For the ABC-based analysis, first focusing on the colonization history of Ireland
435 and Britain, Scenario 1 (Fig. S1) was selected (620/1,000 trees) with the posterior

436 probability estimated at 0.741. The ancestral population for Ireland and Britain was
437 estimated to have split from the European mainland at 17.7 kyrs BP (95% CI: 15.81–
438 18.95 kyrs BP), with the Irish population originating at 16.1 kyrs BP (95% CI: 15.04–
439 18.1 kyrs BP; Fig. 4; Table S2). For Scandinavia, Scenario 1 (510/1000 trees) was
440 selected with the posterior probability estimated at 0.66. The initial split of the Central
441 European and Russian populations was estimated at 23.04 kyrs BP (95% CI: 19.34–
442 26.55 kyrs BP). The current Scandinavian originates from admixture from Russian and
443 Central European populations at 5.36 kyrs BP (95% CI: 1.83–8.75 kyrs BP; Fig. 4;
444 Table S2).

445 **Discussion**

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

451

452 *Phylogeographic structure of the red fox in Europe*

453 Individual- and population-based analyses revealed congruent patterns of genomic
454 structuring at the broad scale of Europe (and Siberia), with certain important nuances
455 being revealed by different approaches (Figs. 1, 2, 3 and S4). Earlier studies had
456 proposed that red foxes may have existed as a single, large panmictic population
457 during the LGM based on a lack of distinct structure at mtDNA markers using modern
458 and/or ancient DNA (Edwards et al., 2012; Teacher et al., 2011). If this was the case,
459 we might have expected to find a more continuous population (excluding the islands
460 potentially) and this is not evidenced here with this greatly expanded dataset in terms
461 of genetic markers, individuals and spatial coverage. In addition, a continuous
462 population over the whole continent at the LGM is not generally congruent with the
463 fossil data and the lack of fossil records beyond the more accepted refugial regions
464 (Sommer & Benecke, 2005; Sommer & Nadachowski, 2006). Our study demonstrates
465 that the observed patterns of genomic variation in contemporary red fox in Europe
466 were mainly shaped by distinct refugial populations, with subsequent post-glacial
467 admixture and isolation when this species had expanded into what is now its current
468 distribution range in Europe (Sommer & Benecke, 2005).

469

470 At lower values of K , the islands of Ireland and Britain are separated from the
471 rest of the European continent. Scandinavia/Russia and Iberia are then separated
472 from the other continental individuals at $K = 3$ and 4 , respectively (Fig. 2). Most of the
473 central European (defined here as those outside of the Mediterranean peninsulas and
474 Scandinavia) and Balkan populations formed a single genomic cluster at $K = 7$ (Figs.
475 1A and 1C). This and the elevated values of genomic diversity (Fig. 3) are likely
476 reflective of more widespread and connected populations occupying the Balkans and
477 Carpathian refugia during the LGM (as is known from fossil records; Sommer &
478 Nadachowski, 2006) and a subsequent expansion into the rest of central Europe in
479 the post-glacial period. A similar scenario has been proposed for other large mammals
480 (Frantz et al., 2014; Stojak & Tarnowska, 2019). At $K = 7$, French, Belgian and Swiss
481 individuals were grouped with other central European populations but population-level
482 analyses (DAPC; Fig. 1B and 3) showed that these populations were distinct from
483 other populations in close proximity (and they formed their own cluster at $K = 9$ in the
484 individual-based Bayesian analysis; Fig. 2). Fossil records of the red fox are known
485 from the Dordogne region in France during the LGM (Sommer & Nadachowski, 2006)
486 so these populations may stem from a previously isolated refugial population in the
487 area, and now show post-glacial admixture with populations stemming from
488 eastern/Balkan and Iberian refugia (Figs. 1B and 1C). The Iberian populations form a
489 distinct cluster/group at $K = 4$ and above (Figs. 1B, 1C and 2) and this is in line with
490 previous findings using fewer molecular markers identifying this as a glacial refugium.
491 Statham et al. (2018) identified mtDNA haplotypes that were endemic to the region,
492 while microsatellites identified Spanish individuals as being distinct from those in other
493 European populations. A similar pattern was found previously in badgers, with Iberian
494 populations having many unique mtDNA haplotypes not found elsewhere on the

495 continent (Frantz et al., 2014). The Pyrenees Mountains have remained a formidable
496 barrier for post-glacial re-colonization, and there appears to be little contribution to
497 subsequent northwards expansion when the ice-sheets receded for many terrestrial
498 species (Bilton et al., 1998). Even though the maximum dispersal capabilities of the
499 red fox are up to 1,000 km in Europe (Walton, Samelius, Odden, & Willebrand, 2018),
500 this mirrors the pattern of mountains acting as significant barriers for the species in
501 North America (Sacks, Statham, Perrine, Wisely, & Aubry, 2010). This is in contrast to
502 red foxes in the other Mediterranean refugium, Apennine (Italy). Although red foxes
503 from central Italy are identified as a distinct cluster in fastSTRUCTURE at $K = 7$ and
504 above, mixed ancestry was identified with neighbouring populations north of the Alps
505 in central Europe and the Balkans (Figs. 1B, 1C and 2). This may rather reflect a more
506 recent divergence of this population given the geography of the region.

507

508 Glaciated regions during the LGM such as the British Isles and Scandinavia
509 present differing problems in terms of how contemporary populations of terrestrial
510 species colonized these areas in post-glacial periods. Ireland has existed as an island
511 for approximately 15,000 years (Edwards & Brooks, 2008) and humans have been
512 proposed as the primary mechanism of transport for its mammalian fauna based on
513 existing fossil data and numerous phylogeographic studies (e.g. McDevitt et al., 2011;
514 Carden et al., 2012; Frantz et al., 2014). Statham et al. (2018) proposed a split of
515 approximately 10 kyrs between Irish and British foxes but with confidence intervals
516 that didn't distinguish between natural and human-mediated colonization scenarios.
517 Both Irish and British populations are distinct from mainland European populations
518 (Figs. 1B, 1C and 2) and have patterns of diversity and structure consistent with
519 colonization and subsequent isolation (Table 1; Figs. 1B and 2). Using an ABC-based

520 approach, a scenario in which Ireland was colonized before humans were known to
521 be present (approximately 15 kyrs BP) was deemed the most likely (Fig. 4; Table S3).
522 Although this conflicts with the current fossil evidence where the oldest known
523 specimen in Ireland is from the Bronze Age (Sommer & Benecke, 2005), this is
524 congruent with previous studies demonstrating high haplotype diversity and the
525 identification of many unique haplotypes at mitochondrial markers on the island in
526 comparison to British and other European populations (Edwards et al., 2012; Statham
527 et al., 2018). The stoat was also proposed to be an early colonizer of Ireland over a
528 post-glacial land bridge (Martínková, McDonald, & Searle, 2007) and several potential
529 prey species (e.g. mountain hare *Lepus timidus* and arctic lemming *Dicrostonyx*
530 *torquatus*; Woodman et al., 1997) were also present in the early post-glacial period. A
531 recent re-assessment of several Irish mammalian fossils has pushed several species
532 (reindeer *Rangifer tarandus*, grey wolf *Canis lupus* and woolly mammoth *Mammathus*
533 *primigenius*) to the LGM/post-glacial boundary (Carden et al., 2020). Given that
534 models of glaciation/de-glaciation patterns are reliant on secure and accurate fossil
535 data, the island may have hosted a larger mammalian community in the early post-
536 glacial period and this may have included the red fox also. While humans were still
537 likely an important factor in determining later faunal assemblages on Ireland (Carden
538 et al., 2012; Frantz et al., 2014), the early post-glacial period clearly warrants further
539 investigation on the island based on the results presented here and newly available
540 fossil data (Dowd & Carden, 2016; Carden et al., 2020).

541

542 Although glacial refugia in Scandinavia during the LGM have been proposed
543 for several species including mammals (Lagerholm et al., 2014; Westergaard et al.,
544 2019), the red fox does not appear in the fossil records in southern Scandinavia until

545 much later (~9,000 yrs BP; Sommer & Benecke, 2005). Here, we examined five
546 different hypotheses for the colonization of Scandinavia (Fig. S2). The most likely
547 scenario for the colonization of Scandinavia is a mixture of foxes from Russian and
548 central/eastern Europe colonizing from the east (Fig. 4). When the ice retreated from
549 northern Scandinavia after the Younger Dryas, a lack of geographic barriers led to
550 later dispersal into the region from the east (Norén et al., 2015; Wallén et al., 2018), a
551 pattern that is evident in other carnivores also (Dufresnes et al., 2018; Keis et al.,
552 2013). Several recent studies have pointed to a pattern of post-glacial colonization of
553 Scandinavia from the south involving individuals from present-day central Europe first
554 crossing temporary land-bridges prior to the Younger Dryas glaciation. This pattern is
555 particularly prevalent in small mammals such as the field vole *Microtus agrestis*
556 (Herman et al., 2014) and the bank vole *Myodes glareolus* (Marková et al. 2020). From
557 all the analyses presented here, this scenario is not supported for the red fox and it is
558 instead apparent that this came later from the east only (Fig. 4). With Scandinavia
559 being one of the last regions of the continent to be re-colonized, the higher genomic
560 diversity observed here than some of the more westerly regions could be due to it
561 being populated from multiple sources from the east (Wallen et al., 2018; Marková et
562 al., 2020).

563

564 Using genome-wide data has allowed us to tease apart broad-scale patterns of
565 structure and diversity in a widespread carnivore in Europe that was not evident from
566 more limited marker sets. The use of genomic data allowed us to identify the
567 importance of refugial regions in terms of both endemism (e.g. Iberia) and sources of
568 post-glacial re-expansion across the continent (e.g. the Carpathians and Balkans). In
569 conjunction with ABC-based analyses, we identified patterns of post-glacial

570 colonization in formerly glaciated regions that contradict previously proposed routes
571 for the red fox and other similar species. Given the genomic resources now available
572 (Kukekova et al., 2018), the application of ancient genomics on the extensive fossil
573 material available for this species (Sommer & Benecke, 2005) should fall into line with
574 other charismatic carnivores (Loog et al., 2020) to fully understand re-colonization and
575 temporal patterns that have not been captured in previous studies of ancient red fox
576 specimens (Edwards et al., 2012; Teacher et al., 2011).

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589

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875

876 **Author contributions**

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

884

885 **Data Accessibility**

886 Raw SNP data and datasets used for individual and population-level analyses are
887 available on a private peer review link on Dryad

888 (<https://datadryad.org/stash/share/lxGNZUG315NeDDYqLZ4srLJGzEqw3U7LLfLztMpaI6E>)

889 **Table 1.** Population-level analyses of 29 pre-defined red fox populations. Populations
890 are named by country of origin with an accompanying label (Pop ID) for illustrative
891 purposes (see Figure 1). Approximate geographic co-ordinates are given in longitude
892 and latitude. The number of individuals in each population is indicated by *N* and
893 population-level measures of allelic richness (AR) and expected heterozygosity (He)
894 are shown that were used to calculate the interpolation of genomic diversity (see
895 Figure 3).

Population	Pop ID	Longitude	Latitude	<i>N</i>	AR	He
Ireland	IRE	53.3927275	-9.9262638	20	1.68	0.237
Northern Ireland	NIR	54.717149	-6.219987	20	1.68	0.238
England North	UKN	54.47445462	-2.680500725	17	1.73	0.254
England South	UKS	51.6839616	0.574959028	27	1.73	0.253
Portugal North	PTN	40.7115386	-7.7495801	11	1.77	0.263
Portugal South	PTS	38.9648718	-9.2992138	18	1.76	0.26
Spain North	SPN	42.829585	-2.700338	29	1.79	0.268
Spain South	SPS	37.14280344	-3.647460938	8	1.71	0.254
France	FRA	48.50605105	2.64715577	20	1.79	0.27
Belgium	BEL	50.589536	6.123152	19	1.81	0.274
Switzerland	SWZ	46.7633251	7.1564597	19	1.79	0.271
Italy North	ITN	45.926389	10.251667	7	1.74	0.269
Italy	ITA	43.32517768	11.31591797	20	1.73	0.254
Germany	GER	52.5200066	13.404954	28	1.81	0.273
Slovenia	SOV	46.31255967	15.50934385	24	1.78	0.265
Croatia	CRO	45.4082114	13.6589423	19	1.81	0.274
Poland East	PLA	53.82505665	23.3406601	16	1.82	0.274
Poland South	PLS	49.7801	20.2505	14	1.81	0.275
Poland West	PLC	52.117714	16.779255	19	1.82	0.275
Serbia	SER	45.83645405	20.43457031	18	1.81	0.271
Ukraine	UKR	47.725824	29.952977	9	1.8	0.274
Sweden South	SWS	57.95217685	13.93725559	17	1.74	0.256
Sweden North	SWN	64.3322949	20.28735358	16	1.77	0.263
Norway	NOR	70.26464	30.34619	14	1.77	0.265
Finland North	FNN	69.9059	27.02396	8	1.76	0.269
Finland South	FNS	60.46667	26.91667	12	1.76	0.259
Russia Komi	RUK	61.8	51.516667	10	1.76	0.262
Russia Kirovskaja	RUV	58.56861045	48.86787415	25	1.78	0.265
Siberia	SIB	135.774568	67.898166	10	1.6	0.211

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897

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

903

904 **Figure 2.** The proportion of admixture in each population based on the ancestry
905 coefficients determined in *fastSTRUCTURE* for K values 2–9.

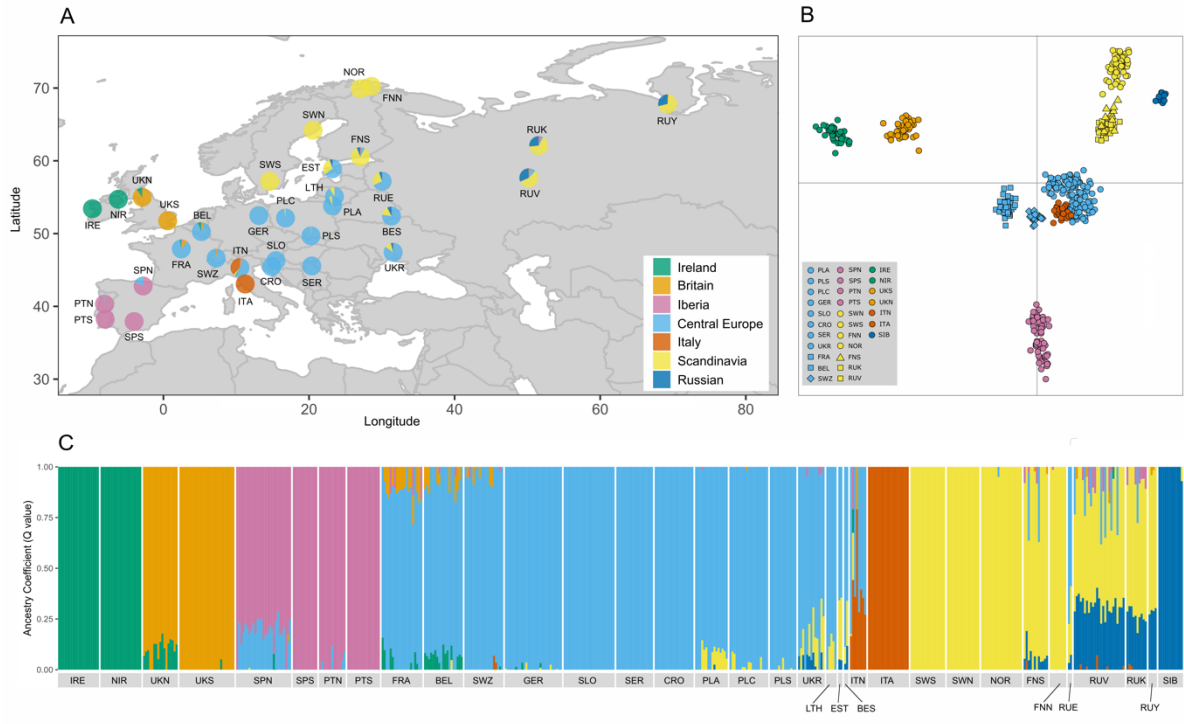
906

907 **Figure 3.** Interpolation of allelic richness and expected heterozygosity in 28 red fox
908 populations (Siberia was excluded in this analysis) in Europe. The interpolated values
909 of both indices are presented in the maps in different colours on a low (blue) to high
910 (red) scale according to the legends. Black circles indicate the population locations.

911

912 **Figure 4.** Graphical representation of the most likely post-glacial colonization
913 scenarios for Ireland and Britain (A) and Scandinavia (B) inferred from Approximate
914 Bayesian Computation.

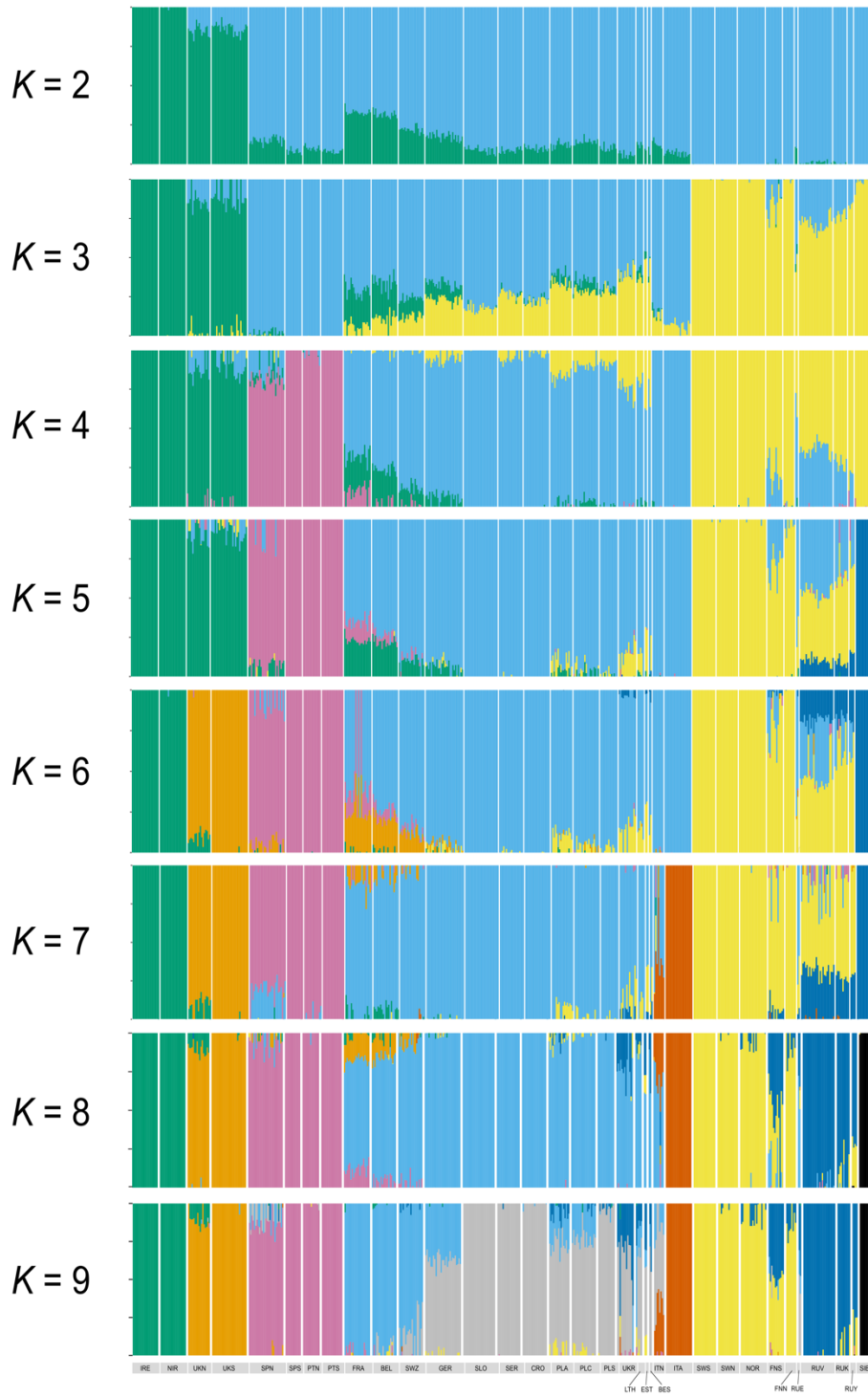
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917 Figure 1.

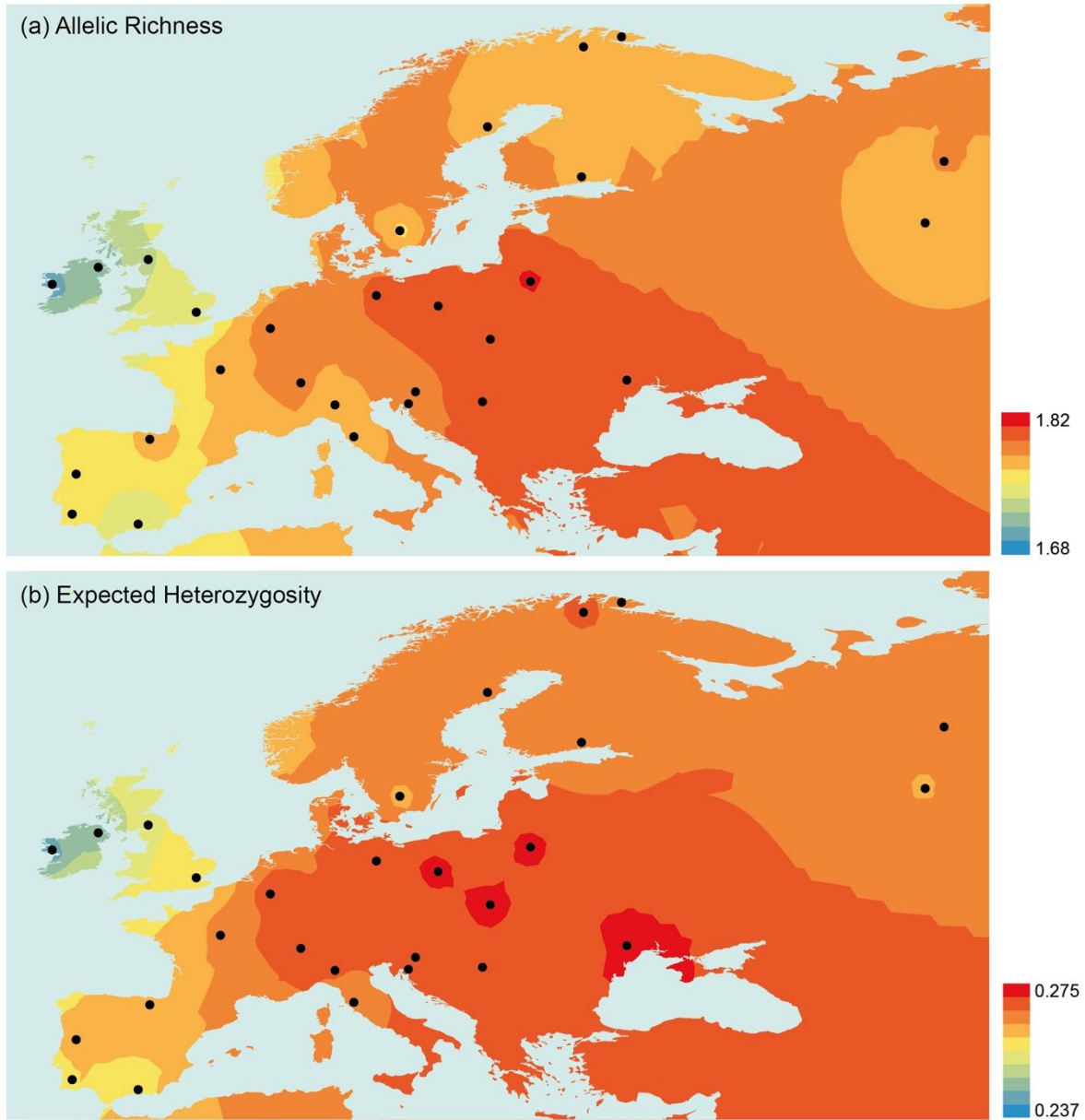
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920 Figure 2.

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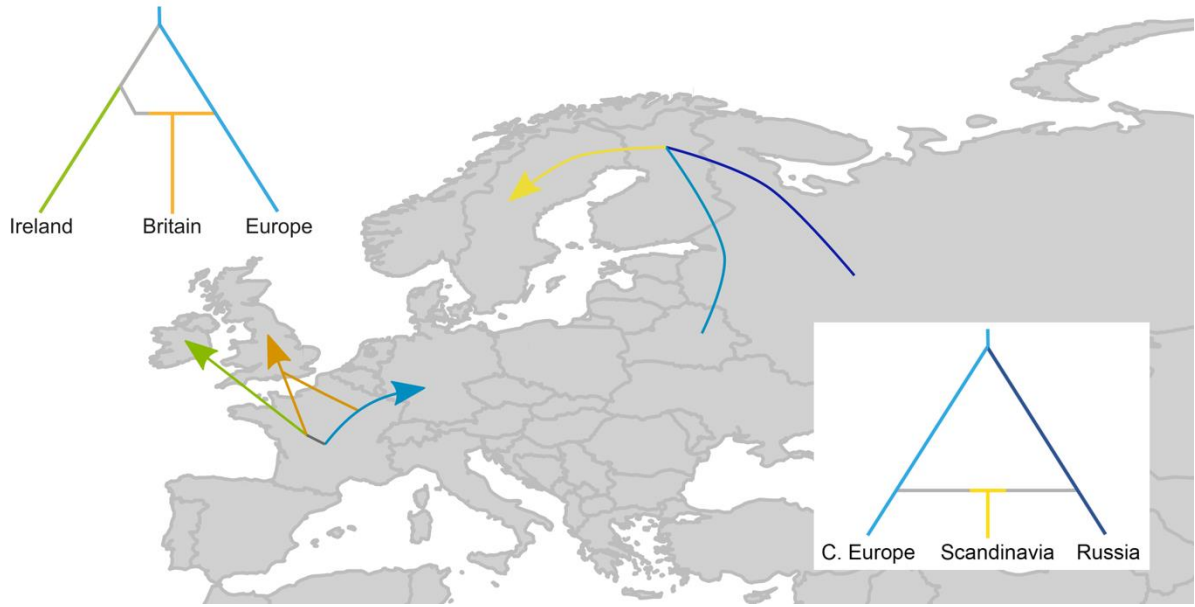


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924 Figure 3.

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928 Figure 4.