

1 The colonisation history of British water vole (*Arvicola amphibius*[L., 1758]):
2 origins and development of the Celtic Fringe.

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17

18

19 **Abstract**

20 The terminal Pleistocene and early Holocene, a period from 15 to 8 KA, was
21 critical in establishing the current Holarctic fauna, with temperate-climate
22 species largely replacing cold-adapted ones at mid-latitudes. However, the
23 timing and nature of this process remain unclear for many taxa, a point which
24 impacts on current and future management strategies. Here, we use an ancient
25 DNA dataset to test more directly postglacial histories of the water vole (*Arvicola*
26 *amphibius*, formerly *A. terrestris*), a species that is both a conservation priority
27 and a pest in different parts of its range. We specifically examine colonisation of
28 Britain, where a complex genetic structure can be observed today. Although we
29 focus on population history at the limits of the species' range, the inclusion of
30 additional European samples allows insights into European postglacial
31 colonisation events and provides a molecular perspective on water vole
32 taxonomy.

33

34 **Introduction**

35 The period from the end of the last (Weichselian/Devensian) glaciation
36 c.14700BP until the mid-Holocene 8200 BP (1) was one of extensive climatic and
37 environmental change, including the presence of two minor temperate-climate
38 episodes (the Bølling and Allerød interstadials) and a full cycle of glacial re-
39 advance and retreat, known as the Younger Dryas (YD), 12800-11500 BP
40 (collectively referred to as the Lateglacial), followed by the rapid climatic
41 amelioration and ensuing vegetation change that accompanied the start of
42 Holocene interglacial warming. The Lateglacial was a period of rapid

43 vegetational change and widespread faunal extinction and translocation across
44 Eurasia (2-3). It may be viewed as the most recent example of the dramatic
45 climatic fluctuations associated with the Pleistocene, where over the last 2.6
46 million years ice sheets periodically spread down from the north, leaving
47 Northern Europe almost fully glaciated and permafrost extending throughout
48 Central Europe with only the southernmost peninsulae ice free (4). During the
49 last full glacial cycle, the maximum extent of glaciation (the last glacial maximum
50 or LGM; c.22500BP) extended across Scandinavia and the British Isles, with large
51 parts of Europe becoming too cold for many mammal species to survive (5, 2, but
52 see 6). Hewitt (7-9) made significant progress in the development of a model of
53 organismal response to Holarctic climate change, proposing that in Europe
54 temperate populations survived periods of climatic deterioration in three
55 refugial peninsulae (Iberia, Italy and the Balkans), before recolonising
56 northwards as glaciers retreated. While this model has been central to our
57 understanding of the population histories of the European biota, subsequent
58 work suggests a more complex pattern of recolonisation that can vary in relation
59 to particular taxa, regions and time points (10). One example is the proposal by
60 Searle and colleagues (10), who found that a general pattern for the
61 recolonisation of Britain can be inferred from studies of multiple small mammal
62 species. Based on a range of genetic markers, they identified pairs of population
63 groups in five different species, and in each case the two populations form either
64 “core” (roughly England, sometimes excluding the south coast) or “peripheral”
65 (Scotland, Wales, and sometimes the south coast of England) populations. This
66 pattern has been referred to as the “Celtic fringe”, since it bears a strong
67 resemblance to the cultural and linguistic distinctions that today separate

68 Scottish, Irish, Welsh, Manx and Cornish peoples from those in central and
69 eastern England (10). While it has been proposed to stem from multiple
70 colonisation events from different populations, the exact timing and nature of
71 this process remains unclear.

72 The application of ancient DNA in reconstructing species history has played a
73 significant role in identifying source populations and postglacial recolonisation
74 events (e.g. 2, 11-12). Here, we focus on one of the ‘Celtic fringe’ species, the
75 northern water vole, *Arvicola amphibius* (also referred to as *A. terrestris*), a
76 widely distributed species found across Europe (excluding Ireland and Central
77 and Southern Spain), east through Siberia to the Lena River Basin, and from the
78 Arctic Sea south to Lake Baikal and North West China through North West Iran,
79 Iraq, North Israel, the Caucasus and Turkey (13).

80 We identified northern water vole as the most suitable organism to
81 explore the formation and origins of the “Celtic fringe”, as it (i) exhibits a very
82 clear spatial pattern of mitochondrial DNA differentiation across the present-day
83 Scottish-English border (14); (ii) is the largest of the small mammal fauna with a
84 proposed “Celtic Fringe” distribution (10), thereby increasing the volume of bone
85 available for each analysis and (iii) both English, and especially Scottish, water
86 vole populations are the focus of considerable conservation efforts, and an
87 improved understanding of the origins of these populations could therefore
88 assist in targeting resources.

89 Water voles are clearly sufficiently polymorphic in both ecology and morphology
90 to present a long-standing taxonomic problem. Membership of the water vole
91 genus has fluctuated, ranging from one all-encompassing species (*terrestris*;
92 (15)), more commonly two (*sapidus* and *terrestris*; (16)), but also four

93 (*amphibius*, *sapidus*, *scherman* and *terrestris*; (17)) and at its peak seven
94 (*amphibius*, *illyricus*, *italicus*, *musignani*, *sapidus*, *scherman* and *terrestris*; (18)).
95 Current taxonomic determinations recognise three species: *A. amphibius*,
96 (Northern water vole, distributed across Eurasia), *A. sapidus* (Portugal, Spain and
97 France) and *A. scherman* (European mountains: Alps, Carpathians, Cantabrian
98 Mountains, Massif Central and Pyrenees) (13), with a caveat that authors
99 anticipate convergence towards the greater diversity recognised by Miller (18).
100 Several studies have sought to resolve the taxonomy and evolution of water vole
101 lineages through molecular analyses (14, 19). Piertney *et al.* (14) specifically
102 targeted water vole from across Britain. The resulting phylogeny identified the
103 presence of two distinct clades, one with haplotypes from England/Wales and
104 the second with haplotypes from Scotland. A geographic and genetic division of
105 this nature suggests that two colonisation events occurred in Britain. Inference
106 from the within-clade association of five representative European samples
107 highlighted that the Scottish population was derived from an Iberian population,
108 and the English/Welsh population from Eastern Europe. However, due to the
109 limitations of an exclusively modern DNA-based dataset, it was impossible to
110 discern whether the two colonisation events were separated geographically but
111 occurred at the same time, or, whether events were temporally distinct, the
112 second colonisers replacing the first, in one or other of the geographical regions.
113 The application of an ancient DNA approach therefore provides an ideal
114 mechanism by which to explore water vole colonisation of Britain. Through the
115 analysis of Pleistocene, early Holocene and additional modern water vole
116 samples, we have tested some of the proposals arising from the Celtic fringe
117 hypothesis of postglacial colonisation of Britain, namely that:

118 (1) There was an initial re-occupation of the mammal fauna after the LGM, in a
119 temperate-climate period dating sometime within the interval 19000 – 12900.
120 Within Britain, this pre-YD population is inferred to have been small and
121 dispersed.

122 (2) The climatic deterioration of the Younger Dryas would have played an
123 important role in the subsequent replacement of these lineages a process that
124 would have taken place prior to the formation of the English Channel (and
125 severance from continental Europe) at 8200-8000 BP (Before Present AD 1950;
126 (20)).

127 (3) The replacement populations came westwards, via Doggerland, presumably
128 from a source population located in either the Balkans or European Russia.

129 (4) The post-YD population would have been prone to replacement by incoming
130 populations from Continental Europe during the Holocene, which would have
131 expanded quickly in size due to more favourable climatic conditions. Thus the
132 displacement of mitochondrial clades is due to drift, rather than any ecotypic
133 advantage for life in lowland environments.

134 Furthermore, and although not the focus of this study, the use of cross-species
135 samples from a range of locations across Europe provides an opportunity to
136 include a molecular perspective on water vole taxonomy and systematics and in
137 particular, to examine the extent to which mitochondrial DNA data are congruent
138 with the currently proposed three species taxonomy.

139

140 **Materials and methods**

141 *Sample Collection*

142 A total of 82 water vole samples were collected from across Europe (*Figure 1 &*
143 *Table S1*). Sample choice was restricted by availability of material for destructive
144 purposes but was designed to source material from the Late Pleistocene through
145 to the present day. Britain and surrounding areas were of highest priority, but
146 sampling, particularly for modern materials, extended throughout Europe to
147 allow a wider comparison with extant European haplotypes. Modern samples
148 were obtained from archived museum sources, collected within the last 100
149 years. Mandibles were used throughout, with species level identifications
150 conducted by the source museums (*Table S1*).

151

152 *DNA extraction and sequencing*

153 All DNA extractions were conducted in a dedicated ancient DNA laboratory,
154 physically separated from the post- PCR laboratory. Mandibles were ground into
155 a fine powder and DNA was extracted using silica spin columns based on Yang *et*
156 *al.* (21), with the inclusion of 1M urea in the extraction buffer. MtDNA was
157 amplified using overlapping fragments spanning 643 base pairs of the control
158 region, tRNA-Phe gene and 12S ribosomal RNA regions. Six primer pairs were
159 designed specifically for this study (*Table S2*), each pair amplifying short (150 –
160 200 base pair) overlapping fragments. PCR reactions, amplicon purification and
161 sequencing were performed as described in (2) with PCR primer specific
162 annealing temperatures ranging from 50°C to 52°C. Standard ancient DNA
163 protocols (22) were followed throughout these extraction procedures to prevent
164 contamination, with repeated PCR amplification and sequencing of fragments to
165 ensure DNA authenticity and the absence of miscoding lesions.

166

167 *Phylogenetic analyses*

168 DNA sequences obtained from this study were aligned with additional sequence
169 data, 27 unique modern haplotypes from the Piertney *et al.* (14) dataset.

170 Phylogenetic relationships were estimated using Bayesian analysis. The DNA
171 substitution model selected with ModelTest3.7 (23) under Akaike Information
172 Criterion (AIC) was General Time Reversible (GTR) with proportion of invariable
173 sites (I) set to 0.6802 and gamma distribution (G) shape parameter 0.8091.
174 Bayesian trees were constructed and approximate posterior probabilities
175 performed using MrBayes 3.1 (24) implementing nucleotide substitution model
176 GTR, four chains (three heated one cold) were run for one million generations.
177 Southern water vole (*A. sapidus*) (sample 208) from Portugal was employed as
178 the outgroup in analyses.

179 Sequence data were partitioned into haplogroups and Southern water vole
180 species to establish sequence divergence between haplogroups and the Southern
181 water vole. These were calculated in Arlequin Ver. 3.11 (25), using pairwise
182 estimates of corrected average population sequence divergence.

183

184 *Radiocarbon dating*

185 Where there was a sufficient mass of sample material, water vole mandibles
186 extracted in this study were also sent for accelerator mass spectrometry (AMS)
187 dating at the Oxford radiocarbon accelerator unit (16 samples). Dates were
188 received as uncalibrated radiocarbon years BP, calibrated calendar ages were
189 generated using Oxcal v4.1 (26) with IntCal09 calibration curve (27).

190

191 **Results**

192 *DNA recovery*

193 In total, 70 of 82 specimens (85%) successfully amplified water vole mtDNA.
194 From the modern samples, 36 amplified DNA (97%), the 14 samples dating to
195 the Holocene returned a 100% success rate and a total of 20 samples from the
196 Pleistocene (65%) also amplified water vole mtDNA. Three of these samples
197 generated insufficient coverage (< 200 base pairs) and were therefore excluded
198 from analyses. Of the remaining 67 samples, 62 amplified the entire 643 base
199 pair region of interest; a further five successfully amplified all but one PCR
200 fragment (*Table S1*). To test whether the un-amplified regions contained
201 informative data, phylogenies were generated using both the entire region of
202 interest and with the un-amplified regions omitted. Trees produced were
203 identical; the five partially amplified samples were therefore included in all
204 further analyses.

205

206 *Phylogenetic analyses*

207 The Bayesian analyses (*Figure 1*) supports three clades of water voles, exhibiting
208 division between samples from the Pleistocene and older Holocene samples.
209 Further to clade identification, sequence data were partitioned to assess the
210 percentage of sequence divergence between the haplogroups identified and the
211 sister species, the Southern water vole (*Table 1*).

212

213 **Discussion**

214 *Confirmation of a Celtic fringe distribution in Arvicola*

215 A primary aim of this study was to test predictions made about the so-called
216 “Celtic fringe” distribution of small mammal haplotypes in Britain, using ancient

217 DNA, with water vole as a model system. We also incorporated original, recent
218 samples from across Britain, to enable exploration of a major (molecular)
219 division between modern water vole in England and those in Scotland (14). The
220 resulting phylogeny (*Figure 2*) places all modern Scottish samples as part of a
221 single clade, identified here as clade 1, and nearly all recent English samples in
222 an additional clade, denoted clade 2. There are two exceptions to this pattern:
223 sample 195 (Read's Island) and 200 (Northumberland) are both from English
224 locations but phylogenetically are placed within clade 1. The Northumberland
225 sample is directly adjacent to the Scottish border and that close proximity likely
226 accounts for its association with the 'Scottish' clade. Read's Island, however, is
227 more of an anomaly. Today, an RSPB reserve situated on the Humber Estuary in
228 East Yorkshire, it is considerably (>200 km) south of the Scottish border, this
229 sample could therefore represent a recent translocation of individuals from
230 Scotland to the Humber. Despite this, the overall trend of a major population
231 division between water vole in the north and the south of Britain persists, with
232 strong support for the nodes that define lineage separation into haplogroup
233 clusters.

234

235 *Post-LGM recolonisation of Britain*

236 Confirmation of the genetic division observed by Piertney *et al.* (14) can be
237 achieved through modern sampling efforts, but to better establish the timing and
238 mode of colonisation, ancient Pleistocene and Holocene water vole samples were
239 included in the analysis. Our AMS dating of these samples shows water vole
240 presence in southern England immediately prior to the LGM (e.g. sample 157;
241 median calibrated date 27955 BP see *Table S1*). While it was not possible to

242 establish a haplotype for sample 157, ancient samples that were successfully
243 haplotyped are indicated in the phylogeny (*Figure 1*) by geographic location and
244 coloured blue (Pleistocene) and pink (Holocene). The phylogenetic placement of
245 these samples is highly informative; Pleistocene samples from England share the
246 same haplogroup as those currently restricted to Scotland, clade 1. In England,
247 only Holocene samples post-dating the Younger Dryas cluster within the modern
248 English water vole clade (clade 2). This supports the two-phase colonisation
249 proposal (10), with members of clade 1 as the pre-LGM colonisers of Britain,
250 subsequently distributed throughout England. Following the end of the
251 Pleistocene, this group was displaced by a second wave of colonisers that
252 remained in England throughout the Holocene to the current day.

253 The combination of radiocarbon dating and phylogenetic inference provides a
254 clear indication that water vole colonised Britain on (at least) two separate
255 occasions, with a resulting population structure that can be attributed to
256 temporally, rather than spatially, distinct colonisation events. This
257 reconstruction also suggests a potential explanation for the anomalous sample at
258 Read's Island, as it could represent a relict population of the initial colonisers, a
259 now isolated remnant of the original colonisers prior to their displacement to
260 Scotland.

261

262 *Timing of the post-YD colonisation*

263 The timing of the second colonisation event can be inferred from the direct
264 dating of samples used in this study (*Table S1* and *Figure 1*). Only three samples
265 from Britain could be directly dated; two of these (187 and 158) were clade 1
266 individuals, with dates prior to the end of the Younger Dryas (median calibrated

267 dates); 14621 and 12081 BP respectively. A third sample, 159, dates to 2900 BP
268 and is associated with the second wave of colonisers, clade 2. Three further clade
269 2 samples (104, 106, 107) are undated, but were excavated from a Bronze Age
270 barrow, which would provide a maximum date boundary at 4500 BP. Thus the
271 second colonisation event occurred after 12081 BP, possibly before 4500 BP, and
272 definitely before 2900 years BP. These dates are thus compatible with natural
273 colonisation (rather than human translocation) via the landbridge between
274 England and continental Europe that is estimated to have been inundated c.8000
275 BP (28).

276

277 *The process of replacement*

278 The most plausible timing for the second colonisation would be before the loss of
279 the landbridge with continental Europe, between 12 and 8 Kyr BP. This was a
280 period of oscillating climate, spanning the end of the last glaciation, the
281 intermediate climatic transitions of the Lateglacial and finally, the start of the
282 Holocene interglacial. Population displacements are most commonly associated
283 with one population outcompeting another, through some ecotypic advantage.
284 Water voles from Scotland are generally considered smaller and typically darker
285 in colour than those from England (29), but morphological studies have shown
286 there to be a continuum across Britain and the variability insufficient to support
287 species or subspecies level differences (16, 30). However, in the early 1900s,
288 water vole in Scotland were considered a subspecies, *A. terrestris reta* (31) based
289 on their darker melanic pelage. Additionally, a study by Turk (32) found Bronze
290 Age water vole skulls from Derbyshire, England to be more akin to the Scottish
291 *reta* subspecies than to the English subspecies, *A. t amphibius*. Turk postulated

292 that either the early Bronze Age population differentiated into two subspecies or
293 that the *reta* population was once common in England, but was replaced by a
294 second population (*amphibius*) after the Bronze Age. This led Van den Brink (33)
295 to allocate two species of British water vole, suggesting that the northerly
296 species had been driven back to the Scottish Highlands by the species from the
297 south. Both authors were derided for their claims; Montgomery (30) asserted
298 that the insufficient sample size of Turk (32) had led to the erroneous
299 identification of two species, and that only one species of water vole had been
300 present in central England during the past 12,000 years. However, in light of the
301 findings from this study, Turk and Van den Brink appear to have been correct
302 with regards to a displacement event due to a second colonisation, even if the
303 proposed timings were inaccurate and the two populations are not sufficiently
304 genetically diversified (Table 1) as to warrant recognition as separate species.

305

306 The evidence from this study indicates that a second wave of colonisers did
307 indeed displace the first populations of water vole in England. As there are no
308 reports of discernable ecological or physiological differences between the two,
309 an alternative supposition, also suggested for other 'Celtic fringe' species (10), is
310 that the colder climate of the Younger Dryas heralded a severe reduction in
311 water vole numbers. As climate warmed, the second colonisers arrived to a
312 region virtually devoid of water voles, resulting in complete genetic replacement
313 in the south. However, as climate warmed, remnant populations could recover
314 and repopulate, meaning that sufficient numbers were in place to avoid genetic
315 replacement.

316

317 *Sources of the two British populations and the possible existence of Cryptic*
318 *Northern Refugia for water voles*

319 Taking a broader geographical outlook, the Pleistocene/Holocene division can be
320 observed across continental Europe (*Figure 1*). Pleistocene samples from
321 Slovakia, Belgium and Germany are found in haplogroup 1, whereas Holocene
322 samples from Belgium and Germany fall within haplogroup 2. This suggests that
323 population replacement in these regions occurred after Younger Dryas. Belgium
324 is an interesting case, as the modern sample reverts to a clade one haplotype.
325 This could indicate that both haplotypes remain in the region, highlighting the
326 possibility of a Belgian suture zone. As only one modern Belgian sample
327 successfully yielded DNA, there is insufficient support to test this line of
328 investigation, but with greater sampling effort in the region, this question might
329 be resolved. Germany also warrants additional discussion, as dated Pleistocene
330 samples from the same site (Fuchsloch im Krockstein) can be found in both clade
331 1 and clade 2. Sample 99, a clade 1 haplotype, dates to immediately prior to the
332 Younger Dryas, while sample 111, a clade 2 haplotype, has a median calibrated
333 date of 11,538 BP; the very cusp of the Younger Dryas/Holocene boundary. The
334 temporal interval between these two samples could therefore represent a more
335 accurate estimate of the timing of the second colonisation event. An alternative
336 interpretation is that Germany also forms part of a suture zone with both
337 haplotypes present, a question as with Belgium that could be resolved with
338 further sampling efforts.

339

340 Although not exhaustive, the inclusion of additional modern samples illustrates a
341 clear lineage division between eastern and western Europe. This is in agreement

342 with the previous proposals (Piertney et al 2005) that major divergent lineages
343 in Europe are derived from Iberian, and eastern refugia.

344 Expansion from an eastern, rather than peninsular, refugia is in contrast to the
345 standard “Hewitt” model of post-glacial recolonisation, and provides further
346 support for the presence of refugia north of the European continental divide (34-
347 35). It also underlines the importance of examining a wide spectrum of species,
348 including small mammals, as water vole appear to exhibit an unusual pattern of
349 genetic diversity. In contrast to almost all other species so far studied (36), the
350 Pleistocene-Holocene transition did not result in a major decline in genetic
351 diversity in this species.

352

353 *Taxonomy and conservation status of European Arvicola*

354 Our results indicate clear confirmation of the species level status of the southern
355 water vole (*A. sapidus*), through the monophyly of samples and the high
356 percentage of sequence divergence between southern and northern water vole,
357 (3.66%; *Table 1*). However, on the basis of mtDNA, we find no basis for elevating
358 the montane water vole (*A. scherman*) to species level. Three modern samples
359 (213, 221, 222) identified as montane water vole failed to form a cohesive
360 monophyletic association - in fact, they were designated to different haplogroups
361 (213 and 221 (France) were clade 1, while 222 (Slovenia) was clade 2. A far
362 clearer lineage separation was apparent in samples from Italy (203 and 215) and
363 southern Switzerland (219). Together, these recent samples form a
364 monophyletic clade, clade 3, with relatively high sequence divergence with
365 respect to either clade 1 (2.42%) or clade 2 (2.49%), and with a divergence not
366 much lower than the species level difference between Northern and Southern

367 water vole (3.66%; *Table 1*). From a mitochondrial DNA perspective, the
368 geographical distribution of this haplogroup, coupled with sequence divergence
369 from other groups, provides a more convincing argument that it should be
370 considered as a separate taxonomic unit distinct from the montane water vole.
371 However, an analysis of the nuclear genome will be required to fully understand the
372 history and taxonomic status of these lineages, as well as the relationship between the
373 lineages currently found in Britain (see e.g 37).

374

375 In summary, this study highlights the benefits and increased depth of knowledge
376 that can be obtained by incorporating ancient DNA approach into studies of
377 population history. While modern data was sufficient to identify molecular
378 distinction between English and Scottish water voles, this study was able to
379 reveal a more comprehensive explanation of lineage separation observed in
380 British water vole today, through phylogenetic analysis of ancient and modern
381 DNA. We also suggest an order and timing of the colonisation of Britain, defining
382 two temporally distinct events, with a second lineage of water vole replacing the
383 first in England ca. 12 – 8 Kyr BP, leaving the initial British colonisers restricted
384 to Scotland.

385

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504 Data Accessibility

505 DNA sequences have been deposited in GenBank
 506 (<http://www.ncbi.nlm.nih.gov/>), with accession numbers XXX. Additional
 507 supporting information may be found in the online version of this article.

509 Author Contributions

510 IB, SB and JS designed the project. DS, JS, RM and MR provided samples and
 511 contextual information. SB performed the research. SB and IB analysed the data
 512 and wrote the manuscript, with contributions from the other authors.

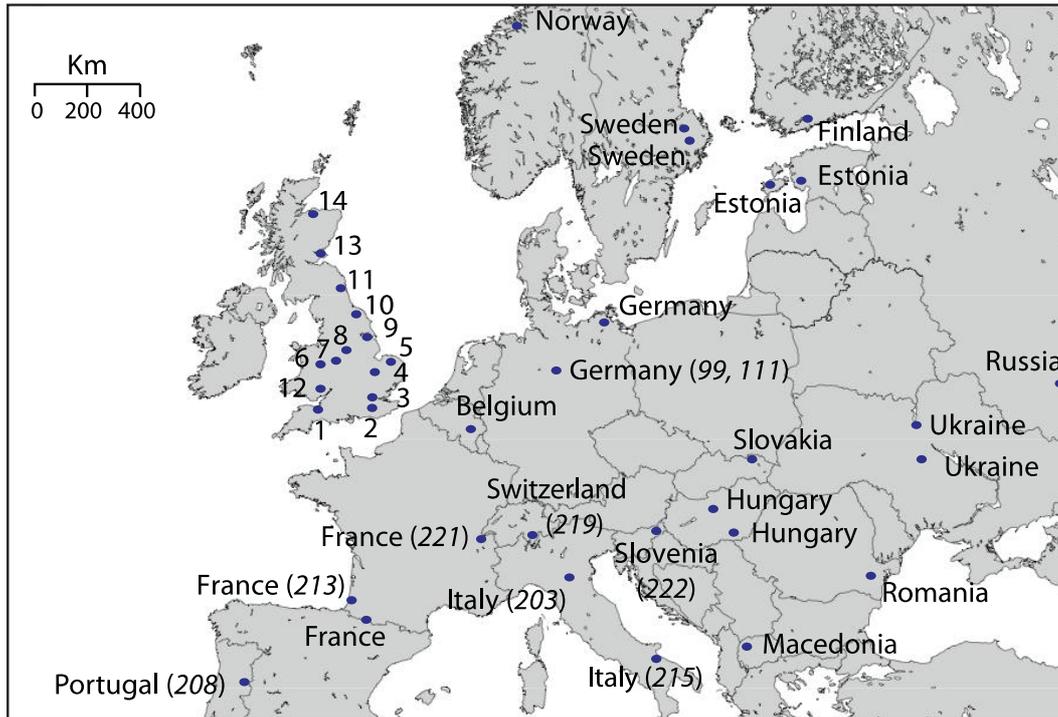
514 Tables and Figures

515 **Table 1** Population average pairwise estimates of sequence divergence with
 516 Kimura-2 Parameter between clades 1-3 as defined by the phylogeny (Figure 2)
 517 and the Southern water vole (*A. sapidus*).

		Clade			All
		1	2	3	<i>A. amphibius</i>
Clade	1				
	2	2.12			
	3	2.42	2.49		
All	<i>A. sapidus</i>	3.9	4.56	3.3	3.66

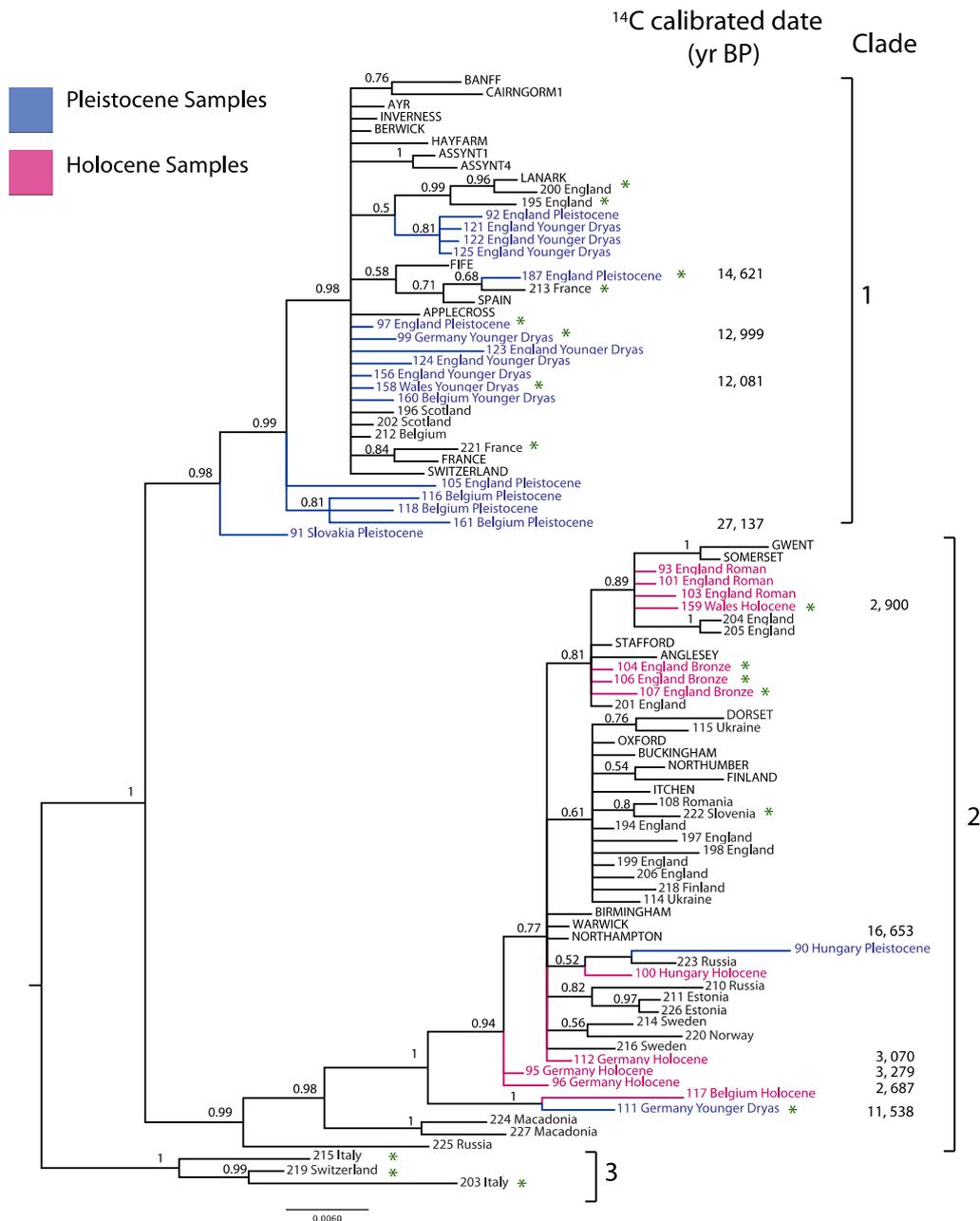
520

521 **Figure 1** Sampling locations for water vole used in this study. British numbered
 522 locations key: England: 1=Somerset (187); 2=Surrey; 3=Hertfordshire;
 523 4=Cambridgeshire; 5=Norfolk; 6=Shropshire; 7=Staffordshire; 8=Derbyshire
 524 (104,106,107); 9=Lincolnshire Read's Island (195); 10=Yorkshire;
 525 11=Northumberland (200). Wales: 12=Gwent (158,159). Scotland: 13=Fife;
 526 14=Morayshire. European sampling locations are denoted by country name.
 527 Bracketed numbers refer to the individual sampling numbers of water vole that
 528 are directly referred to in the text.
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533 **Figure 2** Phylogenetic relationships of water vole samples: Bayesian tree
 534 constructed in MrBayes. Nodal support is shown through approximate Bayesian
 535 probabilities (only values above 50% shown). AMS dates are given as the median
 536 calibrated year BP. Southern water vole used as the outgroup (not shown).
 537 Nomenclature: Sample location written entirely in uppercase = data taken from
 538 Piertney and colleagues (14); all other data are from this study and 1st number =
 539 unique sample identifier, followed by sample location and time period, where
 540 blue = Pleistocene (> 10 Kyr BP, oldest dated sample 27 Kyr BP), pink =
 541 archaeological Holocene (10 Kyr BP to 250 yr BP), black = recent/museum
 542 samples (250 yr to present). Green star denotes that the sample is directly
 543 referred to in the text.
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