

1 Title: Thermal refugia and the survival of species in changing environments: new evidence
2 from a nationally extinct freshwater fish.

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16 **RUNNING HEAD:** Burbot colonisation of English rivers

17 **KEYWORDS:** burbot, colonisation, freshwater fishes, glacial maximum, phylogeography.

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20 *"This is the peer reviewed version of the following article: Worthington, T. A., Van Houdt,*

21 *J. K. J., Hull, J. M. and Kemp, P. S. (2016), Thermal refugia and the survival of species in*

22 *changing environments: new evidence from a nationally extinct freshwater fish. Ecology*

23 *of Freshwater Fish, which has been published in final form at*

24 <https://dx.doi.org/10.1111/eff.12285>. *This article may be used for non-commercial*

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26 **ABSTRACT**

27 Variation in global climate during the Quaternary has helped shape current species
28 distributions. The stenohaline fish fauna of the British Isles is generally thought to have
29 colonised eastern England via a landbridge following the last glacial maximum. This theory
30 is investigated using the nationally extinct burbot, *Lota lota*, as a model species. Samples
31 were collected from 15 museum specimens of known English provenance and analysed for
32 differences in the mitochondrial DNA control region. The DNA analysis produced eight
33 sequences of 270 basepairs, with one sample reaching 420 basepairs in length. Genetic
34 analysis suggests the extinct English population of the burbot was a distinct lineage, differing
35 from those previously described from across the species' global distribution. Despite this,
36 network analysis suggests that the English lineage is closely related to populations in western
37 Europe, supporting colonisation via a post-glacial landbridge. The rate of genetic divergence
38 suggests that the timing of *L. lota*'s colonisation of English rivers was prior to the last glacial
39 maximum. *Lota lota* appears to have survived the last glacial maximum in refugia within the
40 British Isles. This study adds to the evidence for a British freshwater refugia and furthers our
41 understanding of the colonisation history of British freshwater fishes. These results also
42 provide valuable information for conservation strategies for *L. lota* indicating the western
43 European clade as most genetically appropriate for potential future reintroductions to English
44 rivers.

45 INTRODUCTION

46 From the beginning of the Quaternary (2.6 million years before present, ybp) and the
47 formation of the Arctic ice cap to the present day, the Northern Hemisphere has been
48 subjected to cyclical patterns of ice sheet expansion and contraction (Hewitt 2000) which has
49 dramatically influenced the distribution and genetic structure of the current biota (Hewitt
50 1996; Dynesius and Jansson 2000). During the Quaternary glacial periods, temperate regions
51 of the Northern Hemisphere saw extinctions and southward shifts in species ranges, with a
52 corresponding recolonisation into northern areas during the warmer interglacials (Culling et
53 al. 2006). Phylogeographical analysis has suggested many western European species
54 persisted during these glacial periods in refugia located in Iberia, Italy, the Balkans-Greece
55 and the Caspian/Caucasus regions (Hewitt 2004). Terrestrial post-glacial colonisation
56 patterns have been described based on several model species (Hewitt 1999), with
57 geographical features such as mountain ranges and water bodies impacting species' capacity
58 to disperse (Taberlet et al. 1998). Freshwater fishes, however, are restricted in their dispersal
59 ability by the interconnectedness of river catchments (Culling et al. 2006; Reyjol et al. 2007)
60 leading Hewitt (2004) to suggest a 'new paradigm' was required. Post-glacial colonisation of
61 European rivers appears to have been generally from the Black Sea via rivers such as the
62 Danube or Dnieper, although routes and glacial refugia are often species specific (Hewitt
63 2004; Makhrov and Bolotov 2006). More recent studies (e.g., Hänfling et al. 2002; Finnegan
64 et al. 2013) have suggested some species may have persisted in 'cryptic northern refugia'
65 (Stewart and Lister 2001).

66

67 In his paper, 'The origin and distribution of the freshwater fishes of the British Isles',
68 Wheeler (1977) discussed two potential mechanisms for the colonisation of British rivers by
69 stenohaline fishes. The first, proposed by (Scharff 1899) was that the freshwater fish species

70 of southern and eastern England colonised following the last glacial maximum during the
71 existence of a land bridge between Great Britain and Europe. The land bridge is thought to
72 have persisted until 7500 ybp, enabling the connection of the eastern English rivers from the
73 Humber through East Anglia, with the Rhine system as tributaries or through the formation of
74 a shared seasonal floodplain (Wheeler 1977). The second, proposed by Orkin (in Schindler
75 1957), was that certain species persisted in refugia during the last glacial maximum, having
76 established in British rivers during an earlier interglacial. Wheeler (1977) suggests that this
77 glacial refugia theory is unlikely, due to the unsuitable environmental conditions for
78 spawning, although he did advocate the possibility that both mechanisms may have been
79 possible. The landbridge theory would seem to be supported by the present day distribution
80 of freshwater fishes as species richness is reduced to the north and west of the British Isles
81 (Maitland and Lyle 1996), although this pattern is confused by human induced movements of
82 fishes or dispersal through canal systems (Wheeler and Easton 1978).

83

84 The distribution of the freshwater gadoid, the burbot *Lota lota*, stretches across North
85 America and Eurasia (Scott and Crossman 1973). Despite this extensive range many
86 populations, particularly in western Europe, are threatened with extirpation (Stapanian et al.
87 2010), while *L. lota* is thought to have become extinct in English rivers in the early 1970s
88 (Worthington et al. 2010). Recent analysis of mitochondrial DNA sequence variation in *L.*
89 *lota* from across its global distribution suggested division into two sub-species; *L. l. lota*
90 found in Eurasia and Alaska and *L. l. maculosa* from North America, south of the Great Slave
91 Lake (Van Houdt et al. 2005). After separation, the *L. l. lota* population was limited to a
92 glacial refuge from where it colonised Eurasia (Van Houdt et al. 2003). European *L. lota* have
93 been subjected to three or four subsequent glacial cycles, splitting the species into several
94 genetic clades (see Fig. 1 in Van Houdt et al. 2005). It is hypothesized that the English

95 population belonged to the western European clade owing to the likely colonisation of
96 English rivers via the landbridge that existed following the last glacial maximum. *Lota lota*'s
97 former English distribution corresponds well with this theory, as the species was restricted to
98 forty-two rivers in eastern England prior to extinction around the beginning of the 1970s
99 (Worthington et al. 2010).

100

101 The aim of this study was to examine the origin and timing of the colonisation of the British
102 stenohaline fish fauna using *L. lota*. Due to its recent extirpation from English rivers, *L. lota*
103 is subject to an investigation as to the feasibility of reintroduction to the rivers of its former
104 English range (Worthington et al. 2009). As such, understanding the genetic relationship
105 between the former English *L. lota* population and potential source populations is an
106 important consideration in reintroduction planning (Leonard 2008).

107

108 **MATERIAL AND METHODS**

109 **SAMPLES**

110 Tissue samples were collected from *L. lota* specimens known to have been captured in
111 England. Museums and universities with natural history collections were contacted to
112 determine whether they held preserved English *L. lota*, and samples were taken from those
113 with suitable specimens (Table 1). A sample of accessible material, either fin clips or muscle
114 tissue, was collected either by the institution's staff or a trained taxidermist. The samples
115 were either from dried taxidermy specimens (n = 8) or fixed using formaldehyde and stored
116 using Industrial Methylated Spirit (IMS, n = 7). The English samples were then compared to
117 sequences from burbot collected from across the species' global distribution (GenBank
118 accession numbers AY656840–AY656915; Van Houdt et al. 2005).

119

120 DNA EXTRACTION AND SEQUENCE ANALYSES

121 DNA analyses were carried out in a laboratory dedicated to the analyses of archival material.
122 Burbot material had never previously been sequenced within the building that housed the
123 laboratory, removing the possibility of contamination from samples outside the study. To
124 control for cross sample contamination, analyses were duplicated with a maximum of three
125 samples analysed concurrently. All sequencing was carried out in a laminar flow cabinet.
126 DNA was extracted using the NucleoSpin Extraction kit (Machery-Nagel GmbH). The
127 digestion, undertaken over a period of 3-5 hours, was enhanced by grinding the samples with
128 a pestle during the incubation phase, for larger tissue samples a double digestion volume was
129 used when required. The DNA from a single spin column was eluted twice with 50 µl of
130 heated elution buffer and stored separately. DNA quality was assessed by means of agarose
131 gel electrophoresis.

132

133 Glacial lineages of *L. lota* have been described using Domain I, 450 basepairs (bp), of the
134 mitochondrial control region (CR) which has been identified as containing 90% of the
135 variation found within the entire *L. lota* control region (Van Houdt et al. 2005). Domain I of
136 the CR was targeted using the primers L19ProGm (5'-CCACTAGCTCCCAAAGCTAGA-
137 3') and HDL400L1 (5'-GATTTAGGATTTATGTACTCC-3') resulting in an amplicon of
138 approximately 420 bp (Van Houdt et al. 2005). The L19ProGm primer was also combined
139 with the newly developed HDL230L1 primer (5'-CGCTAGATGATCTCTTACTAC-3')
140 specifically to amplify the first 270 bp of the CR. A minimum of two independent PCR
141 amplifications were carried out per sample per marker. The PCR was carried out in 30 µl
142 containing 1x PCR buffer (Invitrogen), 1-20 ng template DNA, 2.0 mM MgCl₂, 0.2 mM
143 dNTPs, 0.6 units of Taq DNA polymerase (Platinum *Taq*, Invitrogen), and 0.4 µM of forward
144 and reverse primer. The PCR profiling commenced with an initial denaturation of 3 minutes

145 at 94 °C, followed by 40 cycles of 45 seconds at 94 °C, 50 °C and 72 °C, finishing with a
146 final 7 minutes at 72 °C.

147

148 PCR products were purified by means of the Nucleofast PCR cleanup (Machery Nagel,
149 GmbH) or with “GFX PCR DNA and Gel Band Purification kit” (GE Healthcare). Cleaned
150 PCR products were sequenced in both directions using the BigDye version 3.1 cycle
151 sequencing kit (Applied Biosystems) on an ABI 3100. Sequences were analyzed and
152 assembled with SeqScape version 2.5 (Applied Biosystems).

153

154 NETWORK ANALYSES

155 A statistical parsimony (SP) network (Templeton et al. 1992) using TCS version 1.3
156 (Clement et al. 2000) was built to map the genetic relationship of the former English
157 population to the global distribution developed by Van Houdt et al. (2005). Network version
158 4.0.0.1 (<http://www.fluxus-engineering.com>) was used to construct reduced median (RM) and
159 median joining (MJ) networks (Bandelt et al. 1999; Bandelt et al. 2000). To estimate the
160 divergence time between English and Continental haplotypes reduced median-joining
161 networks were used to calculate $q \pm r$ (Forster et al. 1996), where q is the average distance
162 from all descendant haplotypes to the ancestral node of the median-joining network and r is a
163 variance estimator. The q statistic was translated into years using a mutation rate for the *Lota*
164 *lota* control region of 2-6% per million years. This value was based on previous research
165 estimating lineage specific mutation rates (Van Houdt et al. 2005). The absolute time since
166 divergence estimations should be interpreted with caution; however, the rough scale of time
167 since divergence can provide valuable insights.

168

169 RESULTS

170 SAMPLES

171 A total of fifteen *L. lota* specimens were sampled (Table 1), three of which had duplicate
172 samples taken (BUR05, BUR10 and BUR15). The samples were almost exclusively from the
173 River Trent (6 samples) or Great Ouse (8) catchments with only a single sample from
174 Yorkshire in the northern extent of the species' former English distribution (Fig. 1).

175

176 DNA EXTRACTION AND SEQUENCE ANALYSES

177 Two DNA extracts were obtained for each of the 15 *L. lota* specimens (n = 36, extraction was
178 duplicated for specimens BUR05, BUR10 and BUR15). A 420 bp amplicon was achieved for
179 a single specimen (BUR05, both samples), however 270 bp amplicons were obtained for
180 eight out of the 15 specimens and no sequence was obtained for the remaining seven
181 specimens (Table 1; GenBank accession numbers KJ381202-KJ381212). Samples that had
182 been taken from the taxidermy specimens more frequently produced sequences (6 sequences
183 from 8 specimens) than those stored in IMS (2/7). For quality control, the obtained sequences
184 were compared to the mitochondrial genome sequence of *L. lota* (AP004412).

185

186 In comparison to the reference sample, three C->T mutations at positions 15718, 15719 and
187 15892 were observed in a single sample for the BUR10 specimen. These mutations, however,
188 were absent from the second BUR10 sample. A C->T variant at position 15785 was observed
189 in BUR07 that was not present in any other specimen and a replicate sample was not
190 available to confirm this mutation. Consequently, Both BUR10 and BUR07 were removed
191 from further analyses. All other variants were observed in at least two independent specimens
192 or the matching samples from the same specimen. Overall, one new English haplotype was
193 detected in the 270 bp amplicon: a haplotype shared by five specimens (BUR01, BUR02,
194 BUR05, BUR06, and BUR15) from the Rivers Trent, Wissey, and Tame. All specimens were

195 characterized by an A->G mutation except BUR11 which was similar to the reference
196 sequence at that position. In the specimen from which a 420 bp sequence was obtained
197 (BUR05), two additional T->C transitions at 16031 and 16081 were observed (positions 330
198 and 380 on the 420 bp amplicon). These variants were confirmed in the two independently
199 analyzed samples of this specimen.

200

201 NETWORK ANALYSES

202 Separate network analyses were constructed for both the 270 bp and 420 bp sequences.
203 Firstly, for the 420 bp sequence that combined the BUR05 haplotype with the relevant known
204 European haplotypes. All network construction methods produced identical results (Fig. 2a).
205 The BUR05 haplotype was clearly different from the previously reported *L. lota* haplotypes,
206 differing by two mutations at positions 144 and 381 from EB01, a variant observed in
207 western Europe. The same analysis was performed on the 270 bp sequence, including the
208 BUR11 haplotype. Due to the limited dataset, the BUR11 haplotype could not be
209 distinguished from the common Eurasian/West European haplotype (Fig. 2b). Nevertheless,
210 the shared BUR01, BUR02, BUR05, BUR06, BUR15 haplotype was still differentiated by a
211 single mutation from all other known variants. Using a divergence estimate of 2-6% per
212 million years to examine the observed divergence in the 420 bp and 270 bp data sets,
213 indicates that the English population diverged from the continental population between
214 80,000 and 240,000 years ago for the 420 bp data set and between 62,000 and 186,000 year
215 ago for the 270 bp data set.

216

217 DISCUSSION

218 Analysis of the 420 bp sequence from BUR05 suggests that the extinct English *L. lota*
219 population was distinct from the clades highlighted by Van Houdt et al. (2003; 2005) who

220 sampled *L. lota* from across its global distribution. In this data set we find the presence of a
221 haplotype in England that is not present in the western/northern Europe samples examined by
222 Van Houdt et al. (2005) which included 84 samples from across the species range in
223 western/northern Europe and yielded 13 distinct haplotypes. Given the sampling effort of
224 Van Houdt et al. (2005), the absence of the English haplotype from western/northern Europe
225 suggests that the English haplotype is indeed restricted to England and not the result of a
226 sampling artifact.

227

228 The six useable samples produced two new English haplotypes in the 270 bp amplicon data
229 set. The 270 bp network provides additional insight about geographic differentiation between
230 England and western/northern Europe. The relationships among these shorter haplotypes
231 illustrates a single shared haplotype with western/northern Europe while none of the other
232 English samples group within any of the other geographic areas; further suggesting a distinct
233 English lineage of *L. lota*. While the results suggest a slight divergence of English *L. lota*, the
234 analysis reveals the extirpated population was closely related to western and central European
235 clades and likely founded through colonisation from western Europe via a land bridge (see
236 Wheeler 1977). Historical literature published from the 12th century onwards suggests that *L.*
237 *lota* was confined to rivers of eastern England (Worthington et al. 2010), which would have
238 been linked to the Rhine system as tributaries or through the formation of a shared seasonal
239 floodplain (Wheeler 1977). The sequences from the English population share much of the
240 genetic code with the haplotypes of the central and western European clades including the
241 ancestral EB30 from central Europe. However, degradation of the samples, owing to age and
242 storage, meant that with one exception, too few base pairs were produced to fully disentangle
243 the relationship between former English population and these two ancestral clades. The sole
244 exception permitted a preliminary evaluation of the relationship, which suggests the English

245 population diverged from the western European clade. However, this result should be treated
246 with caution due to the lack of replication.

247

248 Despite supporting the mainland European origin of *L. lota* in the British Isles, the presence
249 of a distinct haplotype provides evidence that *L. lota* was present in England during the last
250 glacial maximum (~14,000 ybp). Our study gives further support to the hypothesis that
251 certain species survived recent glaciations in northern refuges (Stewart and Lister 2001). The
252 freshwater fish fauna of Great Britain is generally considered to be comparatively young in
253 geographical and evolutionary terms (Hughes et al. 2001), with the widely accepted theory
254 being that stenohaline fishes colonised from the Rhine basin following the last glacial
255 maximum (see Wheeler 1977). However, species with current northerly distributions (>
256 60°N) appear to have ecological and physiological traits that would have allowed them to
257 persist in northern glacial refuges (Bhagwat and Willis 2008). The physiological tolerance of
258 *L. lota* would help to explain the possibility the species survived in English rivers during the
259 Devensian (12,000 – 110, 000 ybp). The extent of the ice sheet during the last glacial
260 maximum indicates a significant proportion of the *L. lota*'s English distribution would have
261 been unavailable (Bowen et al. 2002; Fig. 1). However, *L. lota* are cold adapted (Hölker et al.
262 2004) and able to spawn in temperatures as low as 1°C (McPhail and Paragamian 2000 and
263 references therein). Evidence from the archaeological record also supports the presence of *L.*
264 *lota* prior to the last glacial maximum, with the species recorded from lower Palaeolithic
265 (300,000 – 2.5 million ypb) deposits at Barnham, Suffolk (Ashton et al. 1994). This study and
266 similar phylogenetic analyses for other cold-adapted taxa, bullhead *Cottus gobio* (Hänfling et
267 al. 2002) and brown trout *Salmo trutta* (García-Marín et al. 1999; McKeown et al. 2010),
268 suggest that freshwater fishes may have colonised the British Isles prior to and persisted
269 through the last glacial maximum.

270

271 The study was based on samples collected from fifteen specimens, of which eight provided
272 suitable material for sequencing. The samples analysed are thought to represent the majority
273 of available museum *L. lota* specimens of known English origin (pers. obs.). Analysis of
274 historical material stored in museum collections is one of the only ways to map genetic
275 relationships for extinct populations (e.g., Hammond et al. 2001; Gugolz et al. 2008). As
276 such, analyses consisting of limited samples still provide valuable insight for understanding
277 phylogenetic and conservation questions (e.g., reintroduction; Pages et al. 2009). Future
278 genetic analysis could include data from bone fragments, with those from outside the species'
279 known range (e.g., the River Thames; Astill and Lobb 1989; Hawkes and Fasham 1997)
280 potentially providing greater evidence for a glacial refugium. While this study is underpinned
281 by a modest sample size in the context of modern genetic investigations, the authors consider
282 that the results provide a preliminary picture of the relatedness of the former English stock to
283 the remainder of the global population. The validity of the results is underlined by replicated
284 sequences from two different samples from the same specimen and the close correspondence
285 between sequences from geographically separate river catchments.

286

287 This study suggests that the extirpated English *L. lota* was genetically different from the other
288 populations in both Eurasia and North America. Despite this difference, the English
289 population was closely related and probably diverged from those in central and western
290 Europe. This provides a framework for selecting suitable source populations, should
291 reintroduction of *L. lota* to English rivers be deemed feasible. There appears the possibility
292 that *Lota lota* colonised English rivers prior to the last glacial maximum surviving the ice age
293 in refugia. This information, together with studies of other freshwater fishes, illustrates that

294 the impact of global climatic cycles on species distributions are species specific and linked to
295 the organism's physiological, biological and ecological traits.

296

297 **ACKNOWLEDGEMENTS**

298 The research was funded by the Esmée Fairbairn Foundation (Grant Number: EN/06-0469)
299 and the Trent Rivers Trust. We thank the Wollaton Hall Natural History Museum; University
300 Museum of Zoology, Cambridge; Norwich Castle Museum & Art Gallery; Warrington
301 Museum & Art Gallery; Cawthorne Victoria Jubilee Museum; Calke Abbey; Wisbech and
302 Fenland Museum; the Natural History Museum, London and the Oxford University Museum
303 of Natural History for allowing sampling of their burbot specimens and T. Irwin for
304 collection of the genetic material. We thank P. Osborne for helpful comments on an earlier
305 draft of the manuscript and C. Orgill for his expertise in sampling taxidermied specimens.

306

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420 **TABLES**

421 Table 1: The capture date and location of the fifteen *Lota lota* specimens if known, the current location of the specimen and the success of the
 422 genetic analysis.

Ref	River	Location	Date	Institution	Preservation	Sequenced	GenBank accession numbers
BUR01	River Trent	nr Nottingham	3 Mar 1905	Wollaton Park Museum	Dried	Yes	KJ381202
BUR02	River Trent	-	-	Wollaton Park Museum	Dried	Yes	KJ381203
BUR03	Old West River	Nr. Aldreth Bridge	21/22 Sep 1969	Cambridge University Museum	IMS	No	
BUR04	Great Ouse	Welney	1929	Cambridge University Museum	IMS	No	
BUR05	River Wissey	-	1936	Norwich Museum	Dried	Yes	KJ381204-5
BUR06	River Tame	-	1886	Warrington Museum	Dried	Yes	KJ381206
BUR07	River Thet	-	-	Victoria Museum, Cawthorne	Dried	Yes	KJ381207
BUR08	River Trent	-	-	Calke Abbey	Dried	No	
BUR09	River Trent	-	-	Calke Abbey	Dried	No	

BUR10	Middle Level Drain	-	1884	Wisbech Museum	Dried	Yes	KJ381208-9
BUR11	River Thet	Thetford, Norfolk	-	Natural History Museum	IMS	Yes	KJ381210
BUR12	-	Yorkshire	-	Natural History Museum	IMS	No	
BUR13	River Thet	Thetford, Norfolk	-	Natural History Museum	IMS	No	
BUR14	-	Nr. Cambridge	-	Natural History Museum	IMS	No	
BUR15	River Trent	East Stockwith	3 Nov 1869	Oxford University Museum	IMS	Yes	KJ381211-12

424 **FIGURE CAPTIONS**

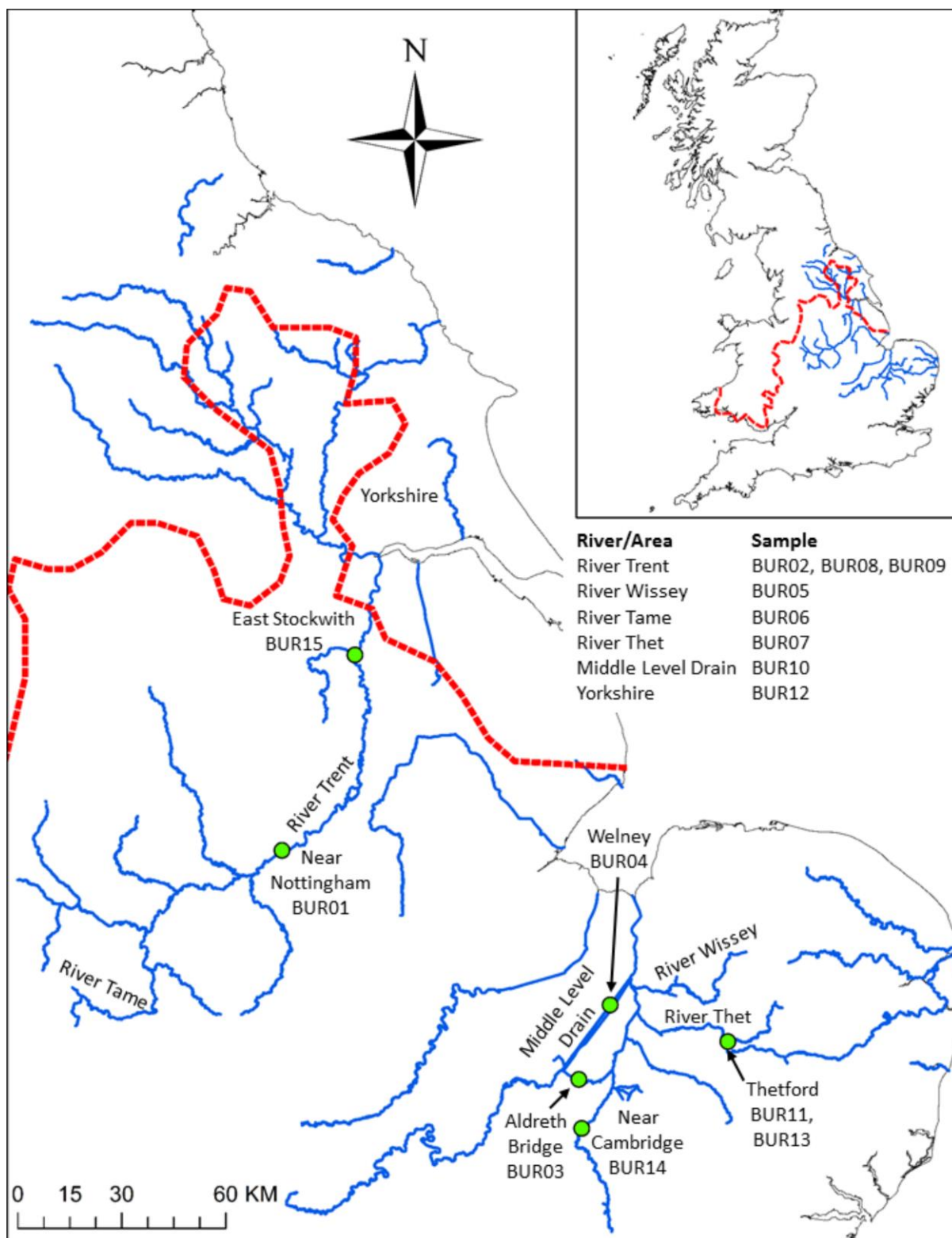
425 Fig. 1. The rivers of the former English *Lota lota* distribution in relation to the maximum ice
426 sheet extent during the last glacial maximum (red dashed line, adapted from Bowen *et al.*,
427 2002). Location of samples with specimen capture site and river information denoted by
428 green circles (numbers relate to sample number in Table I), rivers/areas without site
429 information marked.

430

431

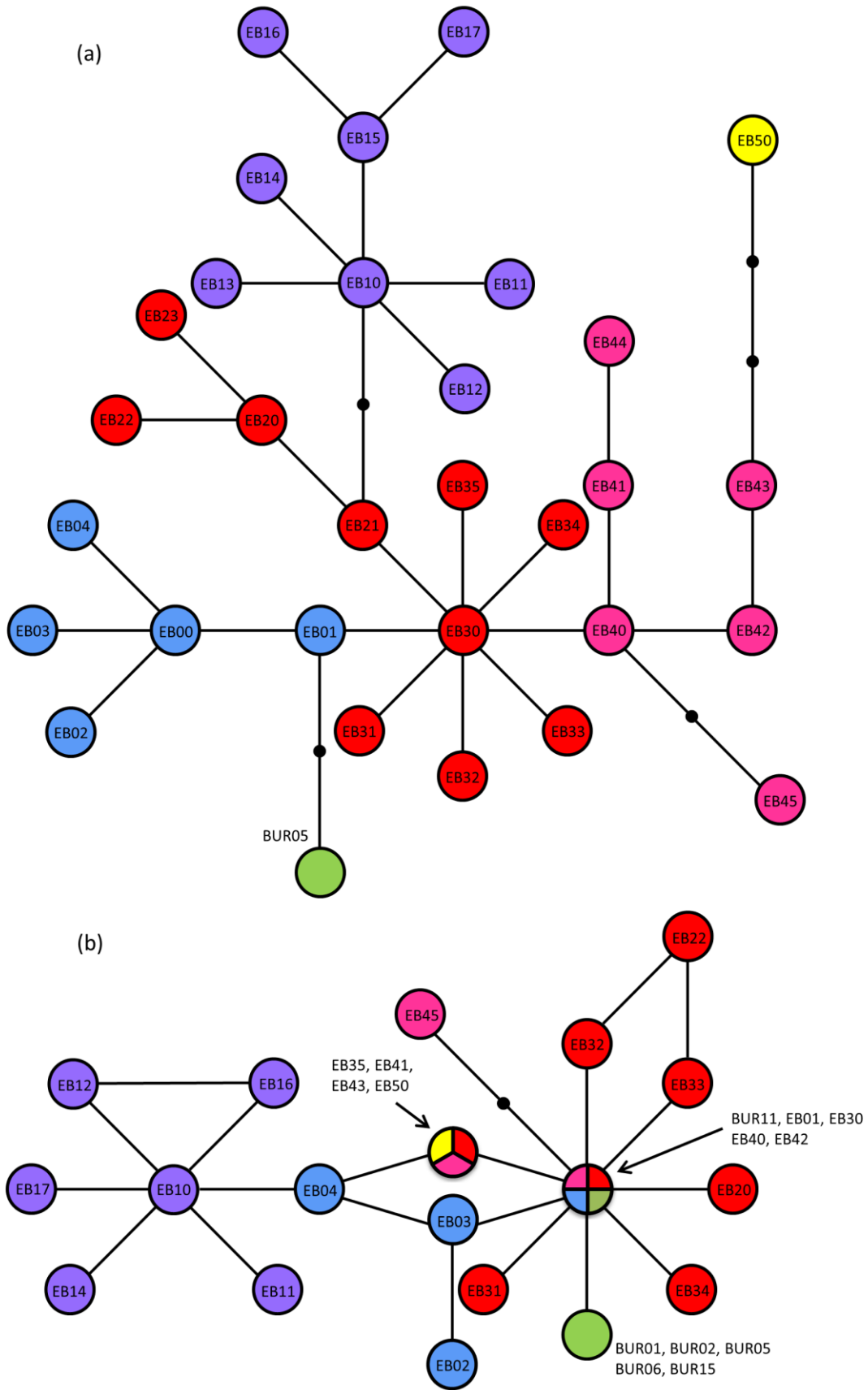
432 Fig. 2. Reduced median-joining network of *Lota lota* control region haplotypes for (a) the
433 420 bp data set and (b) the 270 bp data set showing the relationship between the English
434 haplotypes (green circles) and haplotypes from Alaska (yellow), Beringia (pink), Eurasia
435 (red), West Europe (blue), and North Europe (purple). Each branch represents a single
436 nucleotide change and black dots indicate unsampled haplotypes.

437



438

439 Fig. 1.



440

441 Fig. 2.