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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

MATERIAL AND METHODS

SAMPLES

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

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 HDL400Ll (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 HDl230Ll 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 $MgCl_2$, 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

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

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

NETWORK ANALYSES

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

RESULTS

SAMPLES

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

DNA EXTRACTION AND SEQUENCE ANALYSES

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

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

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

NETWORK ANALYSES

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

DISCUSSION

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

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

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

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

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

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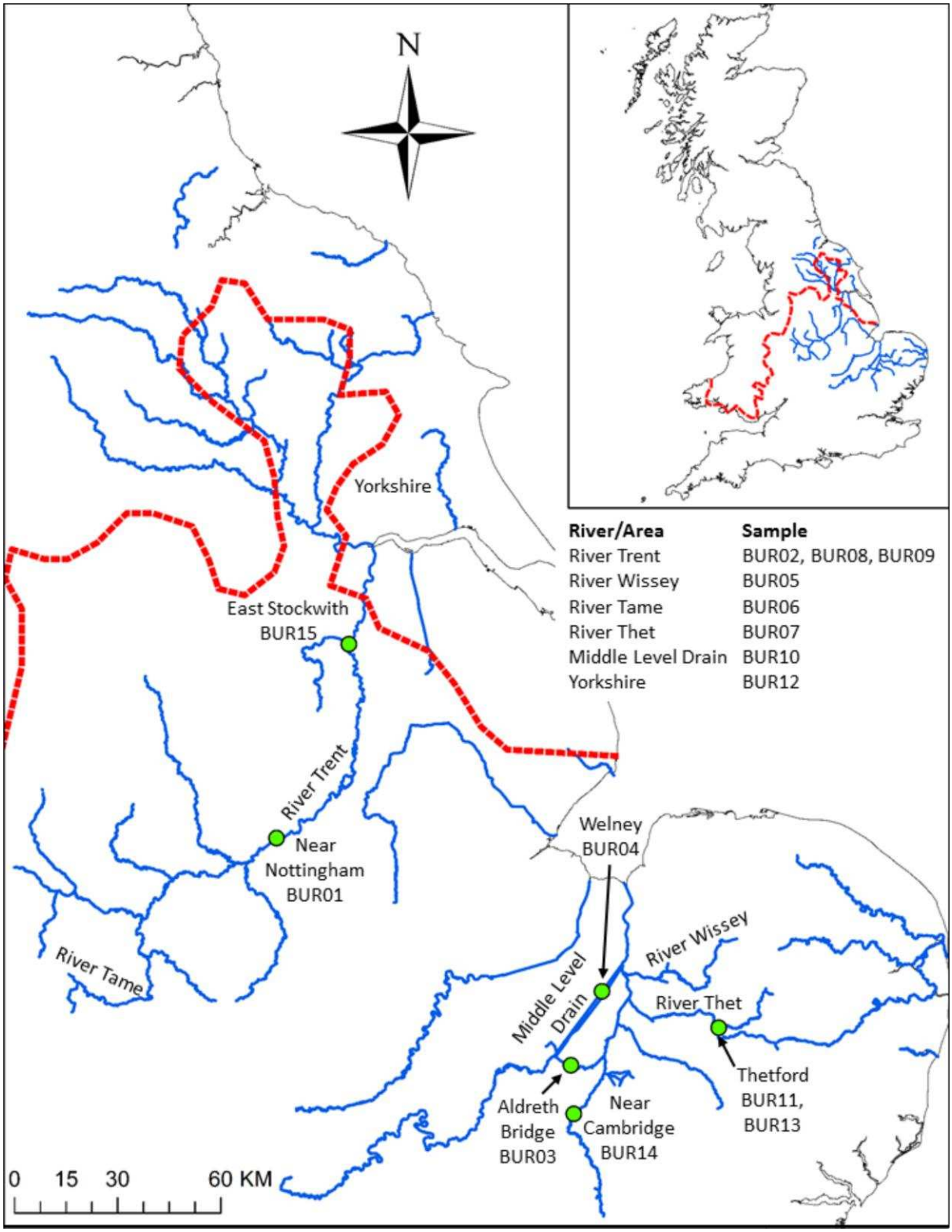
FIGURE CAPTIONS

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

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


N
Yorkshire
East Stockwith
BUR15
River Trent
Near
Nottingham
BUR01
River Tame
Welney
BUR04
River Wissey
Middle Level
Drain
River Thet
Thetford
BUR11,
BUR13
Aldreth
Bridge
BUR03
Near
Cambridge
BUR14
0 15 30 60 KM
River/Area
Sample
River Trent
BUR02, BUR08, BUR09
River Wissey
BUR05
River Tame
BUR06
River Thet
BUR07
Middle Level Drain
BUR10
Yorkshire
BUR12

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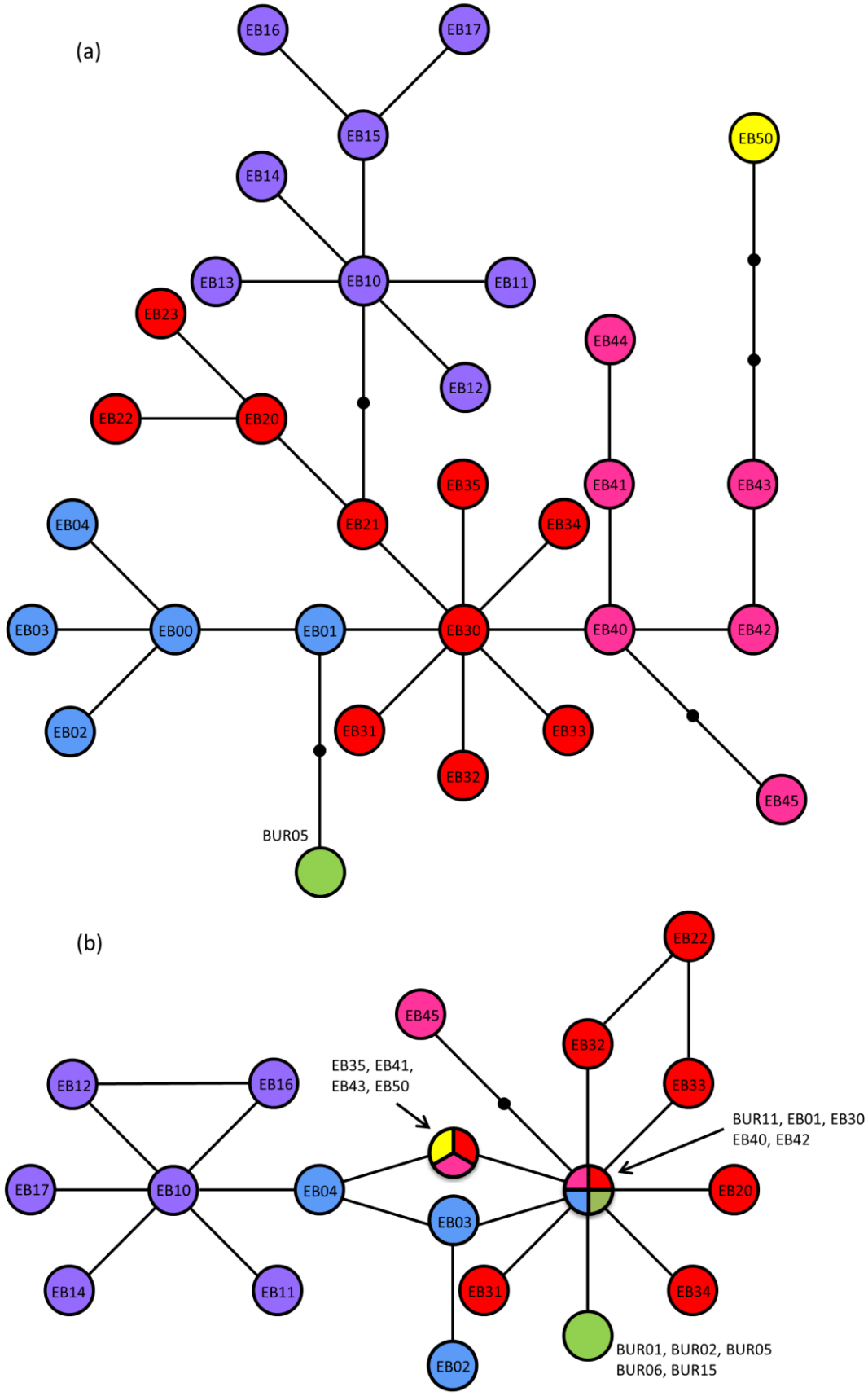
439 Fig. 1.


(a)
EB16
EB17
EB15
EB50
EB14
EB13
EB10
EB11
EB23
EB44
EB12
EB22
EB20
EB35
EB41
EB43
EB04
EB21
EB34
EB03
EB00
EB01
EB30
EB40
EB42
EB02
EB31
EB33
EB32
EB45
BUR05
(b)
EB22
EB45
EB32
EB12
EB16
EB35, EB41,
EB43, EB50
EB33
BUR11, EB01, EB30
EB40, EB42
EB17
EB10
EB04
EB20
EB03
EB14
EB11
EB31
EB34
EB02
BUR01, BUR02, BUR05
BUR06, BUR15

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441 Fig. 2.