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Patterns of genetic diversity in the red squirrel (*Sciurus vulgaris* L.): Footprints of biogeographic history and artificial introductions

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Abstract

British *S. vulgaris* are classified as a separate subspecies, *S. v. leucourus*, to mainland Europe. While *S. vulgaris* is not under threat across most of its Eurasian range, in Britain, Ireland and Italy populations are declining, mainly due to the introduction of the American grey squirrel (*S. carolinensis*). In this study, we conducted an extensive survey of mitochondrial DNA variation in British *S. vulgaris* populations and a preliminary survey of continental European populations. Our main aims were to determine the extent to which any populations of *S. vulgaris* in Britain are partially or wholly the product of artificial translocation of red squirrels from continental Europe, and whether continental population variation will provide information on post-glacial reafforestation patterns in Europe. We found that the majority of extant populations of British *S. vulgaris* are of continental ancestry, many with a very recent (last 40 years) Scandinavian ancestry. The Scandinavian haplotype has rapidly become the most dominant in northeastern Britain, despite not appearing in northern English populations until 1966. This suggests that these squirrels may have an adaptive advantage in the non-native spruce dominated conifer plantations of northern England. Our preliminary examination of continental populations demonstrated that they are sufficiently differentiated to allow a phylogeographic study of this species.

Introduction

The red squirrel (*Sciurus vulgaris*) is native to Britain, continental Europe and Asia (Corbet 1978). Across its geographical range, *S. vulgaris* has been divided into a number of subspecies primarily based on coat colour and morphological variation (Corbet 1978; Wiltafsky 1978), with British populations classified as a separate subspecies (*S. v. leucourus*). However, the status of many of these subspecies is unclear (e.g. see Sidorowicz 1971) and a comprehensive re-assessment of the complex using genetic and morphological techniques is required (Lurz et al. in press). While *S. vulgaris* is not under threat across most of its Eurasian range, in Britain, Ireland and Italy populations are declining, mainly due to the introduction of the American grey squirrel (*S. carolinensis*) (Gurnell and Pepper 1993; Wauters et al. 1997; O'Tangeana et al. 2000). Once ubiquitous throughout Britain, *S. vulgaris* is now restricted to the north of England and parts of Scotland, along with a few small islands, and is the focus of a national biodiversity action plan (http//www.jnc.uk/ukbg/bap/species).

The genetic structure of the British *S. vulgaris* populations is complicated by several introductions from continental Europe over the last 150 years. There is literature evidence for introductions of individuals (probably *S. v. fuscoater* and/or *S. v. varius*) from Europe to Scotland (Harvie-Brown 1880-81), as well as Lancashire (Segar 1968; Lowe and Gardiner 1983) and County Durham (J. Reynolds personal communication) in England. There is no evidence for introductions to the northwest of England (Cumbria), and some individuals from Cumbrian populations display coat colour characteristics similar to those described as common in the 18th century in English populations. Northeastern English populations display a mixture of these and colour patterns attributed to

continental populations (Wiltafsky 1977; Hale and Lurz 2003).

A previous study found no clear geographic pattern in a phylogenetic analysis of *S. vulgaris* mtDNA haplotypes within Britain (Barratt et al. 1999). The authors concluded that the lack of geographical structure was the result of demographic fluctuations and bottlenecks due to habitat fragmentation (Barratt et al. 1999). Given the literature evidence for recent introductions of *S. vulgaris* from continental Europe, we consider that another possible explanation for the lack of a geographic pattern in the mtDNA haplotypes is that there has been substantial artificial movement of individuals from Europe to Britain, and possibly within Britain. In this study, we examine mtDNA haplotype variation from *S. vulgaris* museum specimens collected since 1861 to look for direct evidence of artificial introductions through the appearance and expansion of particular haplotypes over time.

In a previous examination of microsatellite variation in *S. vulgaris* in the extant populations in the north of England, we found substantial population genetic differentiation between three regions: western (Cumbria), eastern (southern Northumberland and County Durham) and northern (northern Northumberland and Scottish Borders) (Hale et al. 2001). The microsatellite variation does not tell us whether this regional variation is the result of contemporary processes, or whether it represents deep historical divisions between populations. Microsatellites have a very fast mutation rate (Hancock 1999), so differentiation may arise after relatively short periods of isolation. Mitochondrial DNA, on the other hand, mutates more slowly and this, combined with a lack of recombination, means that mtDNA is more likely to preserve the genetic signature of historical population processes (Cruzan and Templeton 2000).

Red squirrels are habitat specialists, with their distribution closely linked to the distribution of woodland habitat. This makes them ideal 'markers' of past biogeographic changes in woodland cover. Changes in American flying squirrel (*Glaucomys*) distribution have been used successfully to assess Pleistocene vegetational shifts in North America (Arbogast 1999; Arbogast and Kenagy 2001). MtDNA analysis indicated that the boreal ecosystem in early-to-middle Pleistocene North America was divided into two distinct communities and patterns of mtDNA sequence variation reflect forest fragmentation linked to glacial cycles of the Pleistocene (Arbogast 1999, Arbogast and Kenagy 2001). While British *S. vulgaris* populations are unlikely to provide any useful phylogeographic information due to the many recorded artificial introductions, populations on the European continent may well provide information on glacial forest refugia and post-glacial reafforestation in Europe.

In this study we examined *S. vulgaris* mitochondrial DNA variation to assess whether the population structuring found in British *S. vulgaris* microsatellite variation is representative of underlying historical population divisions, whether continental populations are likely to provide information on post-glacial reafforestation patterns in Europe, and to determine the extent to which any populations of *S. vulgaris* in Britain are partially or wholly the product of translocations of red squirrels from continental Europe.

Methods

Sample collection

S. vulgaris samples were collected primarily from museum pelt specimens. The majority were from squirrels collected in Britain (n = 134). We also received samples from four continental countries; the Netherlands (n = 10), Italy (n = 6), Spain (n = 19) and Sweden (n = 13). All samples comprised approx. 2 mm^2 skin tissue, except for 13 British and six Italian samples which consisted of tail hair. The specimens were collected between 1861 and 2002, with the majority collected since 1960 (Appendix 1).

The British specimens were mainly from the north of England (Cumbria, Northumberland, County Durham and Lancashire), with a few individuals from the Scottish Borders. In addition, eight individuals were from an extinct British population (Dorset). The Dorset specimens were all collected between 1894 and 1895, and were sampled in an attempt to get mtDNA haplotypes of original *S. v. leucourus* for comparison with extant populations.

In a previous genetic study on *S. vulgaris* in Britain we partitioned samples into populations based on an analysis of genetic subdivision and heterozygote deficiency over a range of small-scale distances (1.5 km to 3.0 km), combined with information on woodland cover and landscape connectivity (explained in detail in Hale et al. 2001). This study demonstrated that the majority of population genetic differentiation occurred between three regions: western (Cumbria), eastern (southern Northumberland and County Durham) and northern (northern Northumberland and Scottish Borders) (Hale et al. 2001). We

therefore grouped the British specimens into these three regions except for the Dorset sample which was treated as a separate population (Figure 1). Hale et al. (2001) demonstrated that the genetic structure (based on microsatellite variation) of *S. vulgaris* in the western region changed during the 1980s, as a result of dispersal across the newly-planted Kielder Forest. We therefore split the western sample into two 'populations', western pre-1980 and western post-1980, to reflect this temporal genetic structuring.

DNA extraction, mtDNA amplification and sequencing

Total DNA was extracted from skin tissue samples using a BioRad Aqua Pure Genome Tissue kit. DNA was extracted from hair samples by overnight incubation (55 °C) of a single hair in 200 μl 5% Chelex® 100 (BioRad) with 5 μl 20 mg/ml proteinase K. Mitochondrial DNA was amplified in 25 μl reactions containing 1X *Taq* buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL, 0.01% Tween-20), 2.0 mM $MgCl_2$, 0.08 mM each dNTP, 0.2 μM H16359 (Barratt et al. 1999), 0.2 μM RScont6 (5′-CCTTCAACTCCCAAAGCTGA-3′), 1.0U *Taq* (Bioline), and 0.5 μl template DNA (skin samples) or 5 μl template DNA (hair samples) under the following conditions: 94 °C for 4 min, then 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1.5 min, with a final extention of 72 °C for 10 min. All amplifications were performed in a PTC-100® thermocycler (MJ Research). PCR products were purified using QIAquick® PCR purification columns (Qiagen) prior to sequencing. Purified PCR products were sequenced using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems) following manufacturer's recommended conditions, and sequences detected on an ABI 310 Prism® automated sequencer (Applied Biosystems). Sequences were aligned and edited manually using ProSequence (Filatov 2002).

Fragments were sequenced in both directions for 20 individuals, using each amplification primer in turn as sequencing primer. As there were no discrepancies between each strand, all other individuals were sequenced in one direction only, using the primer H16359. Sequences obtained from hair samples were repeated twice to ensure correct base calling. This was considered necessary because the much lower quantity of DNA obtained from hair resulted in a generally lower quality sequence. Despite this, there were no discrepancies between replicate sequences.

Data analysis

The sequence data were analysed using both population genetic and phylogenetic methods to gain a complete picture of overall genetic structure. For the population genetics, the data were analysed in two sets: British samples only and Britain plus Europe. For each dataset the degree of population subdivision, measured by F_{ST}, was calculated from mtDNA haplotype frequencies and sequence similarities using AMOVA in the program ARLEQUIN (Schneider et al. 2000). An exact test of population subdivision (Raymond and Rousset 1995) was also performed, with a Markov chain length of 10000 steps. The British 'populations' analysed were eastern, northern, western pre-1980 and western post-1980. Each European country was analysed as a separate 'population'. The Dorset sample was not included in the British analysis, as it does not represent an extant population. A neighbour-joining tree was calculated from the matrix of Slatkin's linearised F_{ST} values (Slatkin 1995) for the Britain plus Europe dataset using ARLEQUIN (Schneider et al. 2000) and PHYLIP 3.5c (Felsenstein 1993).

Bootstrapped, unrooted maximum parsimony trees of mtDNA haplotypes were calculated using PAUP* 4.0 (Swofford 2002) for the total data set (Britain plus Europe) and for Europe only.

Results

Sequence data

395 bases of aligned mitochondrial DNA sequence data were collected for each of the 182 individuals sampled. There were 39 polymorphic sites and a total of 28 haplotypes (Table 1). Aligned sequence data are available on GenBank, accession numbers AY178452–AY178479. Only seven of the 28 haplotypes were found in more than one region (Table 2). Haplotype divergence ranged from 0.25% to 3.29% with an average of 1.70%.

Variation in British populations, excluding Dorset

Seventeen of the 28 haplotypes were found in British populations. There were eight haplotypes in the eastern sample (n = 59), seven in the northern sample (n = 24), eight in the western post-1980 sample (n = 34), and only two haplotypes in the western pre-1980 sample (n = 9). There were no

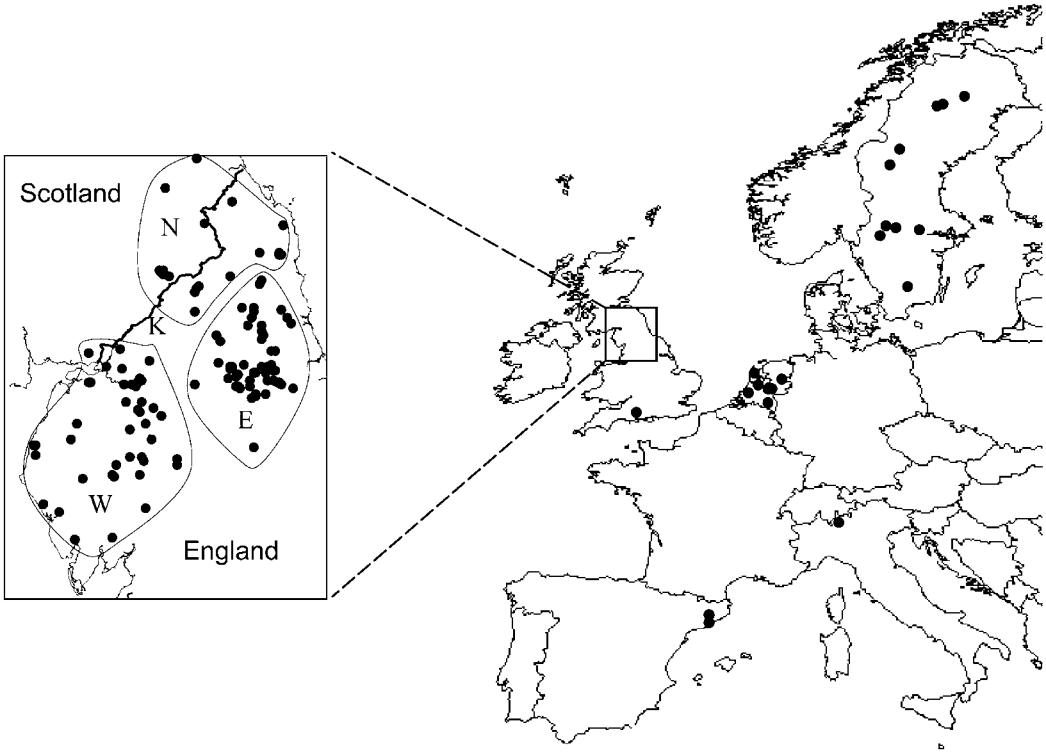

Figure 1. Map of study area. Black dots represent the collection site of at least one *S. vulgaris* specimen in Britain, the Netherlands, Italy, Spain and Sweden. Inset: the three British regions identified in a previous study (Hale et al. 2001) are outlined. W = UK western, N = UK northern and E = UK eastern region. K = position of Kielder Forest. Kielder Forest extends into both the northern and western regions.

shared haplotypes between western pre-1980 and either the northern or eastern regions. Substantial genetic subdivision existed among these four regions (F_{ST} = 0.376, $P < 0.001$), and between all possible pairs of British regions except for western pre-1980 and western post-1980 (Table 3). The majority of the variation was between the western region and the other two. There was no statistically significant genetic subdivision between western pre-1980 and western post-1980 as defined by pairwise F_{ST} (P = 0.092), but a chi-squared comparison of western pre-1980 and post-1980 samples showed a marginally significant difference in haplotype frequencies (χ^2 = 3.85, P = 0.049, df = 1). The lack of a significant difference between the two samples based on F_{ST} may be a result of the small sample pre-1980. The probability of randomly sampling eight H13 haplotypes, and one non-H13 haplotype in any order, from a population with the same haplotype frequencies as Western post-1980 is 0.025. This suggests that the pre-1980 haplotype frequencies were different to post-1980 in Cumbria, and that H13 was more common prior to 1980 than it is now.

From the lack of geographic structure in the maximum parsimony haplotype tree (Figure 2), it appears unlikely that the various British mtDNA haplotypes are the result of historical vicariance. It is apparent from the F_{ST} analysis that there is very little gene flow between the regions, and it seems likely that the variation is due to migration from outside Britain.

European variation

Twelve of the 28 haplotypes were found in at least one of the four continental European samples (Sweden, Italy, Spain and the Netherlands). None was found in more than one European country, and only two (H1 and H14) were found in both Britain and Europe. Population genetic subdivision between the four European countries was substantial (F_{ST} = 0.489, $P < 0.001$). The four countries differed significantly, based on pairwise F_{ST} analyses (Table 3), and also based on an exact test of sample differentiation calculated from haplotype frequencies ($P < 0.01$ in all cases). When the British regions were added to the European analysis, the degree of population genetic

Table 1. Base composition at the 39 polymorphic sites for each of the 28 mtDNA haplotypes. Full aligned sequences are available on GenBank, accession numbers AY178452–AY178479

H1	AAATAGCGACGTACGTGATAAGAGATACGAAGTTTAATG
H2	AAATAGCGACGTNCGTGATAAGAGATACGAAGTTTAATG
H3	AAATAGCGACGTACGTGATAAGAGATACAAAGTTTAATG
H4	AAACAGCAACGTACGTGGTAAGAGGTATGGAGTTTAAGG
H5	GGACAGTAATGTATGTGGTAAGAGGTATGGAGTTTAATG
H6	AAACAGTAATGTACGTAATGAGAGGCATGGAGTTTAATG
H7	AAACAGCAATGTACGTAATAAGAGGTATGGAGTTTAATG
H8	AAACAGCAGTGTACGTAATAAGAGGTATGGAGTTTAATG
H9	AAACAGCAGTGTACGTAATAAGAGGTGTGGAGTTTAATG
H10	AAATAGCAATATATGTAATAAGAGGTATGGAGTTTAATA
H11	AAGCAGCAATGTATGTAAAAAGAGATATGGAGTTCAATG
H12	AAACAGCAATGTATGTAATAAGAGGTATGGAGTTTAGTG
H13	AAATAACAATGTACGTAGTAAGAGGTATGGAGTTTAATG
H14	AAATAGCAATGTACGTAATAAGAGGTATGGAGTTTAATG
H15	AAGCGGCAATGTACGTAATAAGAGGTACGGAGTTTAATG
H16	AAACAGCAATGTACGTAGTAAGAGGTACGGAGCTTAATG
H17	AAATAGCAATGTACGTAGTAAGAGGTACGGAGCTTAATG
H18	AAGTAGCAATGTACATGGTAAGAGGTACGGAGTTTGATG
H19	AAATAGCAATGNACGAAGTGAAAGGTACGGAGTTTAATG
H20	AAATAGCAATGNACATGATGAGGGGTACGGAATTTAATG
H21	AAATAGCGATGTACATAGTAAGAGGTACGGAGTGTAATG
H22	AAATAGCGATGTACGTAGTAAGAGGTACGGGGTTTAATG
H23	AAATAGCAATGTACGTAGTAAGAGATACGGAGTTTAATG
H24	AAATAGCAATGTACGTAGTAGGAGATACGGAGTTTAATG
H25	AAATAGCGATGTACGTAGTAAGAGATACGGAGTTTAATG
H26	AAATAGCAATGTACGCAATAAGGGATACGGAGTTTAATG
H27	AAATAGCAGTGTACGCAATAAGGGATACGGAGTTTAATG
H28	AAATAGCAGTGTACGCAATAAGGTATACGGAGTTTAATG

subdivision decreased slightly (F_{ST} = 0.468, $P <$ 0.001). The relationship between the four European and four British regions can be visualised with an unrooted neighbour-joining tree of Slatkin's linearised F_{ST} genetic distances (Figure 3). The British regions do not group together. The northern and eastern regions of Britain are more similar to Sweden than to the western region of Britain. Four regional samples do not occupy terminal positions on the tree: northern, eastern and western post-1980, all in Britain, and the Netherlands. The internal position of these regions suggests that they may be 'mixed' populations – mixtures of haplotypes that evolved elsewhere.

Unlike the British haplotypes, the maximum parsimony phylogenetic analysis of European haplotypes reflects geographical distribution (Figure 4). Bootstrap support was low, as expected for intraspecific analyses which generally have lower haplotype divergence than interspecific analyses. Despite this, analysis of the data via maximum parsimony, maximum likelihood and distance estimation (Kimura's 2-parameter) all produced the same phylogenetic tree with haplotypes grouped into countries, except for H20 (Italy) which grouped with the two Swedish haplotypes.

Genetic introgression

The similarity between the eastern and northern regions of Britain and Sweden can be explained by the presence and spread of haplotype H1 in Britain (Figure 5). This haplotype was found in all Swedish specimens except one, despite the Swedish individuals being collected over a large area that encompassed most of that country. Haplotype H1 was found in 68% of eastern British individuals, but the frequency and geographic location of this haplotype has not been stable over time. The large sample of British museum specimens allowed us to examine haplotype frequencies across time as well as space. Partitioning these into four time periods (pre-1970, 1970s, 1980s, 1990–2002) allowed the frequency and geographic location of haplotype H1 versus the other haplotypes to be determined. Older specimens were grouped into the category 'pre-1970' because there were relatively few samples collected prior to 1960, so these earlier samples were added to the 1960s decade. Similarly, the 1990s decade was extended to include samples collected up to 2002, as only a small proportion of the 2000s decade was sampled.

Haplotype H1 did not appear in the northern British samples until 1966. The frequency of this haplotype was significantly lower in the pre-1970 sample for the combined eastern and northern regions compared with the 1970s (27% and 76% respectively, χ^2 = 6.60, P = 0.01, df = 1). The western region was excluded from the frequency analysis since haplotype H1 was not found in the western region prior to 2002. The frequency of haplotype H1 did not differ between the 1970s, 1980s and 1990s (1980s H1 = 48%, 1990s H1 = 64%, χ^2 = 4.20, P = 0.12, df = 2). Prior to 1970, it was found only in a small area around Corbridge in the county of Northumberland (Figure 5). During the 1970s, this haplotype was found throughout the southern half of Northumberland. In 1980 it appeared in the northern sample for the first time, and during the 1980s was found as far north as the Scottish Borders. In the 1990s it spread further west, reaching northern Cumbria in 2002.

The introgression of the Swedish haplotype H1 into the northern and eastern regions in Britain does not wholly account for the genetic differentiation of the western region and the northern and eastern regions of Britain. When all British individuals with

Table 2. Haplotype frequencies in each of the four British regions and four European countries. WestA = western pre-1980, WestB = western post-1980

Hap #	Eastern (n = 59)	Northern (n = 24)	WestA (n = 9)	WestB (n = 34)	Sweden (n = 13)	Italy (n = 6)	Spain (n = 19)	Netherlands (n = 10)
H1	0.678	0.458	–	0.029	0.923	–	–	–
H2	0.017	0.042	–	–	–	–	–	–
H3	0.017	–	–	–	–	–	–	–
H4	0.017	–	–	–	–	–	–	–
H5	0.085	–	–	–	–	–	–	–
H6	–	–	–	–	–	–	–	0.400
H7	–	0.083	–	–	–	–	–	–
H8	0.017	0.167	–	–	–	–	–	–
H9	–	0.042	–	–	–	–	–	–
H10	–	–	–	–	–	–	–	0.100
H11	–	–	–	–	–	–	0.526	–
H12	–	–	–	–	–	–	0.474	–
H13	–	–	0.889	0.529	–	–	–	–
H14	0.017	–	–	–	–	–	–	0.100
H15	–	–	–	–	–	–	–	0.400
H16	–	–	–	–	–	0.333	–	–
H17	–	–	–	–	–	0.333	–	–
H18	–	–	–	–	0.077	–	–	–
H19	–	–	–	–	–	0.167	–	–
H20	–	–	–	–	–	0.167	–	–
H21	–	0.042	–	–	–	–	–	–
H22	–	–	–	0.029	–	–	–	–
H23	–	–	–	0.147	–	–	–	–
H24	–	–	0.111	0.088	–	–	–	–
H25	–	–	–	0.059	–	–	–	–
H26	–	–	–	0.088	–	–	–	–
H27	0.153	0.167	–	–	–	–	–	–
H28	–	–	–	0.029	–	–	–	–

Table 3. Pairwise F_{ST} between British and European regions based on haplotype frequency data. Above diagonal: all haplotypes included. Below diagonal: haplotype H1 excluded from British populations. Non-significant F_{ST} values ($P \geq 0.05$) indicated in bold (3000 permutations). WestB = Western region post-1980, WestA = Western region pre-1980

	Eastern	Northern	West B	West A	Sweden	Italy	Spain	Netherlands
Eastern	–	**0.062**	0.464	0.576	**0.043**	0.482	0.608	0.518
Northern	**0.047**	–	0.348	0.474	0.217	0.338	0.514	0.355
West B	0.283	0.340	–	**0.092**	0.644	0.292	0.559	0.440
West A	0.356	0.467	**0.087**	–	0.857	0.514	0.681	0.541
Sweden	0.439	0.603	0.676	0.857	–	0.721	0.807	0.707
Italy	0.220	0.297	0.314	0.514	0.721	–	0.612	0.331
Spain	0.411	0.472	0.577	0.681	0.807	0.612	–	0.447
Netherlands	0.258	0.248	0.460	0.541	0.707	0.331	0.447	–

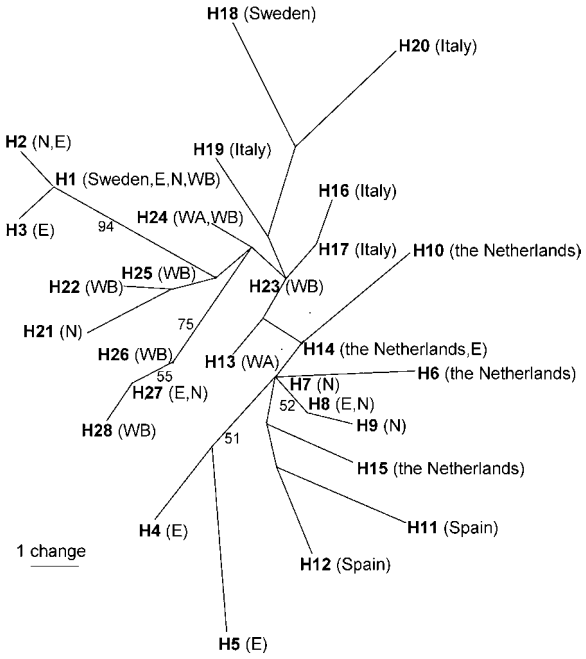

Figure 2. Unrooted maximum parsimony tree of *S. vulgaris* mtDNA haplotypes in Britain and Europe. Bootstrap values greater than 50 are indicated (100 replications). The regions where each haplotype was found is also indicated after each haplotype name. E = UK eastern region, N = UK northern region, WA = UK western region pre-1980, WB = UK western region post-1980.

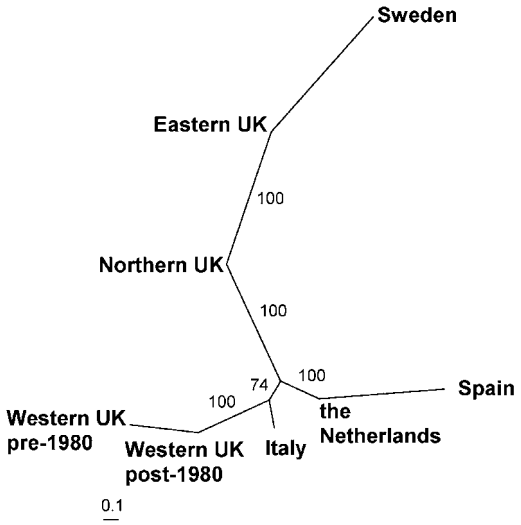

Figure 3. Unrooted neighbour-joining tree of Slatkin's linearised F_{ST} (genetic distance) between British and European regions. Bootstrap values are indicated (100 replications). *S. vulgaris* populations in the northern and eastern regions of Britain were more similar to *S. vulgaris* from Sweden than the western region of Britain.

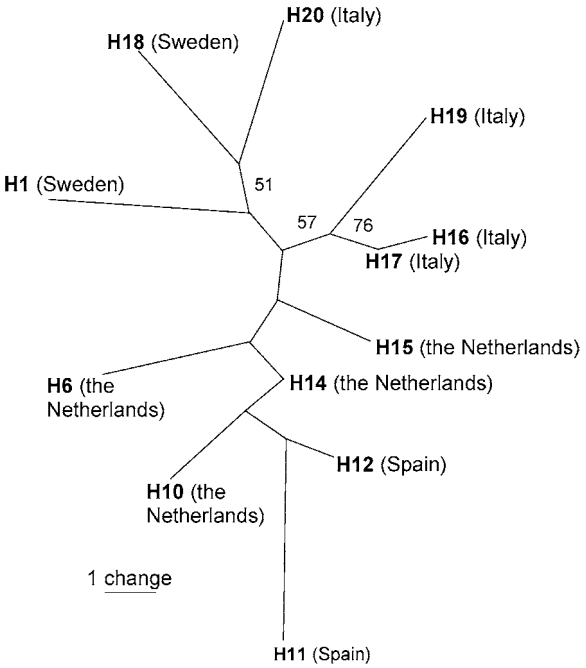

Figure 4. Unrooted maximum parsimony tree of *S. vulgaris* haplotypes in Europe only. Bootstrap values greater than 50 are indicated (100 replications). The haplotypes basically group by country except for H20 (Italy) which groups with the two Swedish haplotypes.

haplotype H1 were removed from the analysis, there was still substantial divergence between the British regions (F_{ST} = 0.268, $P < 0.001$), and the eastern and northern British regions were still more similar to mainland European samples (Italy and the Netherlands) than to the geographically much closer western British region (Table 3). The matrix of Slatkin's linearised F_{ST} values produced essentially the same neighbour-joining tree when H1 was excluded from the British populations as when it was included (Figure 3). The only difference between the two trees was that the branch lengths between eastern Britain and the Netherlands/Italy, and northern Britain and the Netherlands/Italy were shorter, and between eastern Britain and Sweden and northern Britain and Sweden were longer than when H1 was included. This suggests that both the northern and eastern regions were 'mixed origin' populations before the introduction of haplotype H1 to these regions.

All of the evidence collected to date, from examinations of microsatellite variation (Hale et al. 2001), morphological variation (Hale and Lurz 2003) and mitochondrial DNA variation (this study), suggests that of the extant northern English populations, only

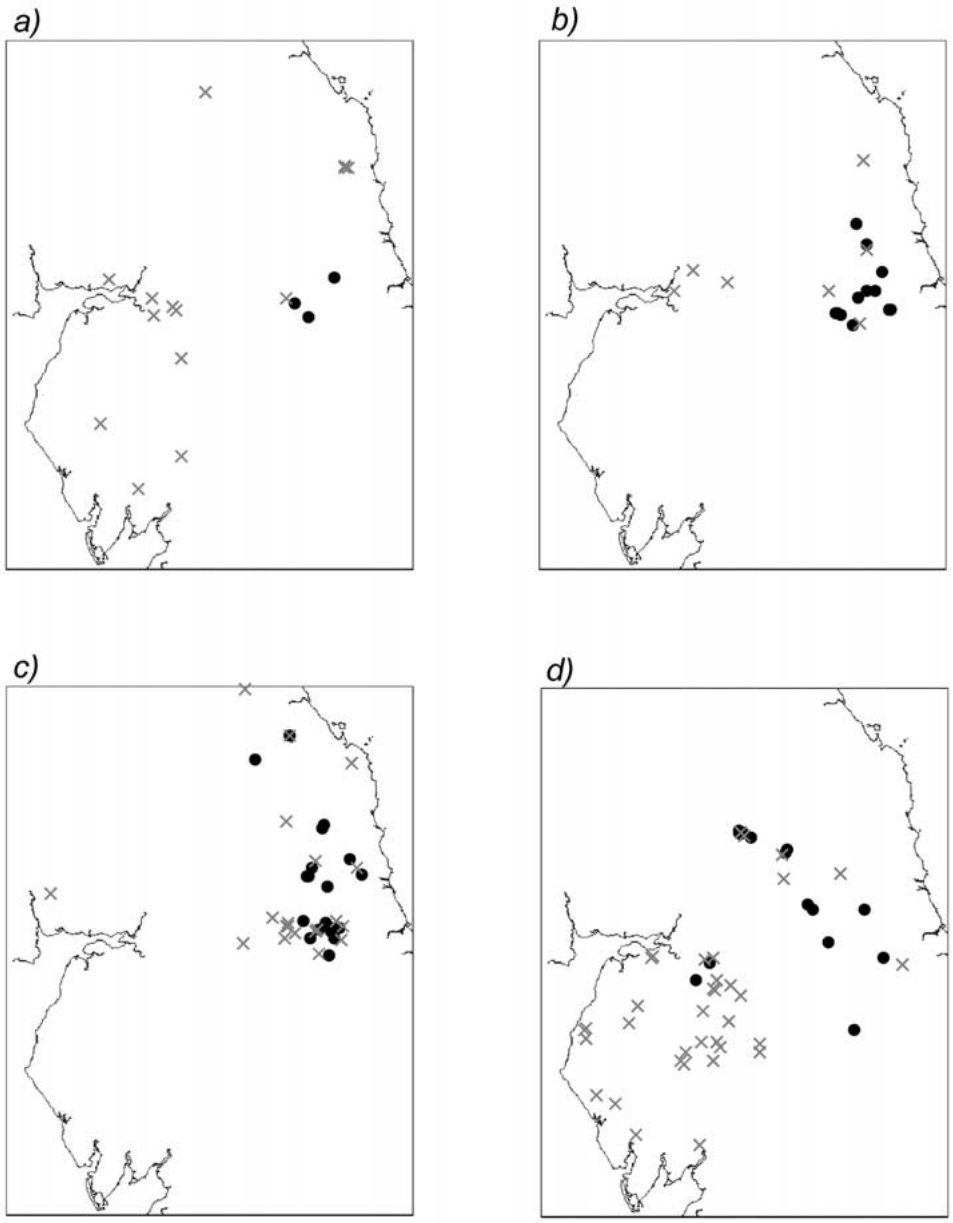

Figure 5. Geographic location of the Swedish haplotype (H1) in British *S. vulgaris* specimens collected (a) prior to 1970, (b) during the 1970s, (c) during the 1980s, and (d) between 1990 and 2002, overlaid on an outline map of the north of England and southern Scotland. Solid circles represent the location of at least one specimen with haplotype H1, grey crosses represent the location of at least one specimen with a haplotype other than H1. Haplotype H1 appears to have expanded from a small area around Corbridge in Northumberland in the late 1960s, to most of the study area except southern Cumbria by the end of the 1990s.

the western region prior to1980 is likely to have contained *S. v. leucourus*. To test this, we compared the haplotypes found in these populations with those from a small sample of *S. vulgaris* individuals collected in Dorset (southern Britain) in 1894 and 1895. Although the Dorset individuals represent some of the oldest specimens in British Natural History Museum's collection, these specimens were collected after the first recorded introduction of European *S. vulgaris* into Britain, but all early recorded introductions were in the north of Britain. However, all eight Dorset individuals possessed haplotype H1.

Discussion

British *S. vulgaris* populations are strongly differentiated genetically, as shown by the high F_{ST} value, but mitochondrial DNA haplotype variation appears random, with no reflection of geographical distribution in the phylogenetic analysis. The continental European *S. vulgaris* populations, on the other hand, displayed both strong population genetic and phylogeographic structure. The lack of shared haplotypes and high F_{ST} values suggest there is almost no gene flow between the eight 'populations' studied, apart from between Sweden and northeastern England (probably artificial) and between eastern and northern England (probably natural). The strong genetic subdivision in *S. vulgaris* microsatellite variation (Hale et al. 2001) was reflected in the mitochondrial DNA variation, but was not solely the result of historical isolation of the British populations. There seems little doubt that the majority of the extant populations of British *S. vulgaris* are of continental European subspecies ancestry. The lack of a clear geographic structure in the phylogenetic analysis of British haplotypes, the internal position of the northern and eastern English regions on the population neighbour-joining tree, and the sheer size of the genetic differentiation between the western and eastern regions (separated geographically only by approx. 20 km of non-squirrel habitat) all demonstrate that the genetic structure of *S. vulgaris* in Britain has been strongly influenced by genetic introgression of continental European haplotypes.

The neighbour-joining tree of all eight 'populations', based on Slatkin's linearised F_{ST} values, formed a triangle with three populations at the extremities: Sweden, Spain and western Britain pre-1980. These three samples had only 2 haplotypes each, and in the case of Sweden and western pre-1980, all individuals bar one had the most common haplotype. This, along with the internal position of eastern, northern, western post-1980 and the Netherlands suggests that these are 'mixed origin' populations, whilst western pre-1980, Sweden and Spain are probably not. Based on microsatellite variation, we know that western post-1980 is of mixed origin, as we observed a significant change in gene frequencies during the 1980s due to migration across the newly planted Kielder Forest (Hale et al. 2001).

The recent introduction and spread of haplotype H1 in Britain, probably from Sweden or a near neighbour, was clear from the direct examination of haplotypes from specimens collected throughout the last century. This haplotype did not appear in the eastern British sample until 1966, the northern sample until 1980, and the western sample until 2002. Kielder Forest did not provide a link between the north-eastern regions and the western region until the 1980s, so it is not surprising that dispersal was first north along older habitat patches, then west through the new conifer plantations. Haplotype H1 was found throughout Sweden (see Figure 1) in five decades over a period of 100 years (1891 to 1991). Although the Swedish sample was small (n = 13), the geographic and temporal range of sampling suggests that the populations throughout Sweden are now, and have been in the past, dominated by haplotype H1. In contrast, the frequency of haplotype H1 has changed both in time and space in the northern and eastern regions of England, which indicates that H1 was introduced from Sweden to Britain, not the other way around. The fact that all Dorset individuals possessed haplotype H1 implies that this population was also introduced. All Dorset samples were collected from the same area over a short period of time (1894 and 1895), so it is much more likely that individuals were introduced from Sweden (or nearby) to Dorset, than from Dorset to Sweden.

In a previous study, Barratt et al. (1999) examined mtDNA variation in British red squirrels using a DNA fragment that overlaps that used in the present study by approximately 50%–75%. Only three haplotypes found in our study were also detected by Barratt et al. (1999), most likely a result of very different sampling locations in the two studies. Haplotype f303 in the study by Barratt et al. (1999) is identical to H1 along the overlapping section of 302 bases. Barratt et al. (1999) did not detect haplotype f303 in any British populations except Spadeadam Forest, which is at the

northeastern edge of our western sample. They did not sample within the areas defined by the northern or eastern regions of the present study, but did sample from a number of southern or western English locations (Formby in Lancashire, Thetford in East Anglia, Isle of Wight, and three locations within Cumbria) as well as several Scottish locations (Fife, Argyll, Isle of Arran, and Torpins in Aberdeenshire). The results of Barratt et al. (1999) support our assertion that haplotype H1 is not a common British haplotype. Rather, it appears to have been a recent addition to the northeastern English populations, and has very recently expanded into the northeastern part of Cumbria.

The frequency of haplotype H1 increased markedly in the decade immediately after it appeared in both eastern and northern samples, rapidly becoming the dominant haplotype in both regions. The rapid rise to dominance of haplotype H1 in northeastern England is intriguing. It suggests that either individuals with this haplotype have some substantial selective advantage, or that many individuals with haplotype H1 have been introduced to Britain. Local adaptation is likely to make residents more fit than migrants in any particular environment, rather than the other way around. However, the large conifer plantations of northern England are dominated by spruce species (McIntosh 1995) not native to Britain and introduced squirrels from the boreal region of northern Europe already adapted to Norway or Common spruce (*Picea abies*) may possess traits more suited to these environments. Thus it is possible that the introduced, probably north European, individuals adapted to spruce forests have a selective advantage in the artificially planted spruce forests of northern England over individuals with a British or southern European ancestry.

Sixty eight percent of eastern British *S. vulgaris* individuals possessed haplotype H1. Even when these individuals were removed from the analysis, the remaining 32% of eastern individuals still appeared to represent a 'mixed origin' population with more similarity to mainland Europe than to western Britain. Therefore, it seems that in the eastern region at least, *S. v. leucourus* has been almost wholly replaced by individuals with European ancestry.

British *S. vulgaris* are classified as a separate subspecies, *S. v. leucourus*, to those found on mainland Europe (Corbet 1978; Wiltafsky 1978). The documentary evidence regarding introductions and the analysis of morphological variation in *S. vulgaris* (Hale and Lurz 2003) indicate that, of the extant British populations, the western region is most likely to contain *S. v. leucourus*. However, without access to specimens that were definitely identified as *S. v. leucourus* prior to recorded introductions in the 19th century, we cannot be sure that the mtDNA haplotype (H13) found in all bar one pre-1980 western individual is characteristic of this subspecies. If haplotype H13 is characteristic of *S. v. leucourus* it should also occur in the Scottish Highlands where there is no literature evidence for introductions. Barratt et al. (1999) found mtDNA haplotype ah178 (identical to H13 along the overlapping section of 296 base pairs) to be very common in Cumbria, as well as in Argyll, in Scotland. This suggests that H13 may well represent *S. v. leucourus* variation, but further surveys of Scottish populations are needed to determine whether H13 is common in northern Scottish populations.

The low variation in Sweden and western Britain pre-1980 can be explained by founder effects possibly as a result of recolonisation after the last glacial period. The low variation in Spain (2 haplotypes among 19 individuals) is surprising as this was expected to be a glacial refuge (Taberlet et al. 1998; Willis and Whittaker 2000), and was therefore expected to contain more genetic diversity than those areas that were recolonised after the last glacial period (Hewitt 2001). The lack of diversity in Spain could be due genetic drift if the refugial habitat was small. The greater diversity in Italy (4 haplotypes in 6 individuals) is more characteristic of glacial refugia. The internal position of the Netherlands on the neighbour-joining (NJ) population tree suggests that this is a 'mixed origin' population, but there is no evidence that this is the result of artificial translocations of individuals. It is more likely that the relatively high diversity in the Netherlands (four haplotypes in 10 individuals) and internal position on the NJ tree are the result of contact between two (or more) post-glacial recolonisation fronts, one from Italy and one from Spain.

In summary, this study demonstrated that (1) northeastern British *S. vulgaris* populations predominantly contain individuals with a recent continental European ancestry. In particular, a large proportion of these British populations comprise individuals with very recent (last 40 years) Scandinavian ancestry. (2) The rapid spread of the Scandinavian haplotype (H1) throughout the British populations suggests that Scandinavian red squirrels may have a selective advantage over those with British or southern European ancestry in the artificially planted non-

native spruce forests of northern England. (3) Our preliminary examination of continental European *S. vulgaris* mtDNA variation demonstrated that these populations have enough population genetic and clear, geographical phylogenetic structure to allow a useful phylogeographic study of this species. As *S. vulgaris* is a habitat specialist, the patterns of mtDNA variation in continental European *S. vulgaris* populations provides a neat method of determining patterns of post-glacial recolonisation of Europe by conifer tree species as well as squirrels. (4) A wider survey of all extant British *S. vulgaris* populations is required to identify populations with unique genetic diversity so that they can be included in the captive breeding program that exists for red squirrels in the UK.

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Appendix 1

Collection location, date, sampling region and mtDNA haplotype of all individuals. Grid reference is British Ordnance Survey Grid Reference for British samples only. Location is the closest town/village or wood/forest to specimen collection location. For Swedish specimens 118–127, location refers to the latitude and longitude of the collection site. Western A = western (UK) pre-1980, western B = western (UK) post-1980.

Specimen no.	Year collected	Grid reference	Location	Region	Haplotype
1	1981	NZ045616	Bywell	Eastern	H5
2	1979	NY980540	Slaley	Eastern	H1
3	1981	NY924583	Whitley Mill	Eastern	H5
4	1967	NY940630	Hexham	Eastern	H27
5	1982	NY035755	Greenlea	Northern	H2
6	1978	NY970550	Slaley	Eastern	H1
7	1980	NZ020580	Healey	Eastern	H1
8	1981	NT810280	Yetholm	Northern	H1
9	1981	NY880660	Wharmley	Eastern	H3
10	1981	NY940630	Hexham	Eastern	H27
11	1982	NY770560	Whitfield	Northern	H27
12	1981	NT944375	Ford Castle	Northern	H8
13	1981	NZ130620	Greenside	Eastern	H1
14	1978	NY960550	Slaley	Eastern	H1
15	1978	NZ080820	Bolam	Eastern	H1
16	1985	NX680500	Kirkudbright	Northern	H21
17	1986	NU180270	Chathill	Eastern	H2
18	1981	NY960600	Dipton Wood	Eastern	H27
19	1970	NZ080640	Ovingham	Eastern	H1
20	1983	NZ170890	Heighley Gate	Eastern	H1
21	1981	NZ100610	Prudhoe	Eastern	H1
22	1982	NY930630	Hexham	Eastern	H5
23	1985	NZ080640	Ovingham	Eastern	H1
24	1966	NZ025556	Minsteracres	Eastern	H1
25	1980	NT944375	Ford Castle	Northern	H1
26	1980	NZ010820	Kirkharle Farm	Eastern	H1
27	1978	NU070150	Glanton	Northern	H8
28	1989	NZ200860	Morpeth	Eastern	H5
29	1982	NZ120650	Close House	Eastern	H27
30	1979	NZ044897	Rothley	Eastern	H1
31	1979	NZ140710	Darras Hall	Eastern	H1
32	1980	NZ090780	Belsay	Eastern	H1
33	1980	NZ040610	Bywell	Eastern	H5
34	1981	NY960600	West Dipton Wood	Eastern	H1
35	1978	NZ170560	Burnopfield	Eastern	H1
36	1984	NZ100620	Prudhoe	Eastern	H1
37	1980	NZ054518	Shotley Bridge	Eastern	H27
38	1978	NZ110640	Wylam	Eastern	H1
39	1978	NZ030500	Minsteracres	Eastern	H1
40	1976	NZ050610	Stocksfield	Eastern	H1
41	1978	NZ170560	Burnopfield	Eastern	H1
45	1891	–	–	Sweden	H1
48	1961	NJ195295	Glenlivet	Northern	H9
50	1982	NT930040	Harbottle	Northern	H7
53	1985	NZ030860	Scots Gap	Eastern	H1
54	1971	NZ052606	Ridley Mill	Eastern	H27
55	1985	NZ110580	Chopwell	Eastern	H1
56	1986	NU070010	Cragside	Eastern	H1
57	1961	NT630430	Lauder	Northern	H8
58	1998	NY990900	Harwood	Northern	H7
59	1985	NZ220830	Hepscot, Morpeth	Eastern	H1
60	1981	NZ012823	Kirkharle	Eastern	H1
61	1997	NY500570	Hayton	Western B	H13
62	1974	NY420720	South Lambhill	Western A	H13
64	1967	NY240140	Rosthwaite	Western A	H13
65	1970	NY553668	Walton, Woodhead	Western A	H13
67	1950	NY430630	Scaleby	Western A	H13
70	1949	NY430630	Scaleby	Western A	H13
71	1970	NY352639	Floriston Station	Western A	H13
72	1963	NY520580	Geltwoods	Western A	H13
73	1993	NY940630	Hexham	Eastern	H1
75	1967	NY540390	Lazonby	Western A	H24
76	1937	NY440560	Scotby	Western A	H13
78	1998	NY218382	High Row, Ulswater	Western B	H13
79	1998	NY460240	Pooley Bridge	Western B	H23
80	1998	NY380160	Patterdale	Western B	H13

Specimen no.	Year collected	Grid reference	Location	Region	Haplotype
81	1999	NY570460	Croglin	Western B	H26
82	2000	NY770880	Sidwood	Northern	H27
83	1997	NZ043283	Hamsterly Forest	Eastern	H1
84	1997	NT646043	Wauchope Forest	Northern	H1
85	1999	NY490550	Headsnook	Northern	H1
86	1999	NY530220	Lowther Estate	Western B	H13
87	1999	NY511461	Armathwaite	Western B	H24
88	1998	NY560320	Edenhall	Western B	H23
89	1998	NY270570	Fingland Rigg	Western B	H23
90	1994	NY510440	Coombsland	Western B	H13
91	1994	NY470560	Warwick Bridge	Western B	H13
92	1992	NY510440	Coombsland	Western B	H24
93	1994	NY500450	Armathwaite	Western B	H13
94	1997	NY400200	Gowbarrow Park	Western B	H25
95	2000	NY680230	Brampton	Western B	H13
96	2000	SD210870	Broughton	Western B	H13
97	2000	SD210870	Broughton	Western B	H25
98	2000	NY680200	Appleby	Western B	H26
99	2000	NY390150	Patterdale	Western B	H13
100	2000	NY060030	Gosforth, Seadale	Western B	H13
101	1994	NY609422	Renwick	Western B	H28
102	1995	NY518483	Holmwrangle	Western B	H26
103	1996	NY464359	Hutton-in-the-Forest	Western B	H13
108	1998	NY020250	Lillyhall	Western B	H22
109	1997	NY506161	Burnbanks	Western B	H13
110	2000	NY506161	Burnbanks	Western B	H23
111	2001	NY883757	Chipchase Castle	Northern	H1
112	2002	NY883757	Chipchase Castle	Northern	H1
113	2001	NZ151572	Lintzford	Eastern	H1
114	2001	NY863783	Blindburn	Northern	H1
115	2001	NZ225544	High Forge	Eastern	H27
116	1980	–	Grimso	Sweden	H1
117	1991	–	Jokkmokk	Sweden	H1
118	1968	–	57′6 N 14′12 E	Sweden	H1
119	1971	–	59′26 N 13′44 E	Sweden	H1
120	1972	–	63′40 N 14′39 E	Sweden	H1
121	1972	–	62′49 N 14′29 E	Sweden	H1
122	1971	–	59′12 N 13′3 E	Sweden	H18
123	1971	–	59′31 N 13′17 E	Sweden	H1
124	1971	–	65′23 N 19′18 E	Sweden	H1
125	1972	–	65′36 N 19′13 E	Sweden	H1
126	1972	–	65′34 N 19′13 E	Sweden	H1
127	1972	–	65′23 N 19′18 E	Sweden	H1
128	2000	–	Cedrasco	Italy	H19
129	2000	–	Cedrasco	Italy	H20
130	2000	–	Cedrasco	Italy	H16
131	2000	–	Cedrasco	Italy	H17
132	2000	–	Bormio	Italy	H16
134	2000	–	S. Antonio	Italy	H17
136	2000	NU160135	Alnwick Moor	Eastern	H14
137	1982	NU075023	Cragside	Eastern	H1
138	1969	NZ120706	Dissington	Eastern	H1
139	1981	NZ144625	Greenside	Eastern	H4
140	1984	NZ095515	Consett	Eastern	H1
142	1984	NY993645	Corbridge	Eastern	H1
144	1966	NY970605	Dipton Wood	Eastern	H1
146	1978	NZ165566	Burnopfield	Eastern	H1
147	1984	NZ055615	Stocksfield	Eastern	H1
148	1982	NT773561	Oxen Dean	Northern	H8
149	1952	NU160145	Hulme Park	Eastern	H27
150	1983	NY945633	Hexham	Eastern	H8
151	1999	NT605065	Wardmoor Hill	Northern	H27
155	1999	NT610058	Wardmoor Hill	Northern	H1
156	1999	NT603068	Wardmoor Hill	Northern	H1
158	1999	NT615063	Wardmoor Hill	Northern	H1
159	1999	NT620050	Wardmoor Hill	Northern	H27
161	2001	–	–	the Netherlands	H15
162	2001	–	–	the Netherlands	H6
163	2001	–	–	the Netherlands	H10
164	2001	–	–	the Netherlands	H15
165	2001	–	–	the Netherlands	H15
166	2001	–	–	the Netherlands	H6
167	2001	–	–	the Netherlands	H15
168	2001	–	–	the Netherlands	H14
169	2001	–	–	the Netherlands	H6
170	2001	–	–	the Netherlands	H6
171	2000	NY785995	Redesdale Forest	Eastern	H1
172	2000	NY775985	Redesdale Forest	Eastern	H1
173	2000	NY765975	Redesdale Forest	Eastern	H27
174	1999	NT603063	Hyndlee	Northern	H1
175	1998	NZ079761	Black Heddon	Eastern	H1
176	2002	–	Centelles	Spain	H11
178	2002	–	Barcelona	Spain	H11
179	2002	–	Barcelona	Spain	H12
180	2002	–	Barcelona	Spain	H12
181	2002	–	Barcelona	Spain	H11
182	2002	–	Barcelona	Spain	H11
183	2002	–	Barcelona	Spain	H12
184	2002	–	Barcelona	Spain	H11
185	2002	–	Barcelona	Spain	H12
186	2002	–	Barcelona	Spain	H12
187	2002	–	Barcelona	Spain	H11
188	2002	–	Barcelona	Spain	H12
189	2002	–	Barcelona	Spain	H11
190	2002	–	Barcelona	Spain	H11
191	2002	–	Barcelona	Spain	H12
193	2002	–	Barcelona	Spain	H11
194	2002	–	Barcelona	Spain	H11
195	2002	–	Barcelona	Spain	H12
196	2002	–	Barcelona	Spain	H12
197	1895	ST888071	Blandford	Dorset	H1
198	1895	ST888071	Blandford	Dorset	H1
199	1894	ST888071	Blandford	Dorset	H1
200	2002	NY516237	Askham	Western B	H13
201	2002	NY441484	Wreay Bridge	Western B	H1
202	2002	NY185315	Highham Hall	Western B	H13
203	2002	NY014288	Workington	Western B	H13
204	2001	SD132994	Muncaster Fell	Western B	H13
205	2002	NY277573	Fingland Rigg	Western B	H23
207	2001	NY018290	Stainburn	Western B	H13
208	2002	NY110240	Bramley	Western B	H24
209	1894	ST888071	Blandford	Dorset	H1
210	1894	ST888071	Blandford	Dorset	H1
211	1895	ST888071	Blandford	Dorset	H1
213	1894	ST888071	Blandford	Dorset	H1
214	1895	ST888071	Blandford	Dorset	H1

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