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Assessing the geographic origin of the invasive grey squirrel using DNA sequencing: Implications for management strategies

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Abstract

The invasive grey squirrel Sciurus carolinensis has become a major pest species causing negative effects to forestry and biodiversity. This study aims to assess the origin of grey squirrel within Cumbria using phylogeographic analysis to aid in management and control. The work reported analysed mitochondrial DNA sequences in the D-Loop gene of 73 grey squirrel individuals from multiple locations in the UK. The results indicate that individuals in north Cumbria are derived from individuals from Scotland and North East England. Other individuals in north Cumbria share a unique haplotype with south Cumbria and Lancashire suggesting a southerly origin and movement around or over the Cumbrian Mountain range which is thought of as a barrier to movements. The assessment of invasive species geographical origin and the identification of potential wildlife transit corridors through natural barriers are becoming more important as species shift range in response to environmental and ecological changes. With the grey squirrel population expansion also occurring in Italy, the European red squirrel may become threatened across its entire range. It is crucial to understand the population origins of the invasive grey squirrel and landscape usage to successfully manage the incursion routes and control the population.

1. Introduction

Invasive non-native species represent one of the greatest threats to biodiversity around the world. The increased accessibility and prevalence of trade and travel has enabled species to be transported around the world and become established in novel sites with a far greater frequency than ever before (Meyerson and Mooney, 2007; Levine and D'Antonio, 2003). A range of different factors (climate and habitat requirements, species characteristics, population dynamics and landscape composition) may influence each stage of the invasion process and ultimately determine whether a species will become invasive (Williamson, 2006; Hayes and Barry, 2008). An invasive non-native species is defined as "any species that is able to spread and causes damaging effects to the environment, economy, or human health and way of life" (NNSS, 2013). A small number of non-native species have negative ecological and economic affects and it is these invasive species that cause conservation concern and should be prevented from establishing and spreading (Manchester and Bullock, 2000).

Where species have arrived and spread to create large populations, more resources are needed to eradicate or control the species (Manchester and Bullock, 2000). Successful eradication, such as the coypu (Myocastor coypus) used a combination of paid trappers and researchers working together, a 10 year management plan, funding and government backing all contributed to the eradication of the coypu in 1992 (Baker, 2006). This exercise was successful as the coypu population was confined to a small area and no immigration from other areas of the UK occurred (Manchester and Bullock, 2000). Invasive species cost Britain over £2 billion annually (Thomas, 2011) and with the threat of new invasive species coming into the UK this cost will increase. Having information on the geographical origin of invasive species is vital to prevent spread and aid eradication of new and established invasive species (Scheffer and Grissell, 2003).

The grey squirrel (Sciurus carolinensis) is probably the most well-known example of an established invasive species in the UK. Individual animals were recorded as being introduced to numerous locations in the UK from 1876 to 1929 (Middleton, 1931). Although originally from the deciduous woodland of North America, many of the individuals released were able to establish viable populations, which then went on to spread very effectively and subsequently became the source for translocations. These founder populations have since dispersed and colonised the majority of England, Wales and parts of Scotland and Ireland (Gurnell et al., 2004). This continuing range expansion places increasing pressure on native species and habitats which subsequently creates need for paradigm changes to existing management regimes to incorporate the growth of this invasive species.

Grey squirrels cost the UK forestry sector over £6m annually in damage (Williams et al., 2010), through bark stripping behaviour (Mayle et al., 2007). Strategic population management of grey squirrel is critical, particularly in areas where the native red squirrel Sciurus vulgaris is present. Grey squirrel and the associated interspecific competition for resources, juvenile presence and transmission of the squirrelpox virus are considered the main causes for the decline and displacement of many red squirrel populations within the UK (Gurnell et al., 2004, 2006). Red squirrels are widely distributed on mainland Europe, but in the UK the main population is located in north England and Scotland.

DNA microsatellite and sequence analysis has been used to show that red squirrels in west Cumbria (North West England), are genetically unique and are suggested to be important to the European population (Hale et al., 2001). This unique Cumbrian population are under threat by the increasing grey squirrel population in the county. In Europe the Italian population of red squirrels are facing similar threats from the advancing grey squirrel population (Bertolino et al., 2014, 2008; Martinoli et al., 2010). With the population expansion also occurring in Italy, the European red squirrel may become threatened across its entire range. In areas where grey squirrels are currently present alongside native red squirrels, it is crucial to understand the population origins and landscape usage to successfully manage the incursion routes and control the population (Stevenson et al., 2013b,c).

DNA barcoding has been shown to be used successfully as a tool for identifying geographical origin (Williams et al., 2012). In essence, DNA barcoding is where a short sequence (barcode) from a specific gene is compared against sequences from known species within a database (Hajibabaei et al., 2007). The sample is then identified to species based on the degree of similarity of the sequence to the ones in the database. Where sequences differ slightly these can reveal new haplotypes or geographical variants (Hajibabaei et al., 2007). Recently DNA sequencing has been used to determine the geographical origin of the invasive Asian longhorn beetle (Anoplophora glabripennis (Carter et al., 2010) and the emerald ash borer (Agrilus planipennis) (Bray et al., 2011). Although these studies were assessing origin at an international scale, the techniques can be used on a regional level to assess population origin. However, it should only be used where founder effects are absent and there is high levels of genetic variation present.

Recent work on landscape connectivity modelling indicated that grey squirrels in Cumbria are separated into two discrete populations (Stevenson et al., 2013a). As there are no recorded grey squirrel introductions in Cumbria (Middleton, 1931), it was hypothesised that the separation was caused by the land covers types and lack of habitat on the Cumbrian Mountain range, which runs west to east through the middle of the county (Stevenson et al., 2013a). Earlier work by Stevenson et al. (2013c) using mtDNA D-loop sequencing to ascertain whether any interspecific variation occurs in the UK provided evidence to support the hypothesis of two separate grey squirrel populations in Cumbria, as distinct genetic variation was detected between northern and southern Cumbrian populations. The grey squirrel population in the UK is known to have high levels of genetic diversity and is thought to have been introduced from multiple source populations and has not been through a genetic bottleneck (David-Gray et al., 1998). It is hypothesised that the current population in Cumbria is derived from different founding populations, Northumberland/

Scottish Borders in the north and from Lancashire in the south (Stevenson et al., 2013c). If this was to be determined as meaningful in a conservation strategy in relation to grey squirrels, samples from neighbouring counties need to be assessed in addition to previous populations to determine patterns of population origin. The work reported here aims to strengthen and build upon the previous research using DNA sequencing to assess the geographical origin of grey squirrel populations in Cumbria, UK.

2. Materials and methods

A total of 73 accessions were collected from five locations within Scotland and 11 within England. Grey squirrel accessions were collected from each of four known introduction points (Middleton, 1931); Balloch near Loch Long; Dalkeith in Edinburgh; Henbury in Cheshire, and from Alice Holt Forest in Surrey as in Stevenson et al. (2013c). In addition, samples were collected from twelve established/emerging populations within the UK; Doune, in Stirlingshire; Lockerbie, Hawick and Berwick on the Scotland/England border; Brampton, Carlisle, Keswick and Melmerby in north Cumbria; Grasmere, Windermere and Millom, in south Cumbria and Bleasdale, in Lancashire (see Fig. 1). All samples were donated by grey squirrel control groups or estate managers and were all humanely dispatched on site by collectors and frozen in a domestic freezer at −20 °C.

DNA was extracted from the leg muscle tissue of the 73 individuals using the QIAGEN DNeasy blood and tissue extraction kit and following the manufactures instructions (QIAGEN Ltd). Following the methodology of Stevenson et al. (2013c)

Fig. 1. Geographical locations of grey squirrel DNA sample locations in the UK.

primers for Sciurus carolinensis Dloop were taken from the published sequence of Barratt et al. (1999) (GenBank Accession no. AF111027) and fragments of the mtDNA sequence encoding D-loop were amplified by polymerase chain reaction (PCR) using 25μl reaction volumes. PCR amplification for Dloop sequence followed the protocol of Trizio et al.(2005), denaturation of 1 min at 94 °C followed by 40 cycles of 30 s at 94 °C, annealing 30 s at 52 °C, extension 1 min at 72 °C, final extension of 5 min at 72 °C. All PCR amplicons were cleaned using the QIAquick PCR purification kit (QIAGEN Ltd). Electrophoresis on 1% agarose gels was used to verify the results at each stage of the methodology. Sequencing of PCR products was carried out at DBS Genomics, Durham, UK, a commercial sequencing facility.

Sequences were aligned manually using BioEdit_R v7.0.4.1 Freeware (Ibis Therapeutics, California, USA) and Sequence Analysis 5.2.0 (Applied Biosystems) using the default settings within the programmes. A sequence from S. vulgaris (GenBank Accession no. AJ238588) was also included as an outgroup accession in the analysis. The sequences were entered into Paup 4.0 Beta 10 (Swofford, Illinois Natural History Survey, Illinois, USA) for phylogeny reconstruction and a 50% majority rule consensus tree was created with 1000 bootstrap and jacknife replicates to provide statistical support. A minimum spanning tree was constructed using Arlequin (ver. 3.5.1.2) and Gephi (ver. 0.8.2.) to illustrate the frequency of haplotypes in the samples.

3. Results

MtDNA was successfully extracted from 73 grey squirrel individuals from selected locations around the UK. The 329 bp Dloop sequence generated showed a 98% similarity match to that of grey squirrel in the Genbank BLAST search tool (Accession no. AF111027). D-loop sequences were aligned in BioEdit and discrete points of variation were detected at 26 unique sites, representing 7.9% of the overall sequence.

Parsimony analysis generated a 50% majority rule consensus tree from the grey squirrel Dloop sequence data which was rooted against red squirrel Sciurus vulgaris (GenBank Accession no. AJ238588). This tree enables the assessment of genetic variation within UK grey squirrel and indicates geographic origin of grey squirrel in Cumbria. Bootstrap and jacknife values were generated and values of significance (>50%) were shown on the tree branch. The consensus phylogenetic tree generated (Fig. 2) indicated that, the accessions analysed can be separated into three distinct clades.

The first clade comprised accessions collected from Scotland, England and north and south Cumbria. All samples from Alice Holt and Henbury (both south of Cumbria) and from Doune and Balloch (both located in Scotland) reside in this clade. Samples from Lancaster the country directly south of Cumbria and Hawick in Scotland are also present; however, samples from Lancaster are also present in clade 2 and from Hawick in clade 3. Samples from two of the three locations in south Cumbria (Windermere and Grasmere) and from one of the four from north Cumbria (Carlisle) are seen within this clade.

Fig. 2. Grey squirrel phylogenetic tree showing groupings of individuals from North and South Cumbria with Lancashire in clade 2, and a grouping of North Cumbria, North East England and Scotland in clade 3. ⁎Note bootstrap and Jacknife analysis above and below each group separation.

Fig. 3. Minimum Spanning Tree illustrating haplotype diversity in UK grey squirrel. HAP5 = North Cumbria, North East England and Scotland, HAP7 = North and South Cumbria and Lancashire, Black nodes = haplotype from only Alice Holt, white nodes = Balloch and light grey representing Henbury.

The second clade groups samples from north and south Cumbria together with Lancashire and has strongly supported bootstrap (89) and jacknife (77) analyses. Clade 2 contains samples from Keswick and Melmerby (north Cumbria) Millom and Grasmere (south Cumbria) and Lancashire. The third clade is comprised from only north Cumbria (Brampton and Melmerby) and northern populations (Lockerbie, Hawick and Dalkeith in Scotland, Berwick on the Scotland/England border) and has strong support from the bootstrap and jacknife analyses.

A Minimum Spanning Tree (Fig. 3) illustrates the frequency of 14 discrete haplotypes found within the 73 samples. Haplotypes are represented by nodes and branches and the number on the branch represents the number of differences within the sequence. K values signify the number of samples sharing this haplotype. Five haplotypes are only found within Alice Holt samples Hap1, 2, 8, AL1 and AL7; shown in black on Fig. 3 (Table 1). Haplotype 10, 11, and 12 are shown to be within the Henbury population and Haplotype four (shown in white) is found within samples from Balloch in Scotland. Haplotypes eight is present in south Cumbria samples (Grasmere and Windermere) and Haplotype nine in England (Henbury and Lancashire). Samples from Scotland, the Scottish/English border and north Cumbria share haplotype 5 and are grouped in Clade 3, suggesting north Cumbria may be derived from Scotland, the Scottish/English border (Table 1). Haplotype 3 is found in Scottish and English samples (Doune and Alice Holt) and Haplotype 6 is seen in North Cumbria and Henbury. Alice Holt and Henbury are two of the source locations used is translocations which may explain these results. Samples from North and south Cumbria, and Lancashire share Haplotype 7 and are also separated in Clade 2, suggesting south Cumbria could have been derived from infiltration from Lancashire which have gone on to spread in to north Cumbria through the mountainous barrier.

4. Discussion

Grey squirrel are successful invaders and are seen to be a major pest species within the UK impacting forestry and native species (Mayle et al., 2007; Gurnell and Mayle, 2003). Although individuals were originally introduced to sites for ornamental purposes, these founder populations quickly became established and either spread to further locations or were chosen to be the source population for translocations across the country (Middleton, 1931). Despite the relatively short evolutionary timeframe in which grey squirrel have been resident in the UK, genetic variation within the population has been detected through DNA fingerprinting (David-Gray et al., 1998) and more recently through the use of DNA sequencing of the D-loop gene fragment in previous and this study (Stevenson et al., 2013c). The variation suggests that the introduced grey squirrel population have been derived from multiple source locations and have not gone through a genetic bottleneck (David-Gray et al., 1998). David-Gray et al. (1998) suggests that the variation reflects the genetic difference in the founding populations or the time period since the first introductions to an area. Due to these levels of variation, this study has been able to use these differences to infer geographical origin of grey squirrel in the county of Cumbria.

For the vulnerable and genetically unique red squirrels in Cumbria, grey squirrel presence in the county is cause for concern. Grey squirrels are not recorded to have been introduced to Cumbria which raises the question of population origin.

Table 1
Grey squirrel samples listed for each unique haplotype obtained through Arlequin and Gephi Minimum spanning 428 tree analysis.

Stevenson et al. (2013c,a) least-cost connectivity modelling and sequence analysis suggests that the Cumbria Mountain range suppresses movements and suggest there are two infiltrates routes into the county from the north and south. Skelcher (1997) also discussed the northerly advance from Lancashire into south Cumbria and noted the risk of grey squirrels colonising Cumbria from the Scotland/England border. However the mountain range is not seen as a complete barrier, it is suggested that small number of individuals may be able to disperse around or over the mountain range Stevenson et al. (2013a).

Our results show that neighbouring counties do share unique haplotypes with grey squirrels found in Cumbria, suggesting potential origin from both north and south directions as suggested by Stevenson et al. (2013a). Individuals from Scotland, North East England and individuals from parts of North Cumbria share a unique genetic haplotype (HAP5, Fig. 3) and are also separated into a discrete clade in phylogenetic analysis with high statistical support (Fig. 2). Individuals were released around Edinburgh from a zoo collection, however, as with Cumbria, no introductions were recorded in Northumberland in North East England (Middleton, 1931). From the 11 sites sampled in England, only two North Cumbria locations and Berwick (North East England) share this haplotype, with three of the five sample locations in Scotland. It is possible that individuals that share this haplotype may have originated from the Edinburgh area and have dispersed south into Northumberland and Cumbria. There may also be the possibility that individuals that were translocated from Woborn, Bedfordshire into North Yorkshire may have then dispersed north into Scotland and northern Cumbria. However, Woborn was used as the main source population for seven recorded translocations (Middleton, 1931) and therefore, any haplotypes unique to this population may be present within the other seven so would not be useful as markers to identify origin. This study, indicates individuals from North Cumbria that share haplotype five have originated from neighbouring counties in the north (Northumberland or/and the Borders).

Individuals from Lancashire, south Cumbria and north Cumbria also share a unique haplotype (HAP7) and form a separate clade with high statistical support (Fig. 2). This suggests that individuals have been moving northwards from Lancashire into Cumbria. It also suggests that some grey squirrel individuals in north Cumbria may originate from Lancashire or have been derived from individuals that had originated there. As it is hypothesised that there is two populations of grey squirrels in Cumbria which are separated by the mountain range (Stevenson et al., 2013c), this grouping suggests that some individuals have either gone around or over the purported mountain barrier which separates north and south Cumbria. This movement may be due to natural dispersal or these individuals may have been assisted by human transportation either directly or indirectly. However, if there are corridors existing through the mountain range which are providing natural dispersal routes, then this will have implications for management. Using natural barriers to aid in management is important to reduce the economic expensive of control and eradication (Sharov and Liebhold, 1998). Further assessment of movement through the mountain range is needed to assess whether there are corridors.

Although haplotype seven shows the movement from Lancashire into Cumbria, no grey squirrels have been recorded as having been introduced into the county of Lancashire (Middleton, 1931, 1935) and therefore these individuals must have originated from elsewhere. Grey squirrel have been introduced to Yorkshire but it has been suggested that the Pennines Mountain range (running south to north down the east of Cumbria and into Lancashire and West Yorkshire, Fig. 1) limit the spread from Yorkshire into Lancashire (Skelcher, 1997). However, individuals may have spread from Yorkshire into other counties then into south Lancashire and then to Cumbria. Haplotype 9 is distinct to individuals from Lancashire and Henbury. Henbury was a large introduction site in Cheshire. Given the grouping some individuals within the Lancashire population may have been derived from Henbury as haplotype 9 suggests.

The results from clade one in the phylogenetic analysis have low statistical support for the initial branching but further branches with high statistical support and can separate this clade into 12 sister clades from multiple geographical locations. As one clade it represents either the dispersal from one or more of the recorded introduction sites (Alice Holt, Henbury, Balloch nr. Loch Long) or, the consequence of translocated individuals from introductions sites to further locations. These sister clades are represented within the minimum spanning tree as discrete haplotypes which support both hypothesis. Alice Holt, Balloch and Henbury are three known introduction points and have discrete haplotypes. Henbury also shares a haplotype (HAP 9) with Lancashire and another with Carlisle in north Cumbria (HAP 6) which possibly occurs through natural dispersal. Alice Holt (England) shares haplotype HAP 3 with Doune (Scotland). The sharing of these haplotype 3 between Alice Holt and Doune is most likely due to translocations from Alice Holt as these two locations are separated by a Euclidean distance of >600 km.

5. Conclusion

Building on initial DNA sequence variation observed previously (Stevenson et al., 2013c), this study examines the sequence variation in the D-Loop mitochondrial DNA of grey squirrels from multiple populations within the UK. Stevenson et al.'s (Stevenson et al., 2013c) suggestion of infiltration routes from neighbouring counties in north and south of Cumbria is further supported in the sequence differences within this study. However, this study has also highlighted that individuals may have been able to overcome the Cumbrian Mountains range. Potentially other areas within the mountain range may also be acting as corridors for grey squirrel movement and other non-native or native species. The use of these corridors needs further study.

Although this study focuses on grey squirrels, genetic sequence analysis can, and has been used to investigate patterns of origin in both native and invasive species dynamics (Scheffer and Grissell, 2003; Carter et al., 2010; Bray et al., 2011). Data gathered from such studies can be used to assess invasive species geographical origin on large and smaller spatial scales and are becoming more important as species shift range in response to environmental and ecological changes brought about by the processes of human development and climate change. For recently arrived species which have had limited spread this technique can be used quickly and with relatively little cost to determine country of origin and aid in biosecurity management. Having knowledge of geographical barriers for a species and knowledge of the origin and incursion routes into an area is essential to inform management and the eradication of invasive species.

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Fig. 1. Geographical locations of grey squirrel DNA sample locations in the UK.

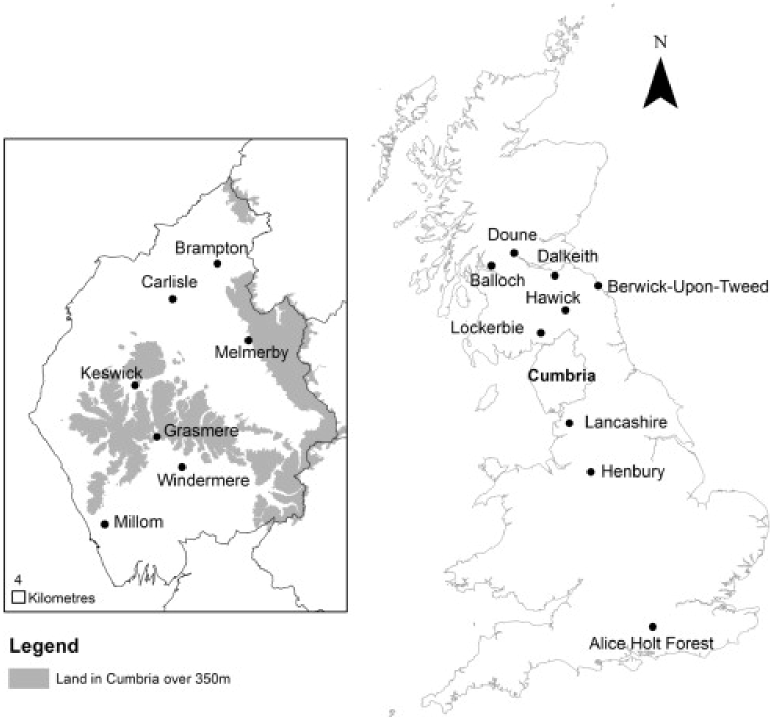

Fig. 2. Grey squirrel phylogenetic tree showing groupings of individuals from North and South Cumbria with Lancashire in clade 2, and a grouping of North Cumbria, North East England and Scotland in clade 3.   Note bootstrap and Jacknife analysis above and below each group separation.

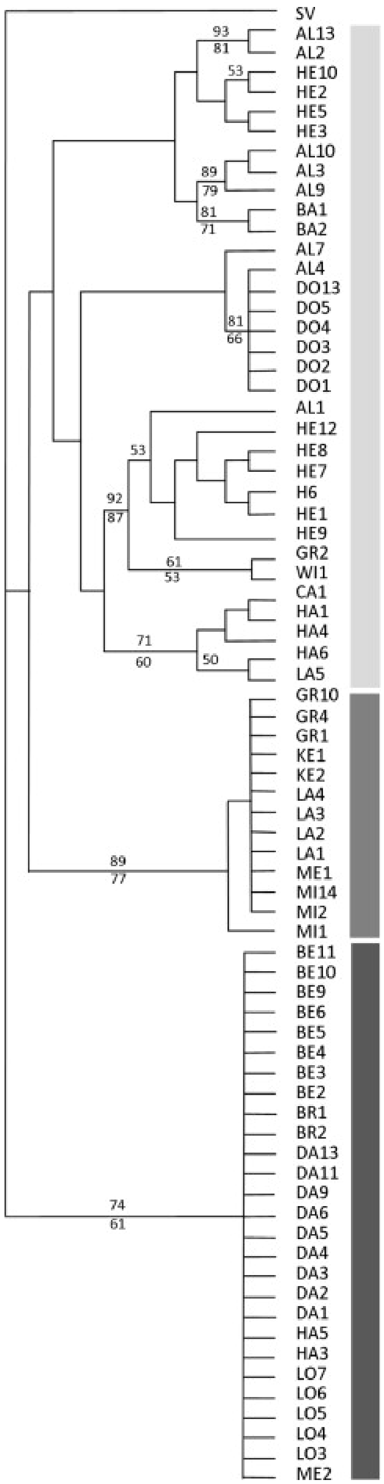

Fig. 3. Minimum Spanning Tree illustrating haplotype diversity in UK grey squirrel. HAP5 = North Cumbria, North East England and Scotland, HAP7 = North and South Cumbria and Lancashire, Black nodes = haplotype from only Alice Holt, white nodes = Balloch and light grey representing Henbury.

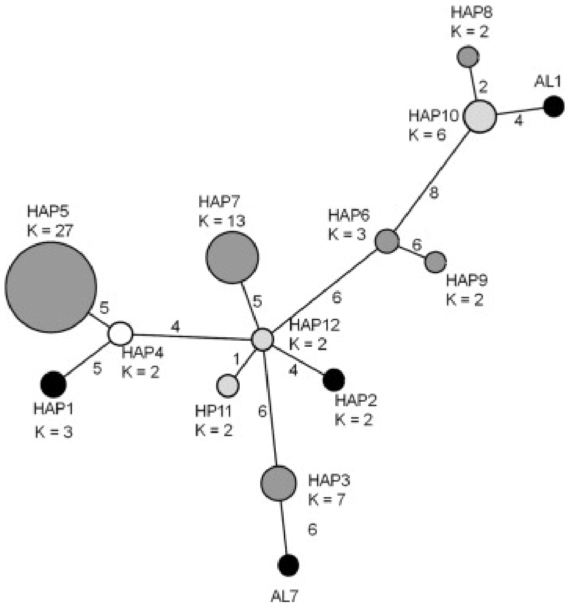

Table 1.
Grey squirrel samples listed for each unique haplotype obtained through Arlequin and Gephi Minimum spanning 428 tree analysis.

HAP1	HAP2	HAP3	HAP4	HAP5	HAP6	HAP7	HAP8	HAP9	HAP10	HAP11	HAP12
AL3	AL2	AL4	BA1	BE2	CA1	GR1	GR2	HA6	HE1	HE2	HE5
AL9	AL13	DO1	BA2	BE3	HA1	GR4	WI1	LA5	HE6	HE10	HE3
AL10		DO2		BE4	HA4	GR10			HE7		
		DO3		BE5		KE1			HE8		
		DO4		BE6		KE2			HE9		
		DO5		BE9		MI1			HE12		
		DO15		BE10		MI2					
				BE11		MI14					
				BR1		LA1					
				BR2		LA2					
				DA1		LA3					
				DA2		LA4					
				DA3		ME1					
				DA4							
				DA5							
				DA6							
				DA9							
				DA11							
				DA13							
				HA3							
				HA5							
				LO3							
				LO4							
				LO5							
				LO6							
				LO7							
				ME2							