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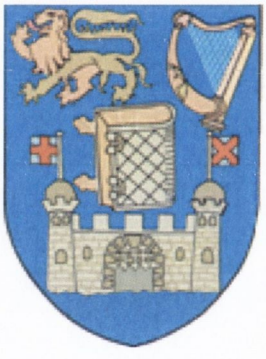
A genetic and morphological investigation of the forces shaping the diversity and distribution of the Eurasian red squirrel, *Sciurus vulgaris*.

by

Laura A. Finnegan



A thesis submitted in fulfilment for the degree of Doctor of Philosophy to Trinity College, University of Dublin

Department of Zoology

School of Natural Sciences

February 2007

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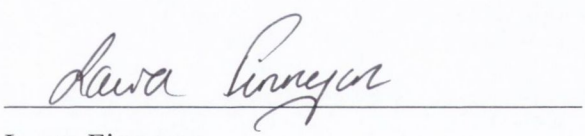

Laura Finnegan

Summary

An understanding of the factors which shape the range of, and distribution of diversity within, a species, is fundamental in understanding both the species itself, but also, the effectiveness, if needed, of conservation measures. The Eurasian red squirrel, *Sciurus vulgaris*, is one of the most widely studied of the Scuiridae, however research into the effects of historical, geographical and local factors which are instrumental in shaping the diversity of the species are scarce. As well as increasing understanding of the species, this information will be critical in designing conservation strategies in the face of ongoing replacement of the red squirrel in the British Isles and Italy, and possibly in the future mainland Europe, by the introduced American grey squirrel, *Sciurus carolinensis*.

In this study molecular markers; microsatellites and mtDNA, and morphological variables were analysed from a number of red squirrel populations from around Ireland, representing animals from two different habitat types (conifer and mixed conifer/broadleaf) and different habitat ages (old and new (planted since 1950)). Data collected from museum specimens added a further temporal range to the data set. It was aimed to use these data, combined with those already published, to investigate postglacial colonisation routes and the effect of landscape on the diversity of the species, and also to investigate the origin of the Irish red squirrel population and assess its subspecific status.

Phylogenetic analysis suggested European red squirrels spread from both an Italian and Iberian refugia, although equally, postglacial spread from more central cryptic refugia would also explain the phylogenetic patterns seen. Although the phylogenetics of the Irish population revealed that it was almost wholly a product of translocations from Britain over the 1800's, haplotype associations between Irish and Iberian individuals were also found, suggesting a Lusitanian origin to Ireland's squirrel population. Also, three extremely divergent haplotypes may indicate red squirrels survived through the last glacial maximum in a refugium in Ireland. Unfortunately, the predominance of British haplotypes in the Irish population means the origins of Ireland's native squirrels will probably never be known.

Using landscape genetics it was found, from the microsatellite analysis, that gene flow between red squirrel populations is restricted by a number of barriers to dispersal, particularly roads and unforested habitat. Red squirrels populations are therefore

particularly susceptible to habitat fragmentation, and its deleterious effects, but equally, may quickly accumulate adaptive traits in response to local environments within geographically restricted areas, explaining the pelage and genetic variations which have been described within restricted areas elsewhere. Within Ireland, although levels of genetic diversity were relatively homogeneous and the phylogenetic analysis had revealed the Irish population is largely the product of translocations, there was significant mtDNA and microsatellite structure in the country which should be preserved in the event of translocations for conservation of the species.

The combination of morphological and genetic data revealed a correlation between the two data sets, with squirrels which had higher genetic diversity having larger body weights, and a correlation between skull length, and snout length was also found, although based on a small sample size. Due to the dependence of reproduction on body size it was unclear whether increased body weight resulted in increased diversity, or vice versa.

Overall, trapping, genetic and morphological data collected in this study revealed that mixed sites are important red squirrel habitat. They supported higher population densities, heavier squirrels and squirrels which were more genetically diverse, which when combined with the landscape genetic results, indicated microevolution to a habitat type, and their importance to the red squirrel as a species should not be discounted. The widespread planting of conifers over the last 50 years in Ireland may have had a substantial effect on the red squirrel population in Ireland, reducing both body size and genetic diversity. Increased broadleaf planting in recent years, if it does not aid the spread of the grey squirrels, may serve to increase diversity within the species.

Although the phylogenetic analysis did not support the subspecific status which has been assigned to the red squirrel in Ireland, the analysis of tail colour found the light colour morph, the defining characteristic of the subspecies, widespread throughout Ireland, showing no regional or seasonal variation, neither was there a temporal variation, with no change in the frequency of light morphs between the museum samples sampled 100 years ago and those sampled recently. There was no association between tail colour and mtDNA haplotypes, and it is unclear why the spread of an introduced Swedish haplotype in Britain has coincided with the virtual disappearance of the light colour morph in that country.

Acknowledgements

The first and biggest thank you has to go to my supervisor Dr. John Rochford for helping, and steering me through, the last four years of fun and (thankfully less frequent) frustration. I'm sure he had his fill of squirrel researchers years ago but still agreed to take me on. Hopefully my inability to catch very many of the furry fiends hasn't put him off taking more on in the future!

Huge thanks also go to my two genetics experts Drs Ceiridwen Edwards and Mark Brown who agreed to read drafts on my chapters and gave me valuable feedback which improved their clarity. I also thank Ceiridwen for help with analysis of my mitochondrial data and for providing computer programs, and thank you to Mark for allowing me to use all his laboratory equipment, without which this project would not have been possible. Thanks also to the varying members of my steering committee: Profs Barry Roberts and Celia Holland and Drs Julian Reynolds and Ken Irvine for their advice over the years. Thanks too to Dr. Peter Lurz for his interest in this study throughout - I'll publish soon I promise.

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1.1. Introduction

'Our ignorance of the laws of variation are profound' (Darwin, 1859). A statement written over a century ago and yet after years of research, for many species, we are as ignorant of the forces which shape diversity today, as when Darwin first published *On the Origin of the Species.* Of course, the large forces behind natural selection are known – environmental variation, the genetic potential for evolution, and isolation for enough time for these effects to manifest themselves as local adaptations. However, some of the more subtle effects of, and forces behind, selection remain unknown and it is these forces which will be of importance in interpreting patterns of diversity, and producing effective conservation strategies, to maintain biodiversity for the future.

Increasingly, conservation of biological diversity is recognising the value of maintaining diversity within species, as well as the species themselves (Barbault & Sastrapradja, 1995; Butlin & Tregenza, 1998; Lesica & Allendorf, 1995; Woodruff, 2001). Intraspecific diversity is the raw material for evolution, and therefore a source of future biodiversity, but it also represents adaptation to a range of environmental conditions, which may be important to the future survival of a species in the face of global habitat change. The advancement of molecular techniques, and their combination with traditional morphological analysis, is allowing investigations of these driving forces behind adaptation. Also, with the speed of microevolution in modern times in response to human interference, so called 'contemporary evolution' (Hendry & Kinnison, 1999), they can track adaptation, and the effect of conservation measures on diversity, in real time.

The Eurasian red squirrel, *Sciurus vulgaris* Linneaus 1758, is the most widely distributed, and one of the most widely studied, of the *Sciurus* species, with 17 subspecies described (Sidorowicz, 1971), and yet little is known of the forces which have shaped the diversity of the species. It is a habitat specialist, occupying boreal forests across much of its range, although, within Ireland, Britain and, to a lesser extent, Iberia, its traditional habitat is broadleaf or mixed broadleaf/conifer forests (Lurz *et al.*, 2005; Mathias & Gurnell, 1998). Therefore, understanding of the forces behind the current patterns of diversity within the species are intrinsically linked to forestry, which in recent years in Britain and Ireland, has seen replacement of many historical broadleaf forests with coniferous monocultures (Neeson, 1991), but in the past, was shaped by postglacial spread of forests after the last

glacial maximum (Hewitt, 1996). Within Britain, Ireland and, most recently, Italy the future survival of the red squirrel is unsure due to the presence of an introduced competitor, the North American grey squirrel, *Sciurus carolinensis* Gmelin 1788.

The widespread distribution of the red squirrel, combined with its habitat specialist nature, makes it an ideal species in which to investigate the factors which have shaped diversity in a widespread temperate species. The red squirrels relatively high reproductive rate (generation time ~ 3 years, Gurnell, 1987), combined with evidence of quick adaptation to changing environments in its North American counterpart, *Tamiasciurus hudsonicus* (Berteaux *et al.*, 2004; Réale *et al.*, 2003a), means that presumably local adaptations would manifest themselves in red squirrel populations relatively quickly. The fact that within the last century, the species has been subject to two of the driving forces behind microevolution in species; competition and habitat change, means it can be used to interpret both the ability of, and speed at which, red squirrels can evolve in the presence of selective pressures. This information can be used to better understand the current distribution of diversity within the species, and the ability of the species to survive into the future, either in tandem with continual habitat change, or through rapid microevolution in response to conservation measures.

Although it is probably one of the most studied *Sciurus* species, with large quantities of research carried out in Britain and mainland Europe, and to a lesser extent, Ireland, revealing much about the ecology, behaviour, genetics and life history traits of the red squirrel (Gurnell, 1987; Lurz *et al.*, 2005, and references therein), together with behavioural and life history responses to an introduced competitor (Bryce *et al.*, 2002; Gurnell *et al.*, 2004; Wauters *et al.*, 2000, 2001, 2002), only a few studies have investigated the forces which have determined the distribution of diversity within the species. Barratt *et al.* (1999) and Hale *et al.* (2004) carried out phylogeographic work on the British, and to a lesser extent, the mainland European, populations, but could draw few conclusions due to the British population being largely a product of recent translocations from Europe. Hale *et al.* (2001a) carried out a detailed investigation of the effect of habitat fragmentation, and subsequent defragmentation, on gene flow among red squirrel populations, while Trizio *et al.* (2005) hypothesised genetic structure in the Italian Alps was shaped by major geographical barriers and rivers, although no statistical analysis was carried out to confirm this. No studies have investigated a range of barriers to dispersal on

gene flow in the red squirrel, and no studies of red squirrels have addressed morphological and genetic differentiation in sympatry.

Also, frequently mentioned is the possible adaptive advantage of red squirrels over greys in conifer forests, due to their smaller body size. Logically, this leads to the question – is coniferous planting, combined with grey squirrel spread, driving microevolution for smaller body size in red squirrels? Although smaller body size of red squirrels in forests where grey squirrels are present has been noted (Lurz *et al.*, 2005), this has been attributed to competition for food, rather than evidence of niche partitioning, and no studies into either phenomena as a driving force behind microevolution for smaller body size in red squirrels, has been carried out.

In this study, molecular and morphological analyses of the Irish red squirrel population, was used to increase understanding of the factors which have shaped, and are shaping distribution of, and distribution of diversity within, the Eurasian red squirrel. Populations were sampled over a temporal and spatial range, and in two habitat types, with two main aims:

- To determine what geographical barriers and/or historical events (e.g. the last glacial maximum) have influenced, and are shaping, diversity in the Eurasian red squirrel
- To investigate whether environmental pressures have influenced microevolution in the Eurasian red squirrel.

1.2. Literature Review

1.2.1. Shaping intraspecific diversity

The same factors, which drive macroevolution and speciation, promote diversity and drive microevolution within species; *i.e.* natural selection to local environment, combined with reproductive isolation, with the magnitude of differentiation between populations increasing with the length of time these factors remain stable (Kinnison & Hendry, 2001). However, other influences can either counter the effects and/or extent of microevolution (e.g. sexual selection (Partridge & Parker, 1999), translocations (Hughes *et al.*, 2003)) or stimulate it (e.g. interspecific competition (Bourke *et al.*, 1999), climate change (Berteaux *et al.*, 2004)).

Therefore patterns of diversity and microevolution are a product of the long term history of the species (e.g. the ice ages), historical and current biogeography, and the extent and level at which they affect the reproductive isolation of the species (insularity), and most recently human interference. When intraspecific variation does occur it can be cryptic, manifesting only at genetic markers such as microsatellites or mtDNA, which may only emerge over time (Lauter & Doebley, 2002), or may be reflected in morphological, behavioural, or life history traits (Magurran, 1999).

1.2.1.1. History: The ice ages and postglacial colonisation

The ice ages, and subsequent postglacial colonisation, have been recognised as the primary factor governing both the distribution of species, and the extent of diversity within these species, in the world today. Although traditionally the role of these cold periods in shaping the distribution and diversity of species was investigated using historical biogeographical studies, and indeed recent examination of pollen records (Bennett, 1986; Kullman, 2002; Willis *et al.*, 2000), and fossil remains (Sommer & Benecke, 2005, 2006) have increased knowledge of the effect of ice ages on species, molecular methods are now also being used to address these questions. This combination of genetics and historical biography, so called phylogeography, allows investigations of patterns of range contraction and postglacial spread at a much finer scale than other methods, with research possible at both the specific, and intraspecific, level (Hewitt, 1996, 1999, 2000; Riddle, 1996; Taberlet *et al.*, 1998).

The ice ages have dominated Earth's history, with cold periods occurring in roughly 100,000 year cycles, interrupted by warmer interglacial periods. The last glacial period, during the Pleistocene, had a large effect on the planet's extant species, particularly in the Northern hemisphere, and is also the ice age from which the largest amount of information is available (Hewitt, 1996). This last glaciation, 70,000 – 10,000 years before present (B.P.), caused the extinction of many species, and the range contraction of others into so called 'glacial refugia', the only areas where temperate species could have existed during full-glacial conditions (Hewitt, 1996, 1999). At that time the Laurentide ice sheet covered most of the northern United States and Canada. In Europe, the Scandinavian ice sheet (Figure 1.1) extended south as far as northern Germany, with some areas in Britain and Ireland remaining ice free. Glaciated areas were also present in the Alps and Carpathian mountains. The area between the ice sheet in the north and the southern mountains of Europe was tundra or cold steppe in nature (Prentice *et al.*, 2000) due to the extension of the permafrost layer.

Within Europe, a number of refugia have been suggested from biogeographical, fossil, and molecular evidence. Due to the presence of both the ice sheet and the southern extension of the permafrost, the main glacial refugia in Europe occurred in the Mediterranean basin; in Italy, Iberia and the Balkans (Figure 1.1). Considerable molecular evidence supports the through glacial persistence of flora and fauna in one or more of these refugia (e.g. woodmouse, *Apodemus sylvaticus* (Michaux *et al.*, 2003); meadow brown butterfly, *Maniola jurtina* (Schmitt *et al.*, 2005), white oak, *Quercus* sp. (Dumolin-Lapègue *et al.*, 1997)). Recently however, other refugia at higher latitudes have come to light (Figure 1.1), with both molecular (Bilton *et al.*, 1998; Deffontaine *et al.*, 2005; Kotlík *et al.*, 2006; Schönswetter *et al.*, 2005) and fossil (Sommer & Nadachowski, 2006; Stewart & Lister, 2001; Willis *et al.*, 2000) evidence revealing that temperate conditions existed in central and more northern parts of Europe.

The consequences of range contraction into, and postglacial spread from, refugia has been described in detail by Hewitt (1996, 2000, 2004). Within refugia, populations of species were isolated from those in other refugia, and this restriction in gene flow has resulted in allopatric differentiation of a number of genetic groups within species. The genetic lineages of these separate refugial groups can be traced today, and are used to track the postglacial spread of populations from the refugia, and determine the barriers that restricted postglacial spread. Although the morphological consequences of range

restrictions into refugia are not as easy to determine, it is expected that different patterns of microevolution did occur in the separate refugia, producing adaptations which may have influenced the ability of populations to expand from the refugium, and therefore determined where so called 'suture zones' between lineages from different refugia occurred (Hewitt, 1996), but further research on the role of refugia in subspeciation, is needed (Taberlet *et al.*, 1998).

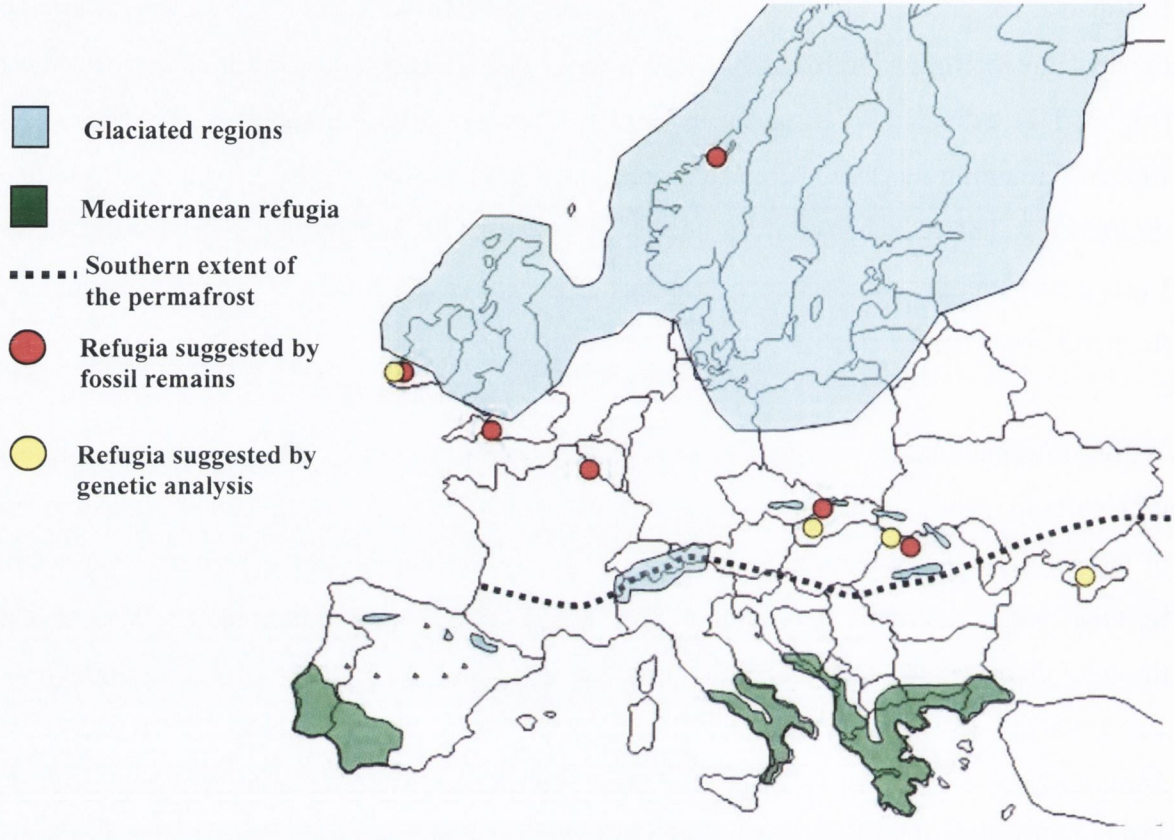

Figure 1.1. Map showing the maximum extension of the ice sheets during the last glacial maximum as well as the traditional Mediterranean refugia, and the extent of permafrost, above which is was presumed the landscape was tundra or steppe (redrawn from Hewitt, 1996). The cryptic refugia at higher latitudes proposed by Stewart & Lister (2001) are also shown, as are the central European refugia (Bilton *et al.*, 1998; Brunhoff *et al.*, 2003; Deffontaine *et al.*, 2005; Jaarola & Searle, 2002), and southwest Irish refugium (N. Martínková, pers. comm.; M.Hughes, pers. comm.; Hamill, 2002; Sinclair *et al.*, 1998), identified through phylogenetic work, some of which have revealed evidence for more temperate conditions, including the existence of forests, above the permafrost line.

Postglacial spread, after the retreat of the ice sheet, was governed by the ecological requirements of species, and the effects of geographical barriers to dispersal on the species. Naturally, species could not colonise new areas before suitable habitat, therefore colonisation patterns of species throughout Europe are intrinsically linked to one another,

with herbivores following plant species, carnivores following prey species etc. Therefore, understanding of one species pattern of postglacial colonisation, once knowledge of the particular barriers which restrict gene flow are known, can be used to interpret patterns in others.

As well as producing divergent lineages in different glacial refugia, rapid postglacial spread is also believed to have left its genetic signatures on the genetic diversity of species today. Hewitt (1996) and Avise (1994) hypothesised that the spread of species from glacial refugia resulted in a series of genetic bottlenecks, with successive founder events producing a cline of reducing genetic diversity as distance from the refugium increased. Therefore, the largest amount of genetic diversity would be expected in the refugia, and this has been found in some species (e.g. tawny owl, *Trix aluco* (Brito, 2005); Scots pine, *Pinus sylvestris* (Sinclair *et al.*, 1998)). Conversely, Petit *et al.* (2003) studied patterns of diversity within a number of different tree species (n = 22) in Europe, and found that the highest amount of diversity was not in the refugia, but instead coincided with the likely locations of suture zones between lineages from the separate refugia. Therefore, patterns of genetic diversity, when being used to track postglacial spread, should be treated with caution, especially with the emergence of a number of refugia at higher latitudes, as diversity which could be interpreted as evidence of refugia, could be a result of genetic introgression or vice versa.

Based on genetic and fossil evidence three widespread colonisation patterns of species from the Mediterranean refugia have been described (Hewitt, 1999), and they are named after the species in which they were first described. The hedgehog, *Erinaceus* sp., colonised Europe from all three Mediterranean refugia; Italy, Iberia and the Balkans. The grasshopper, *Chorthippus parallelus*, spread mostly from the Balkan refuge, with colonisation from Italy and Iberia blocked by the Alps and Pyrenees respectively. The bear, *Ursus arctos*, spread from the Iberian and Balkan refugia, with movement from Italy essentially blocked in a pincer movement, with available habitat already colonised by individuals from the other refugia. However, as already mentioned, colonisation patterns are not as simple as these three, with both refugia at higher latitudes, and refugia further east, produced complicated patterns of dispersal. Taberlet *et al.* (1998) reviewed the phylogeographical data for 10 species, four mammals, a newt, the grasshopper and four species of tree and found that, although patterns of postglacial spread throughout Europe have some degree of interspecific similarity, and a general pattern of postglacial spread and

location of suture zones was mapped (Figure 1.2), each species has a distinct phylogeographical pattern, and the role of refugia in Asia, and the evidence for temperate species survival at higher latitudes (Bilton *et al.*, 1998; Deffontaine *et al.*, 2005), means that further phylogeographical studies, carried out over a wide geographic range, and in a number of species, combined with knowledge of the factors that influence gene flow at the species level, is needed to understand Europe's, and indeed the Northern hemisphere's, current species diversity.

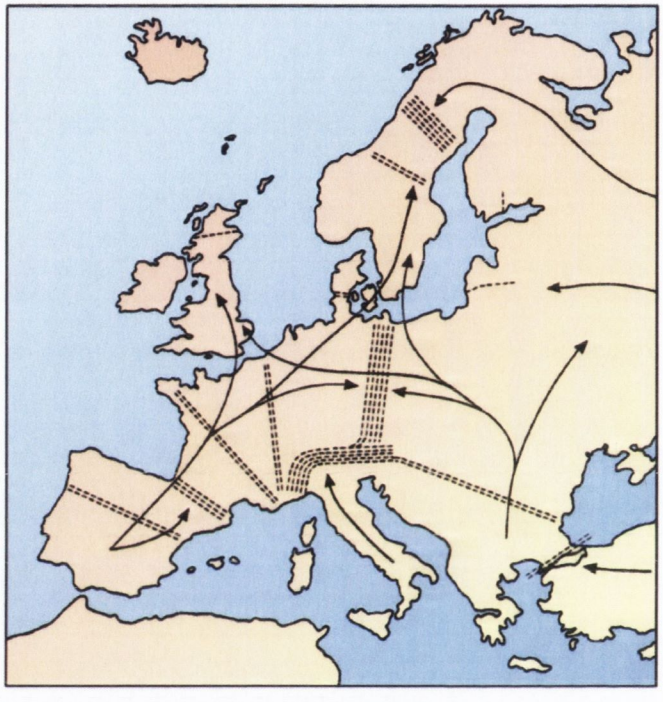

Figure 1.2. Map of Europe (after Hewitt, 2000) showing the main postglacial colonisation routes and the position of hybrid or suture zones between lineages from different refugia. The largest suture zones are in Scandinavia, the Italian Alps and along the western German border.

1.2.1.2. Insularity

Insularity is essentially the genetic isolation of a population. Understanding of species, ecology, behaviour and dispersal capabilities is intrinsic in the definition of the extent of insularity of a species. The most extreme example of insularity occurs on isolated islands, with no opportunity for natural dispersal from, or into an island population. However, insularity can occur at a much narrower scale, as a result of habitat islands separated by

barriers to dispersal, and to a lesser extent, can be experienced at the edge of the ecological or geographic range of a species (peripheral populations).

Islands are probably the best studied of biogeographical factors which drive both micro and macroevolution, with Darwin's theory of natural selection based on the divergent forms driven by isolation of populations on islands. Depending on the age of the founder event, populations on islands (hereafter referring to actual islands and habitat islands, although most cited research was carried out on actual islands) are extremely divergent from their mainland contemporaries, with divergence increasing with increasing island isolation, and decreasing island area (Pergams & Ashley, 2001). Generally, island populations are described as having lower levels of genetic diversity, but higher levels of genetic structure, than mainland populations, due to genetic isolation. As a result of this reduced gene flow, island populations can rapidly accumulate morphological characteristics, in some cases driven by reduced interspecific competition and reduced predation, which may not manifest in larger, less isolated, populations (Meiri *et al.*, 2005).

Morphological divergence on islands has been described in quite subtle forms, in many cases attributed to competitive release, although the forces behind it are probably more complicated (Dayan & Simberloff, 1998). A study carried out on Irish mustelids found differences in canine size between Irish and British populations, possibly as a result of the relatively restricted prey species occurring in Ireland, when compared with Britain. Also, sexual dimorphism was found in Ireland, with males and females of stoat, *Mustela erminea*, and pine marten, *Martes martes*, diverging in size, probably to exploit ecological niches in Ireland filled by the presence of the polecat, *Mustela putorius*, and weasel, *Mustela nivalis*, in Britain (Dayan & Simberloff, 1994). Similar size results were found when comparing pygmy shrews, *Sorex minutus*, in Ireland and Sweden, where, in Sweden, the species co exists with another shrew species (Malmquist, 1985), and in Japan where the Japanese field mouse, *Apodemus argenteus*, occurs on islands, some of which have a congeneric species (Millien, 2004). Likewise, research carried out on the Indian mongoose, *Herpestes javanicus*, on islands where it was introduced, and on which it did not have to compete with congeners, found variation which was compatible with competitive release, but suggested further research into the role of diet on morphological variation (Simberloff *et al.*, 2000).

However it is the combination of morphological and genetic data which will be of most use in interpreting patterns of diversity within species (Dayan & Simberloff, 2005), as morphological diversity on islands, which could be attributed to natural selection, could merely be a product of population isolation and genetic drift. Davis (1983) suggested that founder events and reduced gene flow caused the morphological variation he detected in populations of house mice in Britain and Faroe, but it has only been recently, with the widespread use of molecular markers, that patterns of morphological diversity of island populations have begun to be understood fully. Experimental work, combined with data on the exact founder dates of island populations, led Clegg *et al.* (2002) to conclude that the microevolution observed in a passerine bird, the silvereye, *Zosterops lateralis*, was largely a result of directional natural selection and not genetic drift. Conversely differentiation in recently introduced populations of chaffinches, *Fringilla coelebs*, in New Zealand was attributed to genetic drift and not natural selection (Baker *et al.*, 1990). Clearly, each population, and its response to isolation, is different, and large sweeping statements regarding the forces behind microevolution cannot be made. Instead, explanations should be formulated based on genetic, morphological, geographic and temporal data, combined with knowledge of the ecology, and ecological restraints, of the species and/or population.

As mentioned above, the extreme genetic bottlenecks experienced by populations which are completely isolated, and, depending on the size of the founder populations, subsequent inbreeding, may, in sympatry with increasing morphological diversity, result in decreased genetic diversity, as in some cases the two are not necessarily positively correlated (Brushi *et al.*, 2003; Kjær *et al.*, 2004; Pfrender *et al.*, 2000). Therefore, small levels of gene flow into a population may be necessary to maintain viable levels of genetic diversity. Although one of the criteria for microevolution was previously outlined as population isolation, both computer simulations (Alleaume-Benharira *et al.*, 2006) and molecular studies (Hoekstra *et al.*, 2004; Petren *et al.*, 2005) found that small amounts of gene flow into a population were not detrimental to the accumulation of adaptive microevolution, with low levels of migration serving both to increase genetic diversity and facilitate adaptive microevolution through augmentation of the genetic material available for natural selection.

Peripheral populations, that is populations which occur either at the edge of the ecological or geographical range of a species, essentially experience the same microevolutionary

forces as island populations, albeit to a lesser extent, with higher levels of migration reducing the rate at which adaptive changes accumulate, but also, maintaining higher levels of diversity than those found on islands. These populations have been suggested as important, as they may contain levels of diversity and/or adaptive microevolution, which may be valuable for conservation of the species (Guo *et al.*, 2005; Lesica & Allendorf, 1995), particularly where the populations occur at the ecological edge of species distribution (Hampe & Petit, 2005; Travis & Dytham, 2004).

1.2.1.3. Human interference

Habitat fragmentation, destruction and change are some of the most obvious effects that humans have on species. Fragmentation is subjecting populations to insularity, in some cases stimulating microevolution within species, as previously discussed, but, in other cases insularity is so extreme that populations within fragments are either unable to sustain themselves, due to inbreeding depressions as a result of extremely low genetic diversity, or subjected to marginal effects, i.e. at the edge of habitat fragments, to which they are not adapted. Both cases can lead to population, and in the most extreme cases, species, extinction (Young *et al.*, 1996). Similarly, habitat degradation or destruction can also lead to adaptive diversity (Bone & Farres, 2001; Reznick & Ghalambor, 2001; Macnair, 1987) but, as with habitat fragmentation, can also lead to extinction.

Secondary to habitat destruction, and in some cases an accidental by product of the spread of, and trade between, human populations worldwide, is the introduction of new species into ecosystems. Again, in the most extreme cases, these new species may cause extinction. Only recently are the more subtle effects of biological invasion on native communities and populations coming to light (Kowarik, 1995 in Straus *et al.*, 2006). Introduced species may become part of the ecosystem in the form of predators, pathogens, parasites competitors, mutualists or hosts (Schiffman, 1994). The response of native species to invaders varies and was recently reviewed in depth by Straus *et al.* (2006). In some cases there may appear to be no evolutionary change in natives in response to invaders This may be caused by the lack of insularity of the population of the native species, by the apparent 'preadaptations' the native species may have, most common when the invasive species closely resemble species already part of the ecosystem. Equally it could occur as a result of the genetic mechanisms underlying adaptive evolution, whereby if two traits are adaptive in the presence of an invader, but negatively correlated with each other, the amount of selection acting on each trait will be reduced.

Nevertheless, microevolution of native species as a result of invasive introductions has been recorded at nearly all trophic levels, and over timescales of less than 100 years (see list of examples in Straus *et al.*, 2006). Although the effects of invasive hosts, herbivores and predators on native species are most prevalent in the literature, some examples of microevolution of native species in response to introduced competitors have also been recorded. For example Bourke *et al.* (1999) recorded a change in benthic morphology in the brook charr, *Salvelinus fontinalis*, after introduction of invasive competitors in lakes in Canada, although the length of time over which this change took place was not recorded. Crowder (1986) recorded changes in life history traits by native fish in order to decrease the length of time juvenile fish would have to compete with the invasive fish species for zooplankton, and this change took place in as little as 20 years. Overall, Straus *et al.* (2006) stressed that, although considerable research has been carried out on the diversity and ecological effects of invasive species, focus should now shift to the response of native species to invaders, particularly when species seem to coexist with invasive species, as this information will aid in understanding of the ability of native species to persist into the future.

Finally, microevolution of species can occur as a result of human conservation efforts. This is particularly evident when conservation is being carried out through captive breeding. Depending on the length of time that the species is being subjected to captivity, microevolution can occur, as in the wild, in response to the local, in this instance captive, environment, resulting in genetic or phenotypic changes which are not advantageous to survival in the wild (Frankham *et al.*, 1986), and can reduce fitness in a population which is augmented with captive bred individuals (Ford, 2002; Lynch & O'Hely, 2001).

Overall, the role of humans in microevolution, and the rate at which this process is now occurring, so called 'contemporary evolution' (Hendry & Kinnison, 1999), means that current rates of microevolution and diversification within species are increasingly caused by anthropogenic effects (Stockwell *et al.*, 2003). The question is whether species can adapt fast enough to cope with their changing environment.

1.2.2. Tracing intraspecific diversity

Choosing in what way to assess intraspecific diversity is as complicated as deciding in which way to measure interspecific diversity. Should species diversity be measured simply by counting the number of species? Should rare species be weighted more than

more common ones? How are divergent populations of the same species accounted for? Could 'Evolutionarily Significant Units' (ESU), which recognise diversity as units which are distinct enough from each other to warrant conservation (Moritz, 1994a; Tregenza & Butlin, 1999), be used?

In the case of intraspecific diversity, traditionally morphological variables were used as a measurement of diversity. Through detailed phenotypic examinations, subspecies were defined and comparison of subspecific traits and environmental factors led to explanations for the driving forces behind this phenotypic divergence. The emergence of molecular markers and the relative ease of their use now have made them the variables of choice for measuring diversity within, and divergence among, populations. Although most of these markers are, by definition, neutral in nature, and do not evolve in response to environmental pressures (Nei, 1987) (but see sections 1.2.2.2 and 1.2.2.3.), their diversity can reflect diversity of phenotypic or genotypic advantageous traits which may reflect fitness (Reed & Frankham, 2003).

1.2.2.1. Morphological variation

Morphometric analysis is probably the most ancient form of assessing diversity, with its roots leading back as far as Aristotle and morphological data were the basis of Linneaus' classification system (Blackith & Reyment, 1971). Whereas traditional morphometrics focused on the study of allometic growth, the same techniques, however, which were applied to allometry can also be used to assess morphological variation. In this instance morphological variation refers to both morphometrics (measurements taken to assess variation (e.g. cranial, body weight)) and other phenotypic traits (such as pelage).

The traits which can be used to assess morphological variation are almost infinite. They can be as simple as coat patterns which define populations as separate subspecies, or as complex as tens of cranial measurements analysed through multivariate statistics to assess size and shape variations. The actual genetic mechanisms behind morphological variation are poorly understood (Foote, 1997), with the exception of pelage and coat polymorphisms which, in some cases, have been found to be controlled by a single gene (e.g. Hoffman *et al.*, 2006). In most cases, morphological changes result from the interaction of a number of genes upon which a number of selective pressures act (Wright, 1968; Lande, 1981 in Pfender *et al.*, 2000).

Analysis of diversity using multivariate morphometrics it still quite widespread (e.g. Goheen *et al.*, 2003; Herfindal *et al.*, 2006; Markov, 2001; Pergams & Ashley, 1999) however in recent years a new means of investigating patterns in morphological diversity has emerged. Rohlf & Marcus (1993) introduced this new means of analysis, geometric morphometrics, whereby the entire geometry of the morphological structure is analysed, allowing shape variations and asymmetry to be statistically analysed, and this method is now the most popular means of analysing morphological data (Adams *et al.*, 2004). However, it is a combination of morphological and genetic analysis which will provide the most insights into macro and microevolution in the future (MacLeod & Forey, 2002). Combining molecular and morphological markers can make interpretations of patterns seen in either or both data sets clearer (Prout & Baker, 1993) or can demonstrate the contrasting evolutionary forces which work at different levels within species (e.g. Baker *et al.*, 1990; Kjær *et al.*, 2004; Pesanto & Brown, 1999).

1.2.2.2. Microsatellites

Microsatellites are rapidly becoming the nuclear marker of choice to study gene frequencies within populations. Like allozymes they are co dominant and inherited in a Mendelian fashion; however they have more alleles per locus, and a higher chance of neutrality, making them more useful for fine scale investigations of populations. Microsatellite DNA can be obtained from a wide range of sample types including blood, tissue and hair root cells. They consist of short tandem repeats of base pairs, typically 2-6 base pairs in length, which may be repeated up to 100 times. Overall, microsatellites are widespread and have been found in every organism analysed so far (Hancock, 1999), although they are less common in plants (Zhivotovsky *et al.*, 1997). Perhaps the most useful trait of microsatellites for population genetic studies is the speed at which they mutate. Rates of mutations in microsatellites have been estimated at 10^{-2} to 10^{-5} per locus per generation (Jarne & Lagoda, 1996), in comparison to point mutations which are approximately 10^{-10}, and mtDNA which has a mutation rate of approximately 10^{-9} substitutions per site, per year (Pesole *et al.*, 1999).

These high rates of mutation could be explained by slipped strand mispairing (slippage) during DNA replication, or recombination between DNA molecules. Slippage essentially is the misalignment of complementary DNA strands whereby a mismatch loop, followed by continued DNA synthesis occurring in the replicated strand, results in an addition of a repeat structure, while, if the mismatch loop occurs on the template strand a repeat

structure is deleted. Recombination on the other hand could lead to large changes in the number of repeat units, through the exchange of DNA between homologous chromosomes, or chromatids within chromosomes. However, as most microsatellite mutations involve the gain or loss of single repeat unit, it is generally accepted that microsatellite mutations occur through slippage and not recombination (Hancock, 1999 and references therein).

The theoretical mutation models which should be applied to microsatellites remain, as yet, poorly understood. Classically, the two extreme models are the *infinite allele model* (IAM; Kimura & Crow, 1964) and the *stepwise mutation model* (SMM; Kimura & Ohta, 1978). Under the IAM a mutation can involve any number of repeat units, and always results in an allelic state which has not previously occurred in the population, i.e. an infinite number of allelic states are possible. The SMM is a mutation model which involves the loss or gain of a single repeat unit, with mutations possible which lead to allelic states already present in the population. However many studies have shown that neither of these mutation models totally explain the mutation patterns seen at microsatellite loci.

A third mutation model was suggested by Di Rienzo *et al.* (1994). The *two phase model* (TPM) is basically a mixture of the IAM and SMM. It assumes, like the SMM, that all mutations are single step changes, but also, following the IAM, larger changes can occur, albeit, less frequently. Finally, the *K-allele model* (KAM) is similar to the IAM, although the number of allelic states, K, is limited. Generally there is no consensus as to which of these four models, if any, best explains microsatellite evolution. Balloux & Lugon-Moulin (2002) argued that none of these models can be assumed to be working ubiquitously across all species and, given loci and species variability, it is likely that individual models of mutation, interpreted from analysis of individual species, may be the only means through which accurate interpretation of results can be achieved.

Although generally described as neutral markers, the distribution of microsatellite loci throughout the genome means that in some cases they are subjected to selective pressures (Jarne & Lagoda, 1996). When microsatellite loci are located within expressed regions of the genome, characteristics of these loci (i.e. size ceiling of allele size), suggests that these loci are under selective pressure (Charlesworth *et al.*, 1993; Sutherlands & Richards, 1995). Jarne & Lagoda (1996) thought this issue would not be of concern as the random nature of microsatellite loci, and the low frequency of loci located within selected regions, means the majority of microsatellite loci isolated would indeed be neutral. However, it is

still possible that some microsatellites are under selective pressures, and research has found correlations between variability at microsatellite markers and non neutral markers (Campos *et al.*, 2006).

The emerging dominance of microsatellite markers as a tool in population genetic analysis is clear. Due to their fast mutation rate they are particularly useful in tracking recent changes in population diversity and are therefore of interest to conservation geneticists as the effects of management strategies, recent habitat fragmentation etc can be detected within relatively short time periods (e.g. Hale *et al.*, 2001a; Ogden *et al.*, 2005; Wisely *et al.*, 2002).

1.2.2.3. MtDNA

Before the advent of microsatellites, mitochondrial DNA (mtDNA) was the marker of choice in molecular genetics. However, although it may not be used as extensively for population genetic studies, it has emerged, due to its relatively slow mutation rate in comparison to microsatellites, as an important marker in the expanding field of phylogeography (Lowe *et al.*, 2004). MtDNA can be obtained from the same sources as microsatellite DNA, however, the large amount of mitochondria present in cells makes it easier to obtain from degraded samples (e.g. museum tissue or hair).

The mammalian mtDNA genome consists of a number of distinct regions; a control region, cytochrome b and a protein coding region. Mutation rates vary among regions (Pesole *et al.*, 1999) and species (Gissi *et al.*, 2000), but overall the control region evolves fastest, and the 12S and 16S RNA genes are the most conserved areas of the mitochondrial genome (Lopez *et al.*, 1997; Pesole *et al.*, 1999). Therefore, most genetic studies involve amplification of DNA from the control region of mtDNA, although high rates of mutation in the cytochrome b region, while lower than those in the control region, also has resulted in that part of the genome being used in molecular studies. Base changes which are transitions, a change from purine to purine (A↔G), or pyrimidine to pyrimidine (C↔T), are more common that transversions, from purine to pyrimidine or vice versa, with ratios of up to 10:1 recorded (Gray 1989) but varying widely between studies (e.g. 2.9:1, Iguchi & Nishida, 2000; 2.29:1, Michaux *et al.*, 2003; 9.5:1, Parsons *et al*, 2002; 6.3:1, Riginos & Nachman, 2001).

Furthermore, as well as a high mutation rate, the inheritance pattern of mtDNA makes it particularly useful in tracking historical population demographics. MtDNA is described as maternally inherited, with the paternal mtDNA not passed on to offspring. This pattern of maternal inheritance makes it an ideal marker to trace colonisation routes of species and also, as mtDNA population size is essentially ¼ that of nuclear DNA, means that population bottlenecks will be detected in the mtDNA genome far more than in the nuclear genome.

Again, mtDNA is traditionally described as a neutral marker however, there is considerable evidence that this is not the case. The structure and replication method of the mtDNA genome means that all genes within the genome are subjected to the same evolutionary forces, with polymorphisms becoming fixed within the genome via genetic hitchhiking with advantageous mutations. Evidence for the non-neutrality of the mtDNA genome has been detected through genetic analysis of humans (Rogers & Harpending, 1992), rodents (Nachman *et al.*, 1994) and *Drosophila* (Ballad & Kreitman, 1994; Rand *et al.*, 1994) (see review in Ballad & Kreitman, 1995). As a result of this problems can arise when using some genetic statistical analyses and Ballad & Kreitman (1995) recommend statistical tests of neutrality are carried out when using mtDNA in population genetic and phylogenetic analysis.

MtDNA continues to be used today to address questions of both population genetics (e.g. Parsons *et al.*, 2002; Randi *et al.*, 2000; Small *et al.*, 2003), and phylogeography (e.g. Brito, 2005; Hale *et al.*, 2004; Piertney, *et al.*, 2005). Increasingly, microsatellite and mtDNA analysis are carried out in tandem, with the different mutation rates and inheritance patterns of the two markers allowing more accurate conclusions to be drawn from interpretation of data sets (e.g. Johnson *et al.*, 2003; Marshall & Ritland, 2002; Rassman *et al.*, 1997).

1.2.3. Ireland's biogeography

1.2.3.1. Postglacial colonisation and origin of Ireland's fauna

Ireland was once an island populated by large mammals, with remains of brown bear, *Ursus arctos*, hyena, *Crocuta crocuta* and mammoth, *Mammuthus primigenius* to name but a few, recovered from caves from around the country (Woodman *et al.*, 1997). However, an ice sheet developed which covered much of Ireland from 25,000 to 13,000 B.P., with the exception of the south and southwest of the country (see Figure 1.1). This coincided

with the disappearance of the majority of the large mammalian fauna of Ireland, although giant deer, *Megaloceros giganteus*, and reindeer, *Rangifer tarandus*, did reappear quite quickly in the country, and it is unclear whether they survived through the last cold period, or somehow rapidly colonised the island after than time (Stuart, 1986; Watts, 1986). Nevertheless, these species were extinct in Ireland by 10,000 B.P. and Ireland's current fauna is largely thought to be derived from colonisation after that time, with genetic analysis finding evidence that only two of Ireland's native mammals, the stoat, *Mustela ermimea* (N. Martínková pers. comm.) and mountain hare, *Lepus timidus* (Hamill, 2002; Hamill *et al.*, 2006; M. Hughes pers. comm.), survived through the last glacial period in Ireland.

A feature of Ireland, both before, and after, the last ice age, is the restricted nature of its fauna, particularly when compared to that of Britain (Savage, 1966; Stuart & van Wijngaarden-Baker, 1985). Currently, of the 24 terrestrial mammalian species (i.e. excluding Chiroptera, Cetacea and Pinnipedia) considered native to Britain, only 10 are classified as native and extant in Ireland (Table 1.1) (Yalden, 1982). The majority of the species present in Britain, but absent from Ireland, are small mammals. Two species of shrew, the mole and three species of vole, are found in Britain, but not found in Ireland, although one has since been introduced (the bank vole). Two mustelids, the weasel and the polecat are also native to Britain and absent from Ireland, and this has resulted in morphological divergence of Irish carnivores, which has been discussed elsewhere (see section 1.2.1.2).

Due to this decrease in diversity from Britain to Ireland, the natural conclusion in the past was the existence of temporary land bridges between Ireland and Britain, acting as a filter, allowing colonisation of the island by some species, and preventing colonisation by others. However, the existence of this landbridge, where and when it might have existed, and what mammals could have crossed it, has been the subject of much debate. Synge (1985) proposed this bridge was positioned between Ireland and Wales, but rising sea levels ca. 12,000 B.P. flooded this bridge, limiting colonisation opportunities by temperate flora and fauna. Conversely Devoy (1985) found little evidence to support the existence of a bridge in this area, and instead proposed that if Ireland and Britain were linked, that the connection was further north between Northern Ireland and Scotland. Later work supported both of these arguments. Wingfield (1995) suggested a temporary landbridge existed across the Celtic sea, which moved northwards up the Irish Sea, while modelling

carried out by Lambeck & Purcell (2001) supported a bridge between southeast Ireland and southern England.

If this landbridge did exist, it was probably partially flooded and/or marshy land (Devoy, 1985; Lambeck & Purcell, 2001), and it is unclear why this habitat type, or the open moorland vegetation proposed by Yalden (1981), would have encouraged colonisation by small mammals such as pygmy shrew while excluding more aquatic mammals (e.g. beaver, *Castor fiber*, water vole, *Arvicola terrestris*), or large, relatively long distance, dispersers (e.g. roe deer, *Capreolus capreolus*, weasels and polecats). Also, morphological investigations found no association between Irish and Scottish mustelids, as would be expected if colonisation of Ireland was via a landbridge between Scotland and Ireland (Lynch, 1996).

Table 1.1. Ireland's native terrestrial mammals (after Yalden 1982). Origins of lineages, where known, and determined through what data type, are indicated.

		Origin	Type of data	Reference
Insectivora				
Erinaceus europaeus	Hedgehog	Britain/France	Genetic	Seddon *et al.*, 2001
Sorex minutis	Pygmy shrew	Lusitanian	Genetic	Mascheretti *et al.*, 2003
Lagomorpha				
Lepus timidus	Mountain hare	Preglacial	Genetic	Hamill, 2002; Hamill *et al.*, 2006; M.Hughes, pers. comm.
Rodentia				
Sciurus vulgaris	Red squirrel	Unknown	Morphology	Lowe & Gardiner, 1983
Carnivora				
Vulpes vulpes	Red fox	Mixture	Genetic	C. Edwards, pers. comm.
Mustela erminea	Stoat	Preglacial	Genetic	N. Martínková, pers. comm.
Martes martes	Pine marten	Lusitanian	Genetic	Davison *et al.*, 2001
Meles meles	Badger	Lusitanian	Genetic	C. Edwards, pers. comm.
Lutra lutra	Otter	Unknown	Morphology	Lynch, 1996
Artiodactyla				
Cervus elaphus	Red deer			

1.2.3.2. Ireland's forests – historic data

Although there is evidence for interstadial flora, including some tree species, in Ireland (Watts, 1985), and it has been suggested that Scot's pine may have survived in Ireland through the last cold period (Sinclair *et al.*, 1998), it is generally thought that Ireland's current tree diversity is a product of postglacial colonisation after the last glacial maximum, and human interference after that time (Coxon, 2005; Mitchell, 2006). As already discussed, the actual mechanism of this postglacial colonisation of the country is unclear, but if forest colonisation was *via* a landbridge, it may have resulted in colonisation of the island by temperate forest species. Although there is no evidence to support the latter, genetic investigations, and isochrone maps have found associations between Iberian and Irish tree lineages, and evidence that these trees colonized Ireland around the time the landbridge proposed by Wingfield (1995) existed (Mitchell, 2006). Although it may not be as clear cut as trees colonising a landbridge and fauna following behind, as an important habitat for many mammalian species in Ireland (e.g. pine marten, red squirrel, woodmouse), not to mention avian and invertebrate fauna, an understanding of distribution and changes in species composition of Ireland's forests is essential in understanding the distribution of diversity of the species which inhabit them.

Prehistoric forests began appearing in Ireland after the end of the last ice age around 8,300 B.C. (Fitzpatrick, 1965; Kelly, 1975), and consisted largely of bushy vegetation, and some tree species; birch, *Betula* sp., hazel, *Corylus* sp., oak, *Quercus* sp. and pine, *Pinus* sp. (Fitzpatrick, 1965). By 5,000 B.C. the country was almost completely covered in a mosaic of different forest types, with broadleaf species like oak and birch in the lowlands and valleys, while wetland species like alder, *Alnus* sp., and willow, *Salix* sp, grew near lakes and rivers (O'Carroll, 1984).

The earliest human settlement in Ireland has been dated at 5,725 B.C. and the arrival of humans, probably from the Iberian peninsular (Hill *et al.*, 2000), together with a deterioration of climate, resulted in a gradual decrease in forest cover, and the subsequent stimulation of bog formation, over the next 5,000 years (Kelly, 1975; O'Carroll, 1984). Although 2,000 years after humans arrived on the island, forest cover was relatively undisturbed (Neeson, 1991), and remained that way throughout much of the Bronze Age, by 550 B.C., the wetter and cooler weather, and increased agriculture, resulted in the disappearance of many forests from the west of Ireland. It is around that time that Scot's

pine may have completely disappeared from the island; although there is the possibility that relic stands survived (Fitzpatrick, 1965), and there are records of pine in the mountains of Kerry, and west of the Shannon until 1800 A.D. (Henry, 1914; a map of Ireland's counties and other areas referred to in the text is given in Appendix 8.1). Similarly, the extent of ash, *Fraxinus* sp., and elm, *Ulmus* sp., in forests decreased drastically around that time, with oak and hazel emerging as the dominant tree species within the landscape. Broadleaf species remained dominant in the Irish forests from that time until the conifer plantations in the 20th century.

The Iron Age in Ireland is dated at around 400 B.C. and coincided with a halt of forest clearance for agriculture, possibly as a result of invasion and warfare (Kelly, 1975), and this lull continued until around 300 A.D. when attacks on secondary stands of forest resumed. During Ireland's Pre-Norman (500 - 1,200 A.D.) era the greater part of Ireland's population resided in the east, on the Kildare plain or the river valleys of the Liffey, Slaney and Barrow. Trees were jealously guarded at this time and, in the *Bretha Comaithchesa,* the Laws of the Neighbourhood, dating from 700 A.D., 27 trees and shrubs are listed along with penalties for cutting or harvesting woods from the species listed (Kelly, 1975). There are no comprehensive records of woodland distribution at this time, although, as many Irish place names date from between the 5th and 12th centuries, it could be presumed that any townland or parish with a name including *coil/cill/kill, fidh, ross* (all meaning woodland), *doire* or *derry* (oakwood), were wooded during the early medieval period (McCracken, 1971). Examination of the distribution of such townland names in Ireland today, with 574 parish names and 3375 townland names containing the prefix or suffix cill/coil, and 1600 doire or derry townlands (O'Connor, 2001) countrywide, leads to the conclusion that forest distribution at that time was probably substantial.

In 1160 A.D. the Normans arrived in Ireland and their buildings, land, social and religious practices had a lasting effect on the Irish landscape. Norman control was primarily based in Munster and Leinster, with most of the remoter areas of Ireland, particularly west of the River Shannon, left alone. The Normans introduced a manorial system of agriculture in Ireland that involved a three part crop rotation of corn, oats and fallow in winter, spring and summer/autumn respectively which resulted in the clearance of many forests (Otway-Ruthven, 1968) and, although in the 13th century Gerald of Wales described an island of '*many woods and marshes*' in his travels of Ireland (O'Meara, 1982: p34), this has been

hailed as propaganda, written to encourage further recruits to the invasion, and in reality, forest cover was in no way as extensive as it once was, particularly in the areas around Dublin where agricultural practices were more intensive. There are records of large areas in Wicklow, Wexford and Dublin being clearfelled around that time (Forbes, 1932) but by 1488 the borders of Dublin had no forests at all (Lawlor, 1908).

The Tudor period began when Henry VII became King of England. He initiated a plantation system whereby Gaelic and Anglo Irish lords were granted acreage outside of the Dublin/Kildare area and given power to rule the areas in Henry's name. In 1556 A.D. Mary Tudor initiated a second plantation scheme and records of Irish forest distribution improve from this time. The first detailed topographical map of central Ireland, including details of forests, rivers, mountains and bogs, was produced in 1563/65 to assist this plantation scheme (Fuller, 1990).

The 17th century in Ireland saw the arrival of the industrial revolution and clearing of forests for charcoal works. In 1600 woodland cover varied from area to area. Planters in Donegal had to import timber from Derry, implying there was not a lot of forested land in the northwest of the county at the time. However, further south, and in the western and southwestern counties, woodland cover was more extensive. Sligo's Loughs Gill, Arrow and Key were surrounded by woodland, likewise the shores of Mayo's Lough Mask was forested and these hazel and alder woodlands extended northwards to Castlebar, Co. Mayo, while oak grew between Lough Mask and the foot of the Partry Mountains. In Galway it was said that one could walk on the tops of the trees from Letterfrack to Galway town (McCracken, 1963).

The end of the 17th century saw Ireland with the most restricted forest distribution since prehistoric times. The most extensive woods were in the northwest (Co. Sligo) and southwest of the country. The woodlands along many of the river valleys had been cleared for timber, central Ireland was almost treeless, and the largest and densest areas of woodland were along the upland river valleys and the mountains. There were large woodlands northwest of Lough Neagh, in the Erne basin, along the River Shannon, and other river valleys in the west and south, and also on the eastern slopes of the Wicklow and Wexford hills (McCracken, 1971).

During the 18th century planting by many of Irelands gentry became popular and a lot of estate planting occurred during this time and it has been called Irelands 'planting age' (Forbes, 1933). Coppicing was a common woodland practice at this time and many detailed records exist on the practice of coppicing on woodlands in Wicklow (Edlin, 1970; Jones, 1986; Quinn, 1994). At the end of the 1700's estate planting covered 52,728 ha, and although conifers were once more appearing in the Irish landscape, broadleaves remained dominant with only 1,120 ha of forested land under conifer species (McCracken, 1971). The first editions of the Ordnance Survey maps of Ireland were produced in the 1840's, therefore conclusively mapping the distribution of forests countrywide for the first time. In 1890 the first state forest was planted in Knockboy, Carna, Co. Galway (Durand, 1979), and the change from forests dominated by broadleaves, to coniferous species, began.

In 1903 the Forestry Branch of the Department of Agriculture and Technical Instruction was established in Avondale, Co. Wicklow (Fitzpatrick, 1975). During the first 25 years of its existence, under the leadership of Arthur Charles Forbes, 26,900 acres (10,760 ha) of forestry was planted. By the outbreak of the Second World War this had reached 8,000 acres (3,200 ha) a year (McCracken, 1971). By 1950, Irelands forest cover had increased from 1.4% forested in 1920, to 2% (Freeman, 1950). After 65 years of state planting in 1968, 200,000 hectares of land were acquired for forestry in the Republic of Ireland, and another 24,000 hectares were under private ownership (McCracken, 1971). 96% of all forest plantings in the Republic were sitka spruce, *Picea sitchensis*. In 1979 state planting had increased to around 10,000 ha per annum with 268,000 hectares in total forested (McEvoy, 1979).

1.2.3.3. Ireland's forests – current data

The forests which currently cover 7% of land area in Ireland, the lowest of any EU country (Aalen *et al.*, 1997), bear little resemblance to the forests of the past, with only 1% of land area covered in broadleaf species (Figure 1.3). Forests in Ireland occur in National Parks, private land, with by far the largest area in state ownership (79% in 1985, Review Group on Forestry, 1985 in Higgins *et al.,* 2004) by Ireland's forestry board, Coillte Teoranta (hereafter referred to as Coillte). Within the National Parks, nature reserves and in private ownership, some remnant stands of old and/or native and semi-native species survive, (Higgins *et al.*, 2004), but as the state forest scheme was established 50 years ago, the

majority (88%) of the forests under state ownership are around 50 years old (Garrett, 2001) and currently, 69% of state forest is sitka spruce, (Coillte, 2005).

The status and extent of forests in Ireland which consist of native Irish tree species was recently assessed by Higgins *et al.* (2004) and they found 1% of land area was forested in native species, mostly ash and oak, but only 5% of these stands were larger than 20ha. A similar survey undertaken by Garrett (2001) on the Coillte estate revealed that 6% of current Coillte forest cover was 'Old woodland', defined as continuously forested since 1850, and a further 4% were plantations older than 50 years of age. Although over 60% of the area of these old woodlands were coniferous, they did have a high proportion of broadleaf species (14%), higher than the Coillte national average, but only 3% of these forests were larger than 100 ha in size. Therefore, large areas of old forests, and particularly old forests consisting of native species, are scarce in the Irish landscape. Of note are areas in counties Waterford, Clare, Sligo, Galway and Wicklow (Garrett, 2001; Higgins *et al.*, 2004) as well as the large areas in Killarney National Park, Co. Kerry and Glendalough, Co. Wicklow.

Nevertheless, the predominant tree species in Ireland remains sitka spruce (Figure 1.3). Although a good forest crop, it has been recognised as a poor species for biodiversity conservation at the floral, invertebrate (French *et al.*, 2005) and vertebrate (e.g. Reilly, 1997) level. Over the last 13 years Coillte has increased diversification of planting within its forests, reducing the planting of sitka spruce by 20%, and increasing planting of broadleaves, with current levels at 4% of total forest area and a total 15% of Coillte's forests being managed for biodiversity conservation (Coillte, 2005).

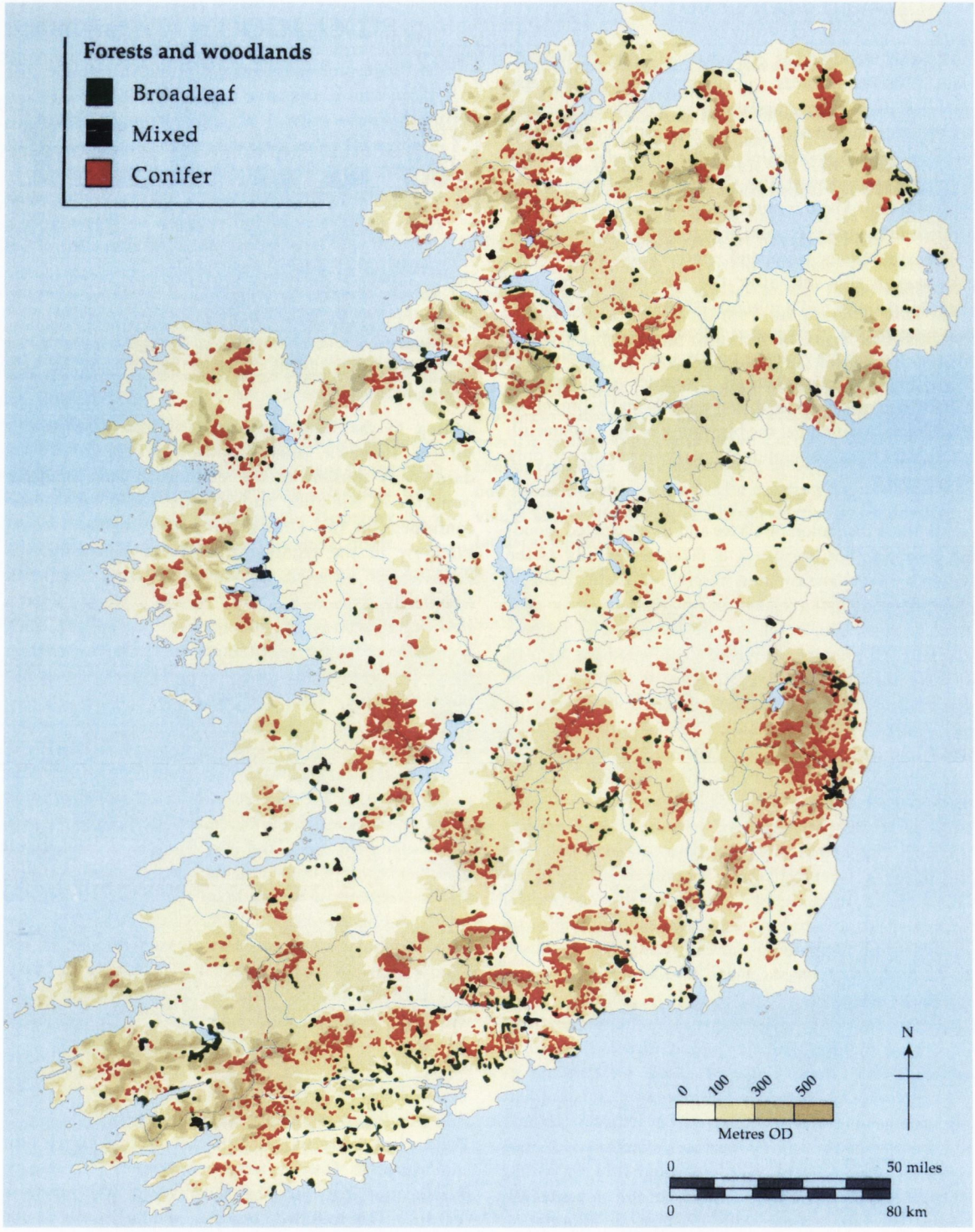

Figure 1.3. Topographical map showing distribution and composition of Ireland's forests (after Aalen *et al.*, 1997). Conifer species are dominant in Ireland's landscape although a number of broadleaf and mixed forests are found in the southwest, south and central parts of the country.

1.2.4. The Eurasian red squirrel

The Eurasian red squirrel is a member of the order Rodentia, a group of mammals characterised by their dentition, with a single pair of incisors in each jaw. The rodent's generalized body plan has allowed their colonisation of almost every land surface on earth and the Family Scuiridae, part of the sub-order Sciuromorpha are found worldwide, with the exception of Australasia. The Eurasian red squirrel is the most widespread of these Sciurid species, found across the Palaearctic (Figure 1.4), with from 17 to 40 subspecies described, based on pelages (Sidorowicz, 1971), which have been attributed to environmental variation (Sidorowicz, 1971; Voipio, 1969; 1970).

It shares its range with one other native squirrel species, the Persian squirrel, *Sciurus anomalus*. Although the Japanese squirrel, *Sciurus lis*, is also found in the Palaearctic, it does not occur on the same island as the red squirrel (Lurz *et al.*, 2005), and in fact genetic evidence has revealed that *S.vulgaris* and *S.lis* may be the same species (Oshida & Yoshida, 1997). Recently, numerous introduced competitors, most notably the grey squirrel, but also two species of *Callosciurus*, are subjecting the red squirrel to increasing resource competition within Europe (Bertolino *et al.*, 1999; Mitchell-Jones *et al.*, 1999).

The species remains common throughout much of its range, and in some areas, is considered a forestry pest and a game animal. However, due to the introduction of the grey squirrel it is of conservation concern in Britain and Ireland, protected under national legislation, and more recently, in northern Italy, although no legislation protects the red squirrel in Italy, or internationally, on a European scale.

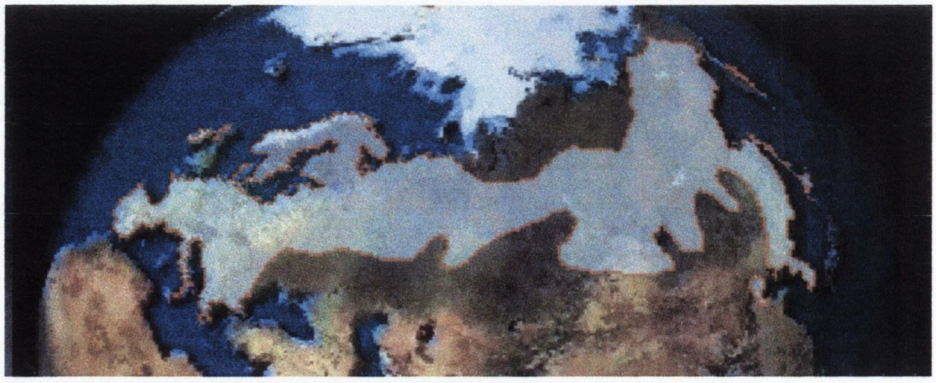

Figure 1.4. The distribution of the Eurasian red squirrel (after Gurnell, 1987). It is the most widespread of the *Sciurus* sp., its range extending from Ireland in the west to Japan in the west.

1.2.4.1. General biology and ecology

The Eurasian red squirrel is a relatively small *Sciurus* with body length of around 450 mm, including the tail (Verboom & von Apeldoorn, 1990), and an average weight of 300g (Dickinson, 1995; Tittensor, 1975). Coat colour can vary from light russet through to dark red, brown and even black, with the ventral area a pale cream colour (Verboom & von Apeldoorn, 1990), although as already previously mentioned, coat colour varies extensively throughout its range (Sidorowicz, 1971).

Across much of its range the species occurs in boreal coniferous forests, but it is found in broadleaf and mixed forests in central and southern Europe (Lurz *et al.*, 2005), and Ireland (Reilly, 1997). Foraging behaviour varies between site types, with squirrels in conifer sites spending much of their time foraging in the canopy (Gurnell, 1987), and ground foraging occurring in the seed depauperate summer months (Wauters *et al.*, 1992). In sites where there is an element of broadleaf species canopy and ground foraging occur in almost equal measures, showing no seasonal variation (Holm, 1990). Although primarily granivorous, red squirrels exhibit the typical generalist rodent diet, particularly when seed crops are scarce, whereby diet is supplemented with green plant material, flowers (Wauters & Dhondt, 1987), buds (Kenward & Holm, 1993), fruit, berries (Moffart, 1923a), fungi, mosses (Moller, 1983), insects (Wauters & Dhondt, 1987) and even animal matter, including eggs, nestlings, carrion and shed deer antlers (Dickinson, 1995; Gurnell, 1987).

Although red squirrels are generally not territorial, they do hold home ranges, with the male home range about five times bigger than the female's (Andrén & Delen, 1994) and overall sex ratios in forests are approximately 1:1 (Lurz *et al.*, 2005). Reproduction and fecundity is intrinsically linked to food availability, with females having to reach a threshold weight of ca. 300g to enter oestrus (Lurz, 1995; Wauters & Dhondt, 1989a), and need to maintain a high body weight throughout lactation to successfully raise a litter (Wauters & Dhondt, 1989a). In a good seed crop year red squirrel females can raise two litters, one in late spring and another in late summer (Nixon *et al.*, 1975), while in a poor seed year only one litter, if any, will be raised. This dual breeding season in red squirrels allows the species to take advantage of mast years and bumper seed crops, and also compensates for the high mortality in juvenile squirrels, whereby it is likely that only two young from each litter will survive to reproductive age (Wauters & Dhondt, 1995). It also allows the species to recover relatively quickly from population crashes or other

demographic bottleneck events, with populations recorded to recover from extremely low densities in as little as 3 years (Gurnell, 1991).

Survival rates of young squirrels are quite low (ca. 20%), but survival increases each year to around 50%, and life expectancy in the wild at 6 months of age is approximately 3 years (Gurnell, 1991). Mortality is influenced by food availability, weather, parasitism, predation and human interference such as habitat fragmentation and road deaths, and the grey squirrel does not directly cause mortality (Lurz *et al.*, 2005).

1.2.4.2. The red squirrel in Ireland

Although the red squirrel is considered indigenous to Ireland (Moffart, 1938), this is based on references to the species in the literature, rather than any archaeological evidence (Stuart, 1982). It is frequently citied that the first reference to the red squirrel in Ireland occurs in the ancient Irish myth *Táin Bó Cuailnge* (The Brown Bull of Cooley), where the hero Cú Chulain kills the two pets of Queen Meabhdh, a hind and a squirrel, as they are sitting on her shoulder. However, the word actually used in the passage is *togmall,* diminutive *togmallán* and *togán*, which are more similar to the old Irish word for marten or ferret, *taghan*, (Eds, 1923), than it is to any of the Irish/Gaelic words which have been described for the red squirrel (feoróg, ora, iora, ir; Scharff, 1922).

Therefore, Augustin possibly made the first reference to the red squirrel in Ireland while writing of his travels around the country in the 7th century (Reeves, 1861), although, again, difficulties in translation make this reference far from definite, with the word *sesquivolos* thought to mean squirrel. There are also a references to squirrels in an old Irish saying, whereby the historical extent of Ireland's forests were described by a squirrel being able to traverse the length of the country without ever touching the ground (Abbott, 1922; McCracken, 1971).

More reliable references to the species occur from the 14th century onwards. Barrett-Hamilton and Hinton (1910-21) wrote of taxes charged on squirrel skins exported from Wexford in the 13th century, and, in 1430, the 'Libel of English Policie', a list of Irish fur bearing mammals, includes a reference to the squirrel (Scharff, 1922). Le Fanu (1922) also found a reference to the squirrel in the book of 'Rates Outward' in 1622, indicating the continued export of squirrel skins up to that time.

However, after this time, it was generally thought that the red squirrel became either extremely rare, or extinct in Ireland, due to extensive tree felling for use in charcoal works (McCracken, 1971), and/or hunting for fur, resulting in its notable absence from export lists (Fairley, 1983). Nevertheless, there are some references to the species in the country up until the late 18th century. O'Flaherty mentions red squirrels in his travels of West Connaught in the late 17th century (O'Flaherty, 1846). There are also references made to squirrels as examples of 'tree climbing animals' in Stringer's '*The Experienced Huntsman*' originally written in 1714 (Fairley, 1977), and they are mentioned as still common countrywide as late as 1739 (K'eogh, 1739 in Barrington, 1880), and present in Luttrelstown, Co. Dublin as late as 1772 (Rutty, 1772 in Scharff, 1922).

However, after this time references to the species did disappear, with the species only reappearing on the export lists in 1829 (Fairley, 1983). There were numerous translocations from Britain to Ireland during the 1800's (Barrington, 1880). A total of ten translocation points were recorded (Figure 1.5), with the majority of them located in the east and centre of the country. Unfortunately, the source and numbers of these introduced red squirrels are largely unknown. In the Carlow centre 'Colonel Bruen introduced some squirrels', the individuals introduced into Offaly 'come from a few brought...from Yorkshire and Sussex', those let loose in Galway by Lord and Lady Clancarty were 'two to four couple of squirrels [were] obtained in London' the only exact figures being the ten and fifteen adults released in Ravensdale (Co. Armagh) in 1851 and 1856 respectively.

The subsequent rapid spread of the red squirrel has been attributed to lack of intraspecific competition from native red squirrel populations (Hamilton, 2006), but equally could be a result of convergence between squirrels from the translocation points with those from remnant populations. There was considerable debate in the literature at the beginning of the 20th century as to whether any remnant Irish red squirrel populations were present in the country at the time of the translocations. Barrington (1880) argued that '*no-one would take the trouble to introduce an animal if it were already in existence in the country*', however, Scharff (1922) countered with '*it is just the people that thoughtlessly introduce animals where they think they do not exist, who frequently are ignorant of the fauna of the country they live in*'. It was accepted that, if these remnant populations did survive, they would have been located in the south and west of the country (Moffart, 1923b; Scharff, 1922, 1923), where human presence was not as prevalent and where large stands of old forests persisted, and where, therefore, red squirrel populations could have remained

undetected and/or unreported until the translocations resulted in either increased sightings, or more likely, increased awareness, of the species. Therefore the red squirrel population in Ireland may be the product of two separate lineages; those present in the country prior to, and during the translocations, and those which were translocated into the country, and it was Scharff (1922) who first suggested trying to determine whether this is indeed the case through morphometric investigation of the Irish red squirrel population.

After the translocations there were numerous reports from various parts of the country referring to the distribution of the squirrel (Abbott, 1922; Barrington, 1880, 1915; Ruttledge, 1924) however the first thorough nationwide survey was carried out by the National Parks and Wildlife Service (NPWS) in 1968, and again in 1973, with both surveys recording the species as widespread in the country. However, the most recent distribution survey (Figure 1.5; Ó Teangana *et al.*, 2000) found that, although the species remained widespread, its range had contracted considerably.

Although the survey techniques employed varied, and its is likely that the most recent survey was the most accurate of the three, as it combined information from both professional and amateur sources, it is unlikely that the large change in distribution was not a result of these different survey techniques, but instead, could largely be attributed to the spread of the introduced North American grey squirrel (Figure 1.5).

Figure 1.5. Distribution of the red (left) and grey (right) squirrel in Ireland mapped after the last distribution survey in 1997 (Ó Teangana *et al.*, 2000). The translocation points of the red and introduction point of the grey squirrel are shown. Red squirrels have disappeared from the centre of the country, coinciding with the areas in which the grey squirrel has been present the longest.

The grey squirrel was first introduced into Ireland at Castle Forbes, Co. Longford in either 1911 (Moffart, 1938; Watt, 1923), or 1913 (Deane, 1964). Later introductions were recorded in 1938 in Co. Longford (Lever, 1977 in Ó Teangana *et al.*, 2000) and in 1928 in Co. Dublin (Deane, 1964), although the accuracy of these records and/or the success of these introductions are debatable, especially regarding the latter record, as grey squirrels have only been recorded in Dublin relatively recently. Since that time, grey squirrel spread has been rapid, with its range extending north and southeast, while its westward and eastward spread, up until recently, was impeded by the River Shannon and the Wicklow mountains respectively (Figure 1.5).

The possible mechanism of replacement of the red squirrel by the grey has been well researched in Britain and, although the exact method is unknown, it is thought that it is a combination of resource competition (Kenward & Holm, 1993; Wauters *et al.,* 2000), and disease (Sainsbury *et al.,* 2000; Tompkins *et al.,* 2003). However, it has been suggested that habitat management may play a role, with grey squirrels possibly having a competitive advantage in forests which had large seeded broadleaf species, grey squirrels being able to digest tannins in unripe seeds which red squirrels cannot (Kenward & Holm, 1993), and red squirrels perhaps having the edge in conifer forests, where their lighter body weight allows them to access seeds in areas of the canopy which grey squirrels cannot reach (Lurz *et al*, 2005).

Although there has been very little research on red squirrels in Ireland, it is believed that a similar process is occurring here as in Britain, although the role of disease in replacement in the Irish population is unknown, as there are no definite records of parapoxvirus in the red or grey squirrel in Ireland, but samples are being tested in an attempt to rectify this (M. McGoldrick pers. comm.). Research in Ireland has focused on distribution surveys (Lawton & Rochford, 2000; O'Neill & Montgomery, 2003; Ó Teangana *et al.*, 2000), ecology and habitat management (Hamilton, 2006; Reilly, 1997) of the red squirrel, and the use of translocations to conserve the species (A. Poole pers. comm.), together with investigations of population genetics (M. McGoldrick, pers. comm.) of, and forestry damage (Lawton, 2000) by, the grey squirrel. However, no studies have investigated the two species in sympatry in Ireland, and there is some evidence that the Irish situation may not mirror that in Britain, with recent reports emerging that red squirrels have replaced grey squirrels in some areas (G. Hamilton pers. comm.), and grey squirrels are no longer

common in what was once the centre of their range (Castle Forbes, Co. Longford; M. McGoldrick, pers. comm.).

The red squirrel in Britain and Ireland is considered a separate subspecies, *Sciurus vulgaris leucourus* Kerr, 1972, based on fading of the ear tufts and tail in summer from a darker coat pelage to cream or white, known as bleaching (Harvie-Brown, 1880-81; Sidorowicz, 1971; Plate 1.1). Although recent morphological investigations have revealed that this colour morph is no longer common in Britain (Hale & Lurz, 2003), only anecdotal evidence of the extent of this phenomenon in Ireland exists (Barratt-Hamilton & Hinten, 1910-21).

Plate 1.1. Illustration by E.A. Wilson showing the typical light tail described for *S.v.leucourus* (after Wilson & Wilson, 2004). Individuals 2 – 5 show the complete bleaching of the hair while individual 1 has a bleached tip and 6 has a bleached tail with a dark dorsal line. Individual 7 has very little bleaching of the tail, with only the tips of the hairs lighter in colour than the rest of the tail.

1.2.5. Summary

Distribution and diversity within species is a product of both historical and current events and biogeography and, increasingly, human interference. Although basic knowledge of ecology and behaviour of species is invaluable, more in-depth knowledge of the more cryptic factors which determine the insularity of a population, and microevolution within species, are needed to greater understand current biodiversity, but also, to best conserve species, when conservation is needed.

The Eurasian red squirrel is a widespread and morphologically divergent species which has been researched extensively, but no in depth investigations of the factors which have influenced this diversity have been carried out. The Irish red squirrel population is a one which can be used to attempt to answer some of the unanswered questions about red squirrel diversity, and this study aims to do this, through utilisation of molecular and morphological data.

As an isolated island population, the Irish population is ideal for the investigation of the factors and barriers which determine gene flow between populations, as island biogeography removes the possibly confusing effect of continual gene flow into the population, therefore adding to the information already provided by Hale *et al.* (2001a) and Trizio *et al.* (2005). As a population at the edge of the species range, through combination of data with previous research, it may be useful in determining postglacial colonisation routes of the species. Also, as a population which has been subject to, within the last century, two of the driving forces behind microevolution in species; competition and habitat change, it can be used to interpret both the ability of, and speed at which, red squirrels can evolve in the presence of selective pressures, and therefore better understand the current distribution of diversity within the species, and the ability of the species to survive into the future, either in tandem with continual habitat change, or through rapid microevolution in response to conservation measures.

Finally at a more localised scale, the results of this study may be useful in determining the postglacial colonisation patterns of both Ireland's forests, and its temperate species, as well as possibly alternative sources of Ireland's current diversity. Also, genetic and morphological analysis combined will hopefully be able to determine whether any remnant lineages of Irish red squirrels have survived in the country in the face of the translocations from Britain.

Chapter 2: Sample collection

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2.1. Introduction

This study aims to address a number of questions relating to the factors which shape the range, and distribution of diversity within, the Eurasian red squirrel. The sampling strategy was designed to encompass the geographical range of the red squirrel in Ireland, within which a more restricted range of ecological and temporal variation in the red squirrel population could be investigated. Two habitats were chosen as representative of the major habitats of the red squirrel in Ireland; conifer and missed conifer/broadleaf ('mixed') forests. These forests were further classified into old (continuously forested since 1850) and new (planted since 1950). It was hypothesised that if any remnant red squirrel populations, or their descendents, survived in Ireland, they would have survived in 'old forests' which had been continuously forested since before the translocations, thus adding a possible temporal range to the data collection. Trapping samples were augmented with cadaver and museum specimens, with the museum samples adding a further temporal element to the sample collection.

2.2. Trapping

Trapping was carried from May 2003 to August 2004, in both conifer and mixed forests around Ireland. These sites were further classified into old and new forests. In order to maximise the range of samples, it was decided to limit the time spent trapping in any one site to seven days, or until 10 squirrels had been caught in the site (whichever period was shortest).

2.2.1. Study site selection

Study sites were selected using a number of different methods. New forests were identified from current Ordnance survey maps (Discovery Series, 2nd Edition, 1999; 1:50,000cm), while possible old sites were chosen after an extensive literature search and examination of first edition (1833-46) Ordnance survey maps of Ireland (6 inch maps, 1:10,560 inches). Although there is no definitive inventory of the locations of old forests in Ireland, numerous sources give detailed descriptions of the locations and distribution of old forests in the country at various points in history (Table 2.1; 2.2). The locations of these old sites were then compared to Coillte records (Garrett, 2001) and mapped onto current Ordnance survey maps to construct a list of possible old study sites in Ireland. A list of possible sites, old and new was then complied and sites were grouped into separate Forest Management Units (FMU) for Coillte employees, and into separate regions for

National Parks and Wildlife (NPWS) staff. These lists were then sent to the respective employees in each FMU/region, with a request to identify any sites which had red squirrel populations resident in them.

Table 2.1. Primary literature sources which mention the locations of woodlands in Ireland and which were used, in conjunction with current OS maps, to identify old forests.

Year referred to	Area	Reference
1000 - 1700	Co. Clare	Westropp (1909)
1100 - 1600	Leinster	Hore (1856-57)
1100 - 1600	Ulster, Muster, Connaught	Hore (1858)
1200 - 1600	Ireland	Forbes (1932)
1600	Ireland	McCracken (1963)
1600 - 1800	North Leinster	McCracken (1969)
1600 - 1840	Donegal	McCracken (1958)
1600 - 1944	Ireland	McEvoy (1944)
1636-1703	Mayo, Galway, Clare	Simmington (1956, 62, 67)
1672 - 1933	Ireland	Forbes (1933)
1702	West Cork	Hodson (1902)
1709	Connaught	Molyneux (1709)
1800	Brackloon, Co.Mayo	Farrell *et al.* (1993)
1850 - 1980	Ireland	O'Carroll (1984)
1900	Galway, Wicklow, Clare	Henry (1914)
Ancient-Present	Central Ireland	Fuller (1990)
Ancient-Present	Raheen, Co.Clare	Leavy (1992)
Medieval	East	Rackham (1995)
Medieval	Munster	Tierney (1998)

Table 2.2. Old maps which depict the locations of woodlands in Ireland as described in McCracken (1959) and Petty (1687).

Year	Name	Reference
1580	The description of the barony of Idrone	
1580	Munster	
1580	Map of the coast of Ireland from Knockfergus to Dundrum	
1585	Mayo. J. Baptiste	
1587	Sligo and Mayo. J. Browne & J.Baptiste	McCracken
1587	Limerick. F.Jobson	(1971)
1587	Munster; Kinsale to Dingle	
1590	County Monaghan. J.Browne & J. Baptiste	
1590	Belfast Lough	
1610	Plott of Ireland, J.Norden from Boazio	
1685	Hiberniae delineatio; Maps covering all Ireland	Petty (1687)

From the sites where red squirrel presence was confirmed, potential trapping sites were chosen, and, when a large number of squirrel positive sites were identified within a single region, a subset of sites was chosen for trapping, based on proximity to other sites (sampling a broad region was given priority), and site size (larger sites would be more likely to have resident squirrel populations while smaller sites may be 'squirrel positive'

based on sightings of transients (Hamilton, 2006)). The final list of 27 sites is given in Table 2.3 and their locations are shown in Figure 2.1.

As squirrel signs are difficult to locate in forests which have a large proportion of broadleaf tree species (Gurnell, 1987, i.e. the mixed sites), positive confirmation of red squirrel presence in those sites was based solely on Coillte/NPWS reports, however all conifer sites which had had red squirrel presence reported in them were personally checked to confirm the existence of red squirrels in those sites. It was envisaged that on average samples from 5 squirrels would be collected from each site, therefore producing a total sample, from trapping, of 135 individual squirrels.

Table 2.3. Trap sites identified from the NPWS, Coillte and personal surveys. Descriptions of these sites are given in Appendix 8.2. The number of squirrels trapped in each site is also given.

County	Grid Ref	Site ID	Wood	Old/New	Species	Size (ha)	No. of Squirrels
Clare	R490600	1	Cratloe	Old	Conifer	22	-
	R720863	2	Lakeside	Old	Mixed	30	2
	R650830	3	Raheen	New	Mixed	100	-
	R570750	4	Violet Hill	Old	Mixed	85	-
	R690870	5	Woodpark	New	Mixed	22.7	-
Cork	W960720	6	Castlemartyr	Old	Mixed	58	-
	W825965	7	Corrin	New	Conifer	100	8
	W760620	8	Curraghbinny	New	Mixed	35	-
	W990780	9	Glenbower	New	Mixed	83.5	-
	W870660	10	Rostellan	Old	Mixed	70	-
Galway	M420030	11	Garryland	Old	Conifer	600	-
	M420195	12	Kilcoran	New	Mixed	100	-
	M660460	13	Mountbellew	New	Conifer	240	-
	M830030	14	Portumna	Old	Conifer	300	-
Kerry	V810005	15	Kilderry	New	Mixed	30	-
	V940860	16	Killarney National Park (KNP)	Old	Mixed	25	7
Sligo	G618273	17	Glenwood	New	Conifer	40	-
	G723345	18	Hazelwood	Old	Mixed	200	-
Waterford	X205970	19	Colligan	New	Conifer	35	-
	X020998	20	Lismore	Old	Mixed	40	-
	S460140	21	Portlaw	Old	Conifer	100	-
	X365982	22	Stradbally	Old	Mixed	25	-
Wexford	T050550	23	Camolin Park	Old	Mixed	174.9	2
Wicklow	T115975	24	Glendalough	Old	Conifer	25	6
	O270125	25	Kindelstown	New	Mixed	50	-
	T150975	26	Laragh	New	Conifer	75	2
	T162962	27	Troopserstown	New	Conifer	56	1

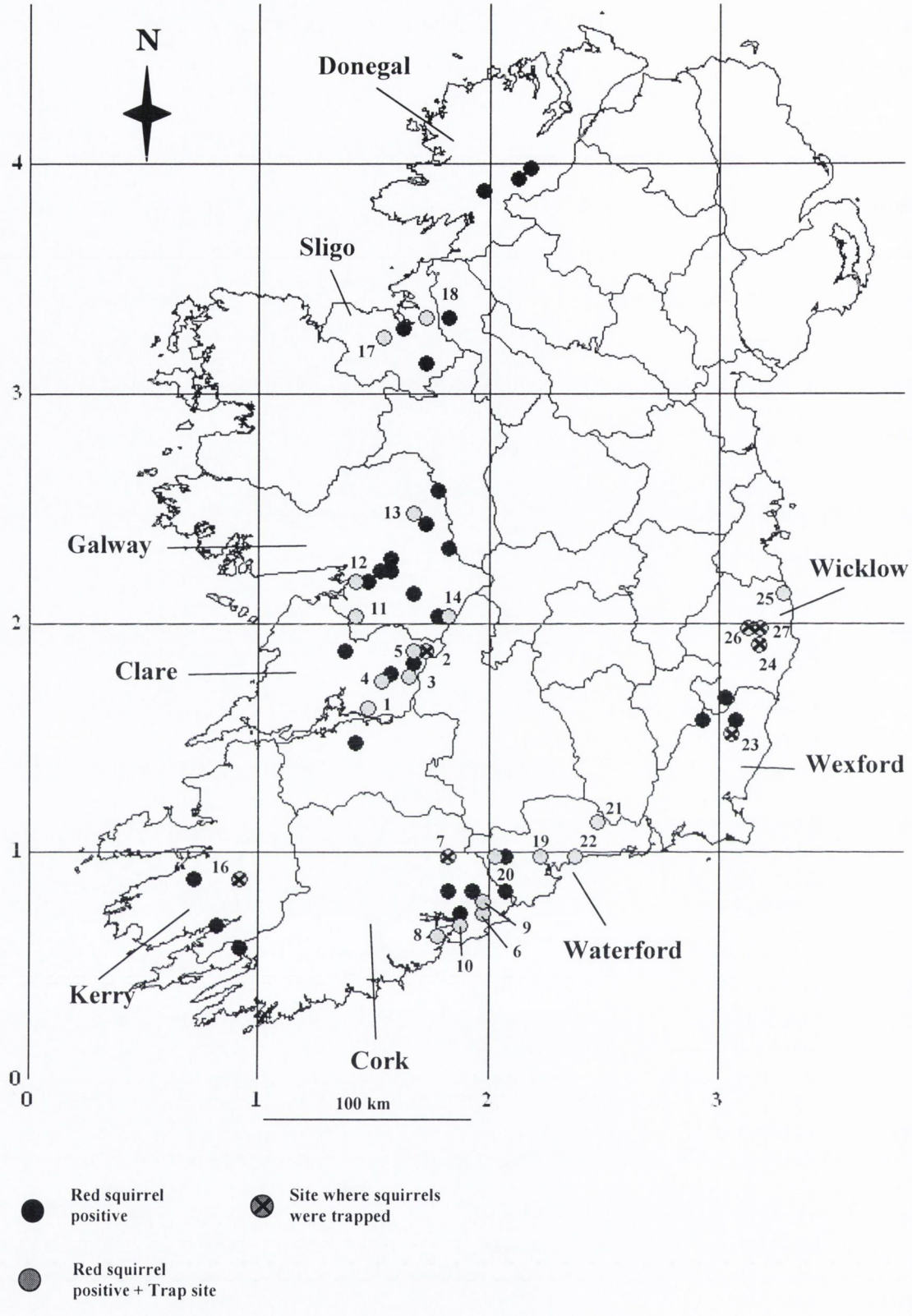


Figure 2.1. Location of red squirrel positive sites and chosen trap sites around Ireland. The ID numbers beside each trap site correspond to those given in Table 2.3.

2.2.2. Trapping regime

The traps used were modified cage mink traps onto which a wooden nest box was attached (Holm, 1990; Tonkin, 1983). The back of the wire mink trap was removed allowing the trapped squirrel access to the nest box for shelter (Plate 2.1). The design of the nest box was slightly modified from that used by Reilly (1997), in that the back of the nest box was removable, allowing easier access to the squirrel in the trap. All wood was treated with non toxic wood preservative prior to assembly. Traps were attached to tree trunks at shoulder height using a thin nylon rope tied with a taut-line hitch (Hamilton, 2006).

Plate 2.1. Trap in position on tree trunk. The treadle is in the 'set' position and bait is visible in and on top of the trap. The self tightening knot prevents it from slipping down the tree trunk.

The trapping regime consisted of a prebaiting period, whereby traps were locked open and baited, and a trapping period. The bait used was a 3:1, peanut:sunflower seed mixture. During prebaiting, bait was scattered around the trap on the forest floor, on the roof of the nest box, at the opening of the trap and within the trap itself, behind the treadle. During trapping, bait was placed within the trap, with a small amount on the roof of the nest box to act as an attractant.

The length of the prebaiting period varied from 4 to 14 days. During prebaiting, traps were visited once a day and the bait replenished when necessary. Due to the time constraints of this study, trapping commenced in each site as soon as evidence of squirrels at the traps was detected – evident from shelled sunflower seeds (small birds readily ate and stole the peanuts) characteristic of rodent feeding, or actual squirrel sightings in and around the trap.

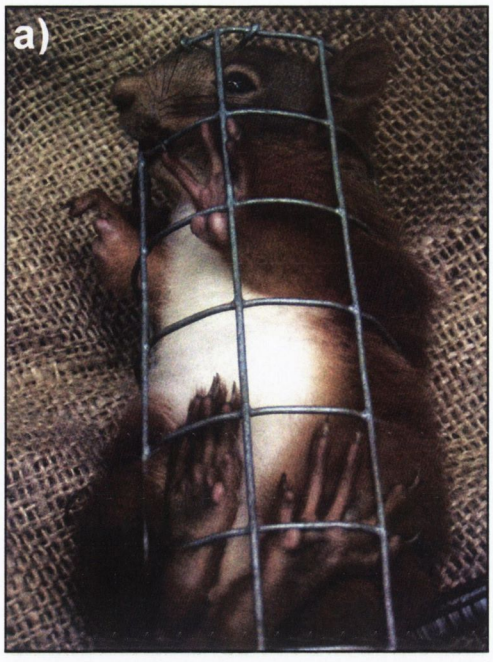
If, after 14 days, no such evidence was detected, trapping was nevertheless carried out, albeit for a shortened period, as red squirrel populations had been confirmed in all the sites. In some cases hair tubes (Garson & Lurz, 1998; Gurnell *et al.*, 2004) were used to detect squirrel presence and if, after two weeks of hair tubing no squirrels were detected with the hair tubes the sites were not trapped.

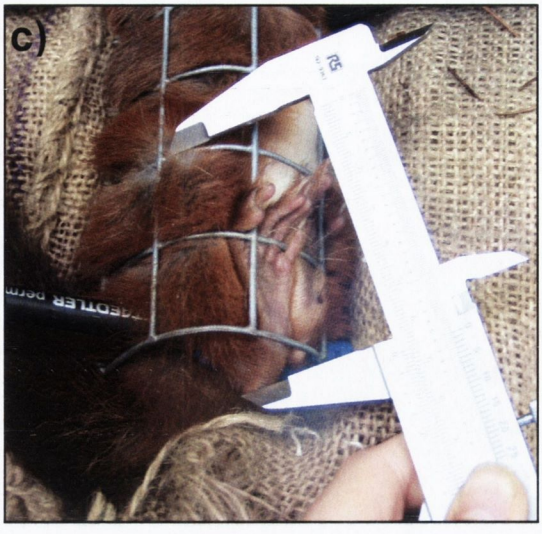
The trapping regime was modified during the course of the study. Initially traps were set at sunrise and checked 6 to 8 hours later, after which the traps were locked open until the next morning. This was later augmented to two checks, at 7 and 12 hours after sunrise, to increase the possibility of catching squirrels.

2.2.3. Squirrel handling and data collection

Trapped squirrels were encouraged to enter the nest box by covering the wire part of the trap with a black bag and, once in the nest box, were blocked inside using a small metal plate inserted between the trap and the nestbox. Squirrels were transferred from the trap into a hessian bag, and then into a 'handling cone' (one inch wire mesh shaped into a cylinder, see Plate 2.2a-d), where it was prevented from escaping by closing the end of the cone with a small stick (Plate 2.2a).

Once in the cone the squirrel could easily be processed without undue stress to the squirrel or the researcher, and body measurements and sex were recorded and genetic samples taken. The squirrels were weighed to the nearest 5g using a 500g Pesola® spring balance (Plate 2.2b), and, as a measure of body size, their right hind shin bones were measured from the joint of the fibula/tibia and femur to the joint of the fibula/tibia and calcaneum (essentially from the 'knee' to the 'ankle') to the nearest 0.1mm using callipers (Reilly, 1997; Plate 2.2c). The sex of the squirrel was also recorded. Males were classified as testes abdominal (TA) or testes scrotal (TS) while females were perforate (Perf) or non-perforate (Nperf). Age classes of squirrels were also determined by using the guidelines outlined by Tittensor (1970 in Reilly, 1997) (adult $\geq$ 240g; subadult 190-240g; and juvenile $\leq$ 180g).





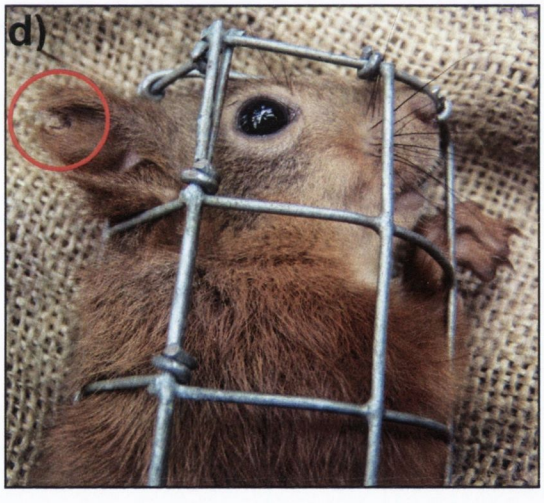


Plate 2.2. Trapping red squirrels: Red squirrels were transferred from the trap into a handling cone (a), squirrels were then sexed, weighed to the nearest 5g using a Pesola® spring balance (b) and their shin bone was measured to the nearest 0.1mm with vernier callipers (c). All trapped squirrels were marked, allowing densities in each site to be calculated, by the removal of a small piece of tissue from their ear (d). This tissue, together with hairs plucked from the tail, was used for genetic analysis.

All squirrels were marked, allowing mark-recapture estimates of population densities, with ear punches (~5mm diameter tissue removed from an ear; Plate 2.2d). Squirrels were marked in their left or right ear in one of three areas (top, middle, bottom), allowing, with the sex divisions, 12 squirrels to be individually marked in each site. The tail colour of each trapped squirrel was noted for use in the morphological study (see Chapter 5). Tissue samples (the ear punches) and hair (~30 plucked tail hairs) were retained for genetic analysis (Chapter 3 & 4).

2.2.4. Trapping success

Sample collection from trapping was poor, with red squirrels caught in only 7 of the 21 sites which were trapped (see Table 2.3). One site (Portlaw) was not trapped at all as it had been almost entirely clearfelled. A total of 28 animals were caught over the entire trapping period. No squirrels were caught in counties Waterford or Sligo, and only 2 individuals were caught in each of counties Wexford (Camolin) and Clare (Lakeside). The largest number of individual squirrels (n = 8) were caught in Corrin, Co. Cork, although similar numbers were caught in Glendalough (n = 6) and Killarney National Park (KNP) (n = 7).

2.3. Roadkill, cadaver and other sample collection.

All Coillte and NPWS staff were requested to collect red squirrel roadkill or cadavers. They were asked to note the locations, providing a six figure grid reference, and send all samples, either the whole squirrel or just the tail, of any red squirrels found in their area, to the Zoology Department, Trinity College. Although it was preferable to receive the entire animal, as this could be used for all aspects of the morphological and genetic study, it was decided to give the option of sending tails, as they would be useful for a large part of the study, were easier and cheaper to post, did not decompose while in transit, and in some cases, particularly when the squirrel was badly decomposed, were the only suitable tissue source for postage. Zoology departments in other universities around Ireland and the Environment and Heritage Service, Northern Ireland were also contacted, requesting that they send any red squirrels that they found.

Twenty seven samples were collected from roadkill and red squirrel corpses from around the country consisting of 12 entire red squirrel bodies, 12 tails and three hair samples. The majority of the samples were collected from Wicklow (n = 12). Only two samples, both tails, were collected from the north of the country (Table 2.4). All of the roadkill samples were used for the genetic analysis (see Chapters 3 and 4). The tails could be used for the

tail colour aspect of the morphological study and entire bodies were used for tail colour, body and skull measurements in the morphological study (Chapter 5). Forty nine hair samples were collected from other graduate studies on red squirrels in Ireland; seven hair samples were collected by Geoff Hamilton, TCD, as part of a trapping study in Curtlestown, Co. Wicklow, while 42 hair samples, as well as information on tail colour were collected from Portumna, Co. Galway, by Alan Poole, NUI Galway as part of a translocation project.

Table 2.4. Samples collected from roadkill, cadaver and other sample collection from around Ireland.

County	Site	Con/Mixed	Grid Ref	Sample Type	# samples
Antrim	Glenhesk	Conifer	J135346	Tail	1
Cork	Glengariff	Mixed	V952555	Tail	2
Down	Newtownards	Conifer	J505737	Tail	1
Dublin	Killiney	Conifer	O263260	Body	1
Galway	Portumna	Conifer	M840030	Hair	42
				Body	2
Kerry	Killarney NP	Mixed	V940860	Body	6
Limerick	Adare	Conifer	R455450	Tail	1
Waterford	Lismore	Mixed	X033995	Tail	1
Wicklow	Laragh	Conifer	T158973	Tail	3
	Glendalough	Mixed	T115975	Tail	2
				Hair	3
	Curtlestown	Conifer	O180170	Body	3
				Hair	7
	Glen of Downs	Mixed	O263110	Tail	1

2.4. Museum Collection

Data and samples were collected from the red squirrel collection held by the Natural History Museum, Ireland and the Ulster Museum, Belfast, and consisted of red squirrels from both Ireland and England. Museum samples were varied; the majority were preserved skins, but there were also skulls, mounts, skeletons and two alcohol preserved specimens (see Table 2.5 and 2.6 for details). Skull measurements and observations of coat colour were recorded for the morphological analysis (Chapter 5) while tissue and/or 10 hairs, plucked from the tail, were taken for genetic analysis (Chapter 3, 4).

Samples were collected from both British (n = 7) and Irish (n = 17) specimens held by the Natural History Museum, Dublin. Hair samples and tail colour for genetic and morphological analysis respectively were collected from dried skins (n = 18) and a single preserved red squirrel. Various cranial measurements (see Chapter 5) were recorded from the skulls (n = 9) and a preserved head and diastema length was measured from the lower

jaw samples. Tissue samples were also obtained from the preserved head. Tail colour (n = 11) and measurements from a single skull were also recorded from the collection in the Ulster Museum, Belfast.

The earliest Irish specimen was collected in Co. Dublin in 1894 with 5 more specimens from 1897. The Irish collection spanned an entire century, with the last squirrel in the collection collected, again in Co. Dublin, in 1987. Red squirrel specimens in the collection were from a widespread geographical distribution with squirrels from both coastal and central counties (Table 2.5; Figure 2.2).

Table 2.5. Irish sample locations, year of collection, sample type and sex (where available) collected from the Natural History Museum, Dublin and Ulster Museum, Belfast. No genetic samples were taken from the Ulster Museum collection.

Museum Id	ID	Date	Location	Grid Ref	Sex	Sample
Dublin						
1894.86.1.	4	1894	Co. Dublin			Skin
1897.6.1.	2	13/07/1891	Freshford, Co. Kilkenny	S417646		Skin
1897.6.2	5	31/07/1897	Co. Wicklow			Skin
1897.6.4	7	1897	Co. Kilkenny			Skin
1897.6.5,	8	02/09/1897	Portarlington, Co. Laois	N542125		Skin
1897.6.6.	9	04/06/1897	Co. Wicklow			Skin
1901.255.1	23	1901	Co. Fermanagh		M	Skeleton
1903.132.1	11	1903	Clonbrock, Co. Galway	M740405	M	Skin
1907.152.1	24	1907	Dundrum, Co. Dublin	O175275		Skin
1908.187.1	14	1908	Dundrum, Co. Dublin	O175275	F	Skin
1936.30.1	20	1936	Big Lagore, Dunshaughlin, Co. Meath	N970525	M	Skeleton
1942.13.1	16	1942	Cong, Claremorris, Co. Mayo	M340750	F	Skin
1964.4.1	15	1964	Clonsilla, Dublin	O055380		Skin
1966.8.118	13	1966	Fota, Co. Cork	W800720		Skin
1966.8.86	18	08/01/1964	Cashel, Co. Tipperary	S078405	M	Skull
1968.43.1	17	1968	Co. Wicklow		F	Skin
1987.6.3	6	1987	Co. Dublin			Skin
Ulster						
M171002	U7	1979	Co. Down			Skin
M171003	U4	1979	Co. Down			Skin
M171005	U3	1977	Co. Down			Skin
M171011	U1	1981	Co. Down		F	Skin and skull
M171012	U2	1980	Co. Tyrone		M	Skin
M171014	U6				M	Mount
1-1951	U5	1951				Skin
	U8	1980	Co. Down			Skin
	U9		Co. Down			Skin
	U10		Co. Down			Mount
	U11		Co. Down			Mount

Figure 2.2. Geographical origin of samples collected from Irish museum specimens held by the Natural History Museum. Dublin and Ulster Museum, Belfast.

The British collection was far more conservative, with squirrels available from an eight year period, 1906 to 1913 and from only 3 locations, one of which was merely identified as 'England'. The majority of the British specimens were osteological with only two skins in the collection, both of which were from Eastbourne (Table 2.6).

Table 2.6. British sample locations, year of collection, sample type and sex (where available) collected from the Natural History Museum, Dublin.

Museum Id	ID	Date	Location	Grid Ref	Sex	Sample
1906.158.1	22	1906	Northamptonshire	SP793686		Skull
1906.290.1	21	1906	Northamptonshire	SP793686	F	Skelton
1906.290.2	19	1906	Northamptonshire	SP793686	F	Lower Jaws
1911.129.1	1	23/01/1911	Eastbourne, Sussex	TV613988	F	Skin and skull
1911.129.2	3	23/01/1911	Eastbourne, Sussex	TV613988	F	Skin and skull
1913.53.1	10	1913	England			Skin
1913.53.2	12	1913	England			Skin

2.5. Sampling distribution

Overall the distribution of samples collected was widespread (Figure 2.3); however it was quite skewed, with some areas only represented by museum samples, some by a single roadkill, and others by a large number of squirrels from both contemporary and museum sources. The largest number of samples collected from a single county was 45 samples obtained from Galway; 44 contemporary and data from one museum specimen from 1903. Wicklow had the second largest number of samples (n = 31), 28 contemporary and data from three museum specimens from 1987 and 1968. Six samples were collected from Dublin – data from five museum specimens and a one contemporary sample and nine; eight contemporary and data from one museum, sample were collected from Cork. Thirteen samples were collected from Kerry, all from KNP, and all of which were contemporary. In Northern Ireland data from 11 individual squirrels were collected from Co. Down; a tail from a contemporary roadkill and data from ten museum specimens, all collected after 1976.

The other counties were represented by much fewer samples. Only one sample was collected from Fermanagh, Antrim and Tyrone in Northern Ireland, with only the sample from Antrim a contemporary sample. A single museum sample was collected from each of counties Tipperary, Mayo, Laois and Meath, and two were collected from county Kilkenny. No contemporary samples were collected from those counties. Wexford was represented by two red squirrels trapped in Camolin, while Limerick and Waterford were represented by a singe roadkill specimen each.

Due to the different nature of the samples collected (tails, bodies, trapped etc) their applicability to different aspects of the study varied. A complete list of the samples collected from each county and their use in the molecular and morphological analysis are given in Table 2.7. Only 11 samples were able to be used in all aspects of the study; all of which were contemporary; two from Galway, six from Kerry and three from Wicklow. Data on tail colour were collected from 119 squirrels; 29 of which were museum samples, from a widespread distribution in Ireland (Table 2.7, Figure 2.3). Body measurements (shin length and body weight) were recorded from all 28 squirrels trapped in study as well as from the squirrels which were trapped in other graduate studies which provided hair samples for this study (n = 55 in total). Microsatellite analysis was able to be carried out on all contemporary samples (n = 100) while mtDNA could be extracted from both contemporary and museum samples (n= 117). The largest number of samples which could

be used in both the genetic and morphological analysis was 110 samples from which information on tail colour and mtDNA could be obtained, consisting of a mixture of both museum and contemporary samples, while 91 contemporary samples could be analysed with respect to tail colour, mtDNA and microsatellite DNA (Table 2.7).

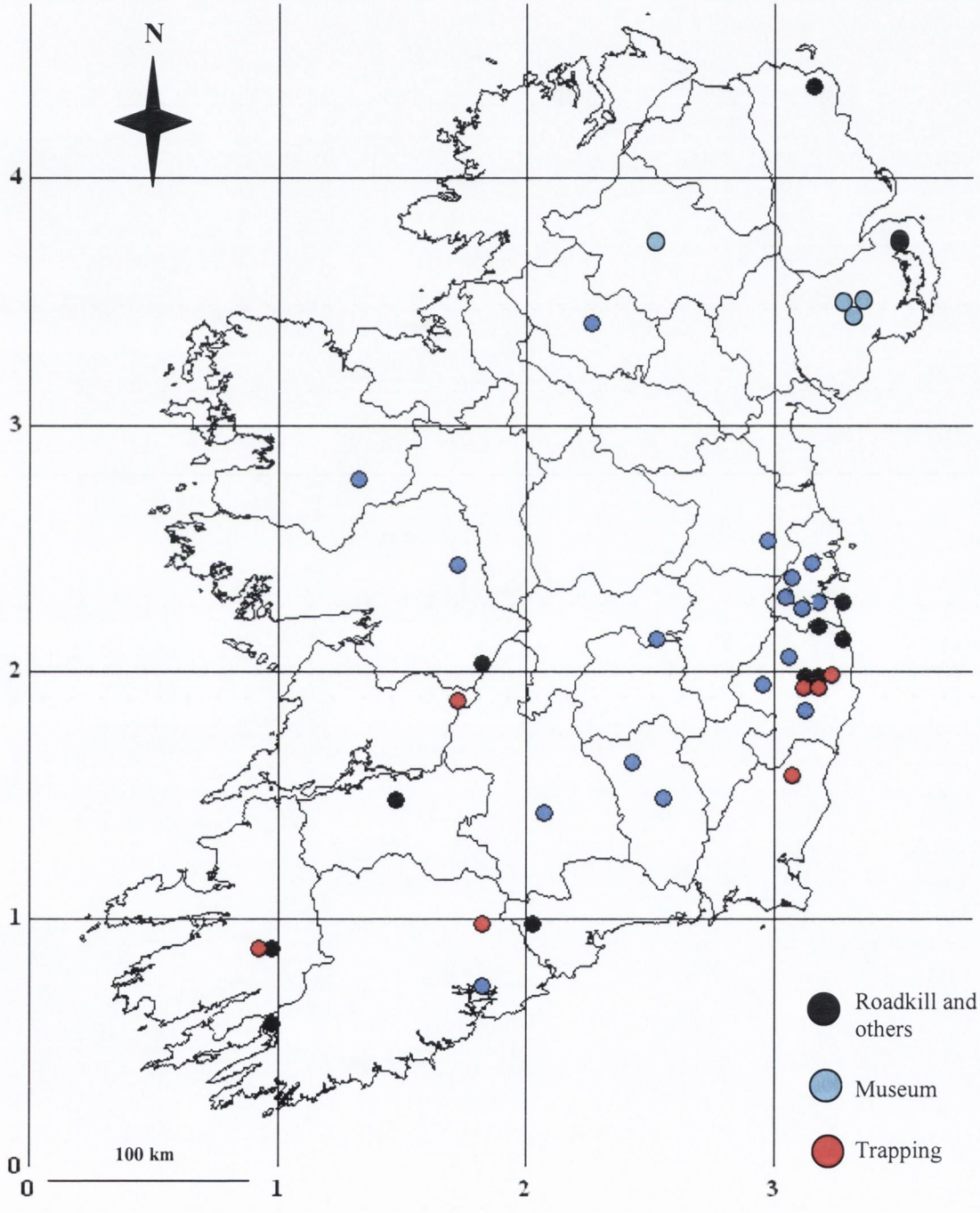


Figure 2.3. Locations of all samples collected from trapping, roadkill and museum specimens from Ireland

Table 2.7. Complete list of samples and their relative use to different aspects of this study. Shaded cells indicated where the sample is of use in that particular analysis. Body under morphological analysis indicates body measurements: shin length and body weight. Cranial indicates the skull measurements outlined in detail in Chapter 5. * Hair samples collected from other graduate projects. Information on body measurements was available for all these samples, while data on tail colour were available for the 42 samples from Galway. † One of the skulls was not entire and consisted of a lower jaw only. $^{\Delta}$ Only intact lower jaws were recovered.

County	Museum/ Contemporary	Sample Type	N	Morphology: Body	Morphology: Cranial	Morphology: Tail Colour	Genetics: MtDNA	Genetics: Microsat
Antrim	Con	Tail	1			■	■	■
Clare	Con	Trapped	2	■		■	■	■
Cork	Con	Tail	2			■	■	■
		Trapped	8	■		■	■	■
	Mus	Skin	1			■	■	
Down	Con	Tail	1			■	■	■
	Mus	Skin/Mount	10			■		
Dublin	Con	Body	1	■	■	■	■	■
	Mus	Skin	5			■	■	
England	Mus	Skull†	3		■			
		Skeleton	1		■			
		Skin and skull	2		■	■	■	
		Skin	2			■		
Fermanagh	Mus	Skeleton	1		■			
Galway	Con	Hair*	42	■		■	■	■
		Body	2	■	■	■		
Galway	Mus	Skin	1			■	■	
Kerry	Con	Body$^{\Delta}$	6	■	■	■	■	■
		Trapped	7	■		■	■	
Kilkenny	Mus	Skin	2			■	■	
Laois	Mus	Skin	1			■	■	
Limerick	Con	Tail	1			■	■	■
Mayo	Mus	Skin	1			■	■	
Meath	Mus	Skeleton	1		■			
Tipperary	Mus	Skull	1		■			
Tyrone	Mus	Skin	1			■		
Waterford	Con	Tail	1			■	■	■
Wexford	Con	Trapped	2	■		■		
Wicklow	Con	Tail	3			■		
		Hair*	10	■				
		Body	3	■	■	■		
		Trapped	9	■		■		
	Mus	Skin	3			■	■	

Chapter 3: Postglacial colonisation of Europe by red squirrels, with particular reference to the Irish population.

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3.1. Introduction

The retreat of the ice sheets at the end of the last glacial period has been recognised as the main influence behind the distribution and phylogeographic structure of extant species in the northern hemisphere (Hewitt, 2000). During the last cold period, approximately 70,000-10,000 BP, the Scandinavian ice sheet covered most of northern Europe, and ice caps also existed in the large mountain ranges such as the Alps and Pyrenees (Frenzel, 1973 in Taberlet *et al.*, 1998). Much of the rest of the continent has traditionally been described as tundra or steppe, confining temperate species to the remaining areas of habitat in the Mediterranean and Balkan regions (so called glacial refugia). However, a number of smaller, more cryptic refugia, at higher latitudes, have also been described, based on both floral and faunal remains (Denton *et al.*, 1971 in Willis *et al.*, 2000; Larsen *et al.*, 1987; Stewart & Lister, 2001), and phylogenetics (Bilton *et al.*, 1998; Brunhoff *et al.*, 2003; Deffontaine *et al.*, 2005; Jaarola & Searle, 2002; Kotlík *et al.*, 2006), the latter even finding evidence that temperate forest species, and therefore forests, existed through the last glacial maximum in central areas of Europe (Deffontaine *et al.*, 2005; Kotlík *et al.*, 2006; Willis *et al.*, 2000).

During cold periods, and subsequent postglacial spread, populations underwent repeated range contractions and expansions which left their genetic signatures on the species today (Furlong & Brookfield, 2001; Hewitt, 1999). As a result of this, specific genetic similarities from populations that arose from the same glacial refugia, and followed the same pattern of spread, occur, allowing the locations of glacial refugia to be determined, as well as postglacial recolonisation routes to be traced. Based on phylogenetic results, a number of paradigms of postglacial spread have been described in Europe (Hewitt, 1999), all based on expansion from Mediterranean refugia. These map postglacial expansion from one, two or three of the refugia, with the Pyrenees and Alps representing barriers to dispersal for some species, while for others, expansion from one refugium was blocked by colonisers from other refugia (Hewitt, 2000), and these patterns of expansion created numerous areas of introgression in Europe, particularly in western Germany and Scandinavia (Hewitt, 2000; Taberlet *et al.*, 1998). However, the increasing evidence for refugia other than those described in the Mediterranean basin, means that postglacial spread of species may be far more complicated than these studies suggest.

Although numerous patterns of postglacial spread through mainland Europe have been described, spread to and/or recolonisation of the British Isles has been an issue of debate for many years. The traditional explanation was a land bridge connecting the islands to each other and then to mainland Europe, logically explaining the notable decrease in faunal diversity from mainland Europe, to Britain, to Ireland. Evidence for periodic rises and falls in sea level between Britain and Europe seem to support a landbridge between those areas after the Devensian (Sutcliffe, 1995), however evidence for such a bridge between Ireland and Britain has been contested. Devoy (1985) suggested a landbridge may have existed between Northern Ireland and Scotland. Wingfield (1995) suggested a temporary landbridge existed which moved northwards through the Irish sea while modelling carried out by Lambeck & Purcell (2001) supported a bridge between southeast Ireland and southern England. However the nature of this bridge (Devoy, 1985; Lambeck & Purcell, 2001) would have excluded the colonisation of Ireland by many temperate species, and evidence for its existence is far from definite.

Phylogenetic studies have revealed a second possible colonisation route of Ireland. A 'Lusitanian' element to Ireland's biodiversity has been detected (Davison *et al.*, 2001; Mascheretti *et al.*, 2003), with close associations between Irish species and those found on the Iberian peninsula, explained by either deliberate or accidental introductions either with the original spread of humans from that area (Hill *et al.*, 2000), or afterwards *via* trade.

However, survival of species in a glacial refugium in the southwest of Ireland has also been suggested, particularly for species adapted to tundral conditions (Stewart & Lister, 2001). This may explain the subspecific status of the stoat, *Mustela erminea hibernica*, and hare, *Lepus timidus hibernicus*, in Ireland and, in fact, recent phylogenetic work both of these species has produced strong evidence for their persistence in an Irish refugium during the last cold period (Hamill, 2002; M. Hughes, pers. comm.; N. Martínková, pers. comm.). It has also been proposed that Scots pine, *Pinus sylvetris*, survived in this refugium (Sinclair *et al.*, 1998), suggesting the persistence of more temperate species, not just those which were adapted to cold or tundra conditions, in Ireland over the last ice age.

Although morphological (Lynch, 1996; Lynch & Hayden, 1995) and archaeological (Woodman *et al.*, 1997) investigations have been carried out on the postglacial origin of Irish species, research using phylogenetic methods has only been carried on a few species (pine marten, Davison *et al.*, 2001; pygmy shrew, Mascheretti *et al.*, 2003; stoat, N.

Martíkóva, pers. comm.; fox and badger, C. Edwards pers. comm.; mountain hare, Hamill, 2002, Hamill *et al.*, 2006; M. Hughes, pers. comm.). However, despite the evidence for survival of Scot's pine in the refugium in Ireland (Sinclair *et al.*, 1998), no research so far has revealed the persistence of temperate species in Ireland through the last glacial maximum.

The red squirrel is a temperate forest species that is widespread throughout Europe (Gurnell, 1987). As a temperate forest species, its presence can be used to determine not just the existence of forests in refugia, besides those in the Mediterranean, but also whether substantial forests, large enough to support a red squirrel population occurred in these refugia. Preliminary European phylogeographic work was carried out by Hale *et al.* in 2004, and they suggested that, although the mainland European population could be utilised as a phylogeographic model for postglacial forest spread, the British population was too much a product of translocations from Europe to be of any applicability in interpreting phylogeography in the species as a whole.

Although within Ireland the species is considered native, the Irish population has also been subjected to numerous translocations, in this case from Britain, during the 1800's (Barrington, 1880), and it is unclear whether the current Irish population is wholly made up of these translocated individuals or if the population is a mixture of native and reintroduced stock. If some native lineages of red squirrels in Ireland survived they may be useful in investigating whether temperate conditions existed in the Irish refugium, or support other faunal colonisation events of the island.

In this part of the study, a detailed phylogeographical study of the red squirrel in Ireland was carried out. Results were compared with those from all of the published literature on genetics of red squirrels in Europe (Barratt *et al.*, 1999; Hale *et al.*, 2004; Ogden *et al.*, 2005) and there were two main aims:

- To reinvestigate European red squirrel phylogeography, including the data collected from Ireland in this study, to investigate whether the inclusion of Irish data reveals more about the likely refugial areas and postglacial expansion routes of the red squirrel in Europe.
- To investigate to what extent the Irish red squirrel population is a product of translocations from Britain, and determine whether the resulting data could be used to increase understanding of the faunal colonisation history of the island.

3.2. Materials and Methods

3.2.1. Sample collection

Samples were collected from both contemporary and museum Irish specimens as outlined in Chapter 2. Figure 3.1 shows the distribution of these samples as well as the 10 recorded points of translocation of the red squirrel into Ireland in the 1800's (Barrington, 1880). Samples were allocated into regions as shown on Figure 3.1. Regions were separated by major geographical barriers (see Appendix 8.1); distance (north from all regions, east from south and southwest), mountains (south from southwest) and rivers (west from all other regions).

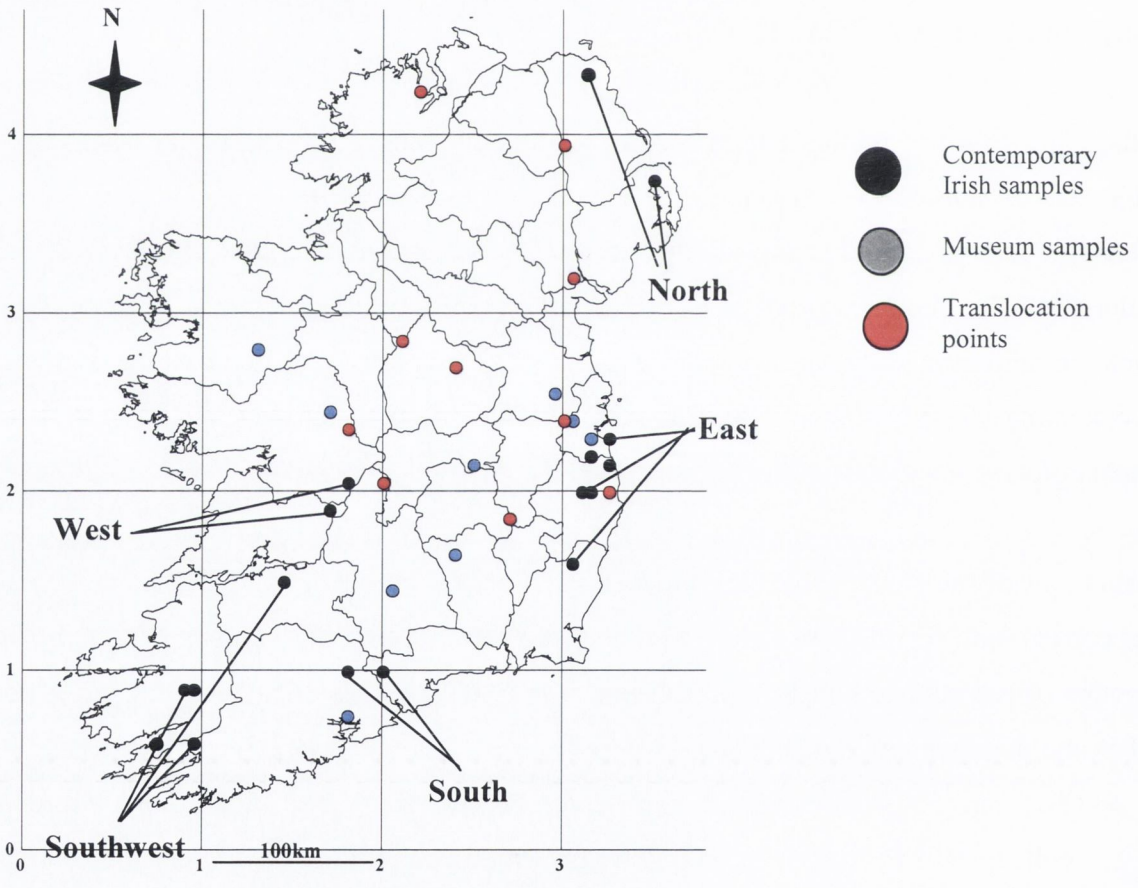


Figure 3.1. Map showing the locations of samples collected from around Ireland from both contemporary and museum sources, and the regions to which these samples were allocated and referred to in the text (see Chapter 2 for more detail). The 10 translocation points recorded by Barrington (1880) are also shown.

3.2.2. DNA extraction

3.2.2.1. Extraction from tissue

Prior to extraction, tissue samples that had been stored in ethanol were hydrated by immersion in decreasing concentrations of ethanol, 75%, 50%, 25% and 0% over the course of two hours. Total genomic DNA was extracted from tissue samples using a Qiagen DNAeasy Tissue Kit following the manufacturer's protocol.

3.2.2.2. Extraction from hair

DNA was extracted from hair using a modification of the Chelex®/proteinase protocol outlined by Sambrook *et al.* (1989) and M. Hale (pers. comm.). Five hair root tips (~5mm) from each individual squirrel were incubated overnight in solution of 5 µl of 20 mg/ml proteinase K and 400 µl of 5% Chelex® 100 (Biorad), at 56 °C in a shaking incubator. Samples were then vortexed for 15 seconds and boiled at 100°C for 8 minutes, followed by additional vortexing of 20 seconds. The Chelex was spun down by centrifugation, and the supernatant removed to a new tube and stored at -20 °C until amplification.

3.2.3. MtDNA amplification and sequencing

A 395 bp section of the control region of the mitochondrial DNA genome was amplified in 25 µl reactions following the protocol outlined by Hale *et al.* (2004), using 0.5 µl of tissue, or 5 µl of hair/museum, DNA (Table 3.1), and the following red squirrel specific primers: H16359: 5'-GGAAGCGGATAGTCATTTGG-3' (Barratt *et al.*, 1999) and RScont6: 5'-CCTTCAACTCCCAAAGCTGA-3' (Hale *et al.*, 2004). PCR conditions were those described by Hale *et al.* (2004), although the number of cycles was increased from 30 to 35 for museum and hair samples as DNA quality and/or quantity may be expected to be reduced in those sample types. Amplification was carried out in a Biometra® T1 Thermocycler under the following conditions: 94 °C for 30 seconds, 30 cycles (35 cycles for museum and hair) of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 90 seconds, with a final extension step of 72 °C for 10 minutes. Positive (red squirrel DNA provided by M. Hale) and negative (dH_20) controls were used in all PCR reactions. PCR products were visualised after staining with ethidium bromide and running on a 1.3% agarose gel alongside a suitable size standard to determine the success of the amplification and to assess the purity of the amplification products. Using the amplification primers H16359 and RScont 6 as direct sequencing primers, PCR products were sequenced by Lark Technologies Inc. (Hope End, Takeley Essex, CM22 6TA).

Table 3.1. The PCR reaction mix used to amplify mtDNA.

Reagent	Stock Conc.	Volume in Reaction	Conc. in Reaction
PCR Buffer	10 x	2.5 µl	1 x
dNTP mix	10 mM	0.2 µl	0.08 mM
Primer FWD	10 µM	0.5 µl	0.2 µM
Primer REV	10 µM	0.5 µl	0.2 µM
$MgCl_2$	50 mM	1 µl	2 µM
Taq	5 units/µl	0.2 µl	1 unit
Sterile Distilled Water		15.1 µl	
Template:			
Tissue		0.5µl (+ 4.5 µl dH_20)	
Hair		5µl	
Total		25 µl	

3.2.4. Data analysis

Forward and reverse sequences from each individual were compared in GENEDOC version 2.6.002 (Nicholas & Nicholas, 1997), and, where an unknown base occurred in one strand the base was determined through comparison with the complementary strand (there was no instance where an unknown base occurred at the same site in both strands). Sequences were then aligned with published red squirrel sequence data (AF1110001-AF111027, Barratt *et al.*, 1999; AY178452-AY178479, Hale *et al.*, 2004; AY372270-AY534120, AY534120-AY534121, Ogden *et al.*, 2005) in CLUSTAL_X (Thompson *et al.*, 1997), and novel haplotypes were identified with the MS EXCEL SEQUENCE TOOLKIT (Stephen Park, UCD). Although only the data described by Hale *et al.* (2004) resulted from amplification of the exact same region of the mtDNA genome as in this study, the 175bp region of overlap between all the published haplotypes encompassed 100% of the variable sites and only this length of sequence was used in all further analyses. Measurements of mtDNA diversity were calculated in MEGA version 3.1 (Kumar *et al.* 2001). The number of polymorphic sites, parsimonious sites, nucleotide composition, mean number of pairwise differences, and % divergence between haplotypes were calculated in MEGA version 3.1 (Kumar *et al.*, 2001).

3.2.4.1. European phylogeography and historical demographics

The relationship between Irish haplotypes and those found in Europe was investigated using population genetic, phylogenetic and demographic analysis.

Population genetics

Population genetic comparisons were carried out between the contemporary Irish haplotypes and those from other European countries to determine the extent of divergence

of the Irish population/Irish regions from other European red squirrels. In addition to the samples generated by this study, haplotypes were available from Britain (n = 52), Spain (n = 2), Belgium (n = 1), The Netherlands (n = 4), Germany (n = 7), Italy (n = 4) and Sweden (n =2; Barratt *et al.*, 1999; Hale *et al.*, 2004; Ogden *et al.*, 2005). Due to the widespread sampling carried out in both Ireland and Britain, those countries were further divided into regions according to where haplotypes were found. Irish haplotypes were grouped into 5 regions: south (n = 2), southwest (n = 6), north (n = 2), west (n = 14) and east (n = 12). After combining data from the published material there were a total of 10 regions in Britain: Wales (n = 6), Scotland (n = 10), West A (n = 2), West B (n = 13), East (n =7), North (n = 6), Anglia (n = 2), Lancashire (n = 2), Jersey (n =2) and the Isle of Wight (n = 2; see appendix 8.1 for map of British regions). Six different hierarchial AMOVA analyses were carried out in ARLEQUIN 3.01 (Schneider *et al.*, 2000), to determine at which level division among Irish and other European haplotypes occurs (Country, Regional etc.), and these are shown in Table 3.2.

Division among countries, and among regions in countries, was also investigated with an exact test of population subdivision (Raymond & Rousset, 1995a) using a Markov chain of 10,000 steps. Regions within Ireland and Britain were treated as separate 'populations' and each European country analysed as a 'population', in ARLEQUIN 3.01 (Schneider *et al.*, 2000).

Table 3.2. 'Populations' used in the Analyses of Molecular Variance (AMOVA)

AMOVA	No. of Groups	Populations
1	8	Ireland, Britain, Spain, Belgium, The Netherlands, Germany, Italy, Sweden
2	12	Ireland south, southwest, north, west, east, Britain, Spain, Belgium, The Netherlands, Germany, Italy, Sweden
3	2	Ireland, Europe (Britain, Spain, Belgium, The Netherlands, Germany, Italy, Sweden)
4	20	Ireland south, southwest, north, west, east, Wales, Scotland, WestA UK, West B UK, East UK, North UK, Anglia, Lancashire, Jersey, Isle of Wight, Spain, Belgium, The Netherlands, Germany, Italy, Sweden
5	3	Ireland, Britain, Europe (Spain, Belgium, The Netherlands, Germany, Italy, Sweden)
6	6	Ireland south, southwest, north, west, east, Britain, Europe (Spain, Belgium, The Netherlands, Germany, Italy, Sweden)

Phylogenetics

Phylogenetic relationships among haplotypes were assessed using Neighbor-joining (NJ), parsimony and Bayesian phylogenetic trees, and a haplotype network. NJ and parsimony consensus trees of 100 bootstrap replicates were constructed and visualised in MEGA version 3.1 (Kumar *et al.*, 2001). The distance measure *p* was used in the NJ analysis as it has low variance, making it especially suitable for phylogenies of very similar sequences, like those in this study (Nei & Kumar, 2000). Parsimony trees were constructed after 10 random jumbles of the data set.

Prior to Bayesian analysis, the optimum model of nucleotide substitution was identified from the 28 most common biological models, for each set of haplotypes, in FINDMODEL (http://gluttony.lanl.gov/content/hcv.db/findmodel.html) using the Akaike Information Criterion (AIC; Akaike, 1974). The model chosen was the General Time Reversible (GTR) + Gamma, and the estimated parameters under this model were: $\Gamma = 0.85$, transition:transversions ratio (ti/tv) = 2.9. Bayesian analyses were conducted with MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001). Metropolis-coupled Markov Chain Monte Carlo sampling was performed with 1000 chains and run for 500,000 generations starting from random trees. Markov chains were sampled every 100 generations resulting in a total of 5,000 sample points. Bayesian trees were visualised in MEGA 3.1 (Kumar *et al.*, 2001). All phylogenetic trees were rooted with the mtDNA control region of the North American grey squirrel, *Sciurus carolinensis* (AF111027).

The relationship between haplotypes was also visualised with a haplotype network, which are increasingly being used to represent the mutational differences between sequences. Networks are useful in intraspecific studies, where the low level of divergence between haplotypes results in phylogenetic trees that have low bootstrap support. A statistical parsimony network of haplotypes (Templeton *et al.*, 1992) was constructed. This method joins together sequences which have one, two, three etc. nucleotide differences, until all the sequences are included in the network, or the parsimony connection limit has been reached. This limit is the maximum number of nucleotide differences between sequences that could, with 95% confidence, have been produced by single nucleotide substitutions. This network was constructed with TCS 1.21 (Clement *et al.*, 2000).

Historical demographics and divergence

Possible expansion after a glacial bottleneck, and colonisation patterns within Europe, were also investigated. Due to the small sample sizes from most countries in mainland Europe, these were grouped into regions, whereby The Netherlands, Belgium and Germany became the 'Western European' region, Spain and Italy were 'Southern Europe' and Sweden was 'Northern Europe'. Ireland and Britain were each classified into separate regions. Population growth of each region was assessed using a number of different methods. Fu's F_S (Fu, 1997) was calculated to test whether sequences confirm to neutral expectations. θ_0 and θ_1, calculated from $2\mu N_0$ and $2\mu N_1$ respectively (where μ is the mutation rate, and N_0 and N_1 are the relative population sizes of females before and after the expansion respectively) were also calculated, differences between these values indicating whether populations have expanded over time. Mismatch distributions for each region were tested against the sudden expansion model (Schneider & Excoffier, 1999), with goodness of fit assessed using a parametric bootstrap of 1000 replicates. Mismatch distribution shows the observed number of differences between pairs of haplotypes within a population. Where populations are at demographic equilibrium (i.e. not expanding after a bottleneck), this distribution has been shown to be multimodal, while populations expanding after a bottleneck will more usually have a unimodal distribution (Rogers & Harpending, 1992). Estimates of time (in generations) since population expansion were obtained from τ. All these population growth analyses were carried out in ARLEQUIN 3.0 (Schneider *et al.*, 2000).

Population expansion was also assessed with the program FLUCTUATE 1.3 (Kuhner *et al.*, 1998) which estimates θ_f ($\theta_f = 2\mu N_F$, where μ is the mutation rate per nucleotide and N_F is the current effective population size of females) and the exponential growth rate of the population (g). θ_f is used as an estimate of the distribution of coalescence times (the times at which two sampled individuals have a common ancestor), and is positively correlated with g. Positive values of g indicate population growth, while negative values indicate decline. As estimates of g may be biased upwards (Kuhner *et al.*, 1998), a conservative approach in testing for significant decline or expansion was used, with only g values larger than three standard deviations from 0 regarded as significant. Transition/transversion ratios for each data set was calculated in MEGA version 3.1 (Kumar *et al.*, 2001) and the program run several times with different numbers of long and short chains to assess consistency of results. The final analysis was run with 10 short chains of 200 steps and 2 long chains of 20,000 steps, with trees sampled every 20 steps.

MDIV (Nielson & Wakeley, 2001) was used to investigate divergence time between regions, therefore distinguishing between recent gene flow between areas and ancestral polymorphism. MDIV uses a Bayesian approach to estimate divergence time and migration between populations that are assumed to have diverged from a common ancestor. As haplotype H99 (see section 3.3.2) in Britain is known to be a recent Swedish introduction into the country (Hale *et al.*, 2004), this was excluded from the all comparisons using British data. The analysis was run three times for each comparison with different random seeds to assess the stability of the results.

The final analysis was carried out using the HKY model with ti/tv estimated from the data. A Markov chain length of 5,000,000 steps was used, discarding the first 500,000 steps as burn-in, and prior distributions of M (migration rate) and t_{pop} (scaled divergence time) between 0 and 10 were used. The modes of the posterior distribution of θ and t_{pop} were used to estimate divergence time, T_{pop}, between populations. T_{pop} was estimated from the formula $T_{pop} = [(t_{pop}. \theta)/2L)/\mu]$, where L in the sequence length (395 bp) and μ is the mutation rate per site per generation (Brito, 2005). A wide range of mutation rates were used (between 0.5 and 10% per Myr) and generation time was set to 3 years (Gurnell, 1987). The modes of posterior distribution of M were used as a graphical representation of the patterns of migration between populations, where M is 2Nm, and N is the effective population sizes of the two populations between which migration is occuring and m is the proportion of individuals of each population exchanged between populations.

3.2.4.2. Irish phylogeography and historical demographics

A more in-depth investigation of phylogenetics, phylogeography and the possible historical changes in population size and divergence times was carried out on the Irish population using methods similar to that used in the European comparison.

Phylogenetics

Phylogenetic relationships among haplotypes were assessed using the same methods as used in the European comparison using only contemporary Irish haplotypes and both Irish and British museum samples in the analysis. Again, the GTR model + Gamma was chosen with $\Gamma = 0.54$, ti/tv = 1.21.

Phylogeographic relationships

Unlike the European comparison, the shared haplotypes between regions in Ireland allowed a nested clade phylogeographical analysis (NCPA; Templeton *et al.*, 1995) to be carried out, statistically testing for associations between haplotype distribution and geography. In this method, haplotypes are grouped together into clades of one step, two step etc. changes as outlined by Templeton *et al.* (1992). These clades are then nested within each other; the one step clades will be nested within 2 step, the two within 3 step etc., until all of the individual clades are nested together in a single clade. By inputting the geographical origin of each of the haplotypes in the network, NCPA calculates the geographical range of each clade, and measures how each clade is geographically distributed in relation to its closest evolutionary sister clades (Templeton *et al.*, 1995). Four distance measures are calculated. Clade distance, D_c, is the average distance between the locations of the members of each clade and the geographical centre of the clade. Nested clade distance, D_n, is the average distance between members of each clade and the geographical centre of the entire nesting clade (Templeton *et al.*, 1995). Where clades are located at either the tip, with only one connection to the entire cladogram, or interior, with two or more connections to other clades, two other distances are calculated. D_cI-D_cT is the difference between the clade distance of tips and interior clades within the nested clade, and D_nI-D_nT is the difference between nested clade distance of tip and interior clades. NCPA was carried out in GEODIS version 2.4 (Posada *et al.*, 2000) using a parsimony network constructed from only contemporary Irish haplotypes in TCS 1.21 (Clement *et al.*, 2000). Where the null hypothesis of no geographical association between haplotypes was rejected, the biological causes for this association were determined using Templeton's (2004) inference key.

Historical demography

Population growth was assessed within each of the five regions (east, south, southwest, west, north) and samples collected from Ireland before 1915, using the same methods used in the full European comparison. Additionally, Slatkin's linearised F_{ST} (Slatkin, 1995) was calculated between each region in Ireland (n = 5), Irish museum samples pre 1915 and each region (n = 10) in Britain, again excluding haplotype H99, to try and ascertain the British geographic origin of the introductions into Ireland. Bootstrapped neighbour joining trees of these distances were constructed using ARLEQUIN 3.01 (Schneider *et al.*, 2000) and PHYLIP 3.5c (Felsenstein, 2004) and visualised in TREEVIEW version 1.1.6. (Page, 1996).

3.3. Results

3.3.1. Irish mtDNA sequence diversity

MtDNA was successfully extracted from 87 individual contemporary red squirrel samples (individual data is given in Appendix 8.3) and 14 museum samples, 10 of which were Irish. 29 novel haplotypes were identified in the contemporary samples (Table 3.3), and 11 in the museum samples (Table 3.4). Only two haplotypes, SW+W+E and IRL, were found in both the contemporary and museum samples. SW+W+E was found in a number of contemporary samples (Table 3.3) and was amplified in a museum specimen from relatively recent Irish specimen from Dublin from 1964, and also from an English sample collected from 'England' (no further geographical information was available) in 1911 (Table 3.4). IRL was also found in a number of contemporary samples (Table 3.3), and was also amplified from an Irish specimen collected from Galway in 1903 (Table 3.4). The regions in which each haplotype occurred, and its frequency within that region, are shown in Table 3.5. A graphical representation of the distribution of these contemporary haplotypes within Ireland is shown in Figure 3.2.

Overall, in the haplotypes amplified from Irish samples, there were a total of 37 polymorphic sites, 24 of which were parsimony informative. Nucleotide composition had the following relative values: C: 10.83%, T: 32.32%, A: 31.10% and G: 25.75% amongst all haplotypes. The ti:tv ratio of 1.2:1 was considerably lower than that described for the red squirrel elsewhere (e.g. 5:1, Todd 2000a; 16:1, Trizio *et al.*, 2005). This may be a result of the extent of divergence between sequences. Moritz *et al.* (1987) found that divergence rates between sequences decreases once the sequences are approximately 15% diverged. After this point evolution is still occurring at a much slower pace due to 'saturation', where once a mutation has occurred at a site, further mutations are not detectable through comparison with another sequence. Although mean divergence in this study was 9%, there was a large range in the data set (2.9-17%), encompassing the 15% divergence threshold suggested by Moritz *et al.* (1987). Therefore, it is possible that the ti:tv ratio within the Irish sequences was higher in the past, however, with the relative isolation of the Irish population (150 years) in comparison to populations examined in other studies, saturations at variable sites have occurred and mutations which were once transitions have since become transversions, resulting in the almost equal ratio detected in this study.

Table 3.3. The 29 novel haplotypes amplified from the contemporary Irish samples. Mutation position, labelled with respect to the number of mutations after the primer, for each of the 37 polymorphic sites is shown. The number of individuals with each particular haplotype are also given.

	0 0 8	0 0 9	0 1 0	0 1 1	0 1 2	0 1 3	0 1 4	0 1 5	0 1 6	0 1 7	0 1 9	0 2 0	0 2 2	0 2 3	0 2 4	0 2 5	0 2 6	0 3 1	0 3 2	0 3 4	0 3 5	0 3 6	0 3 8	0 4 5	0 4 7	0 4 8	0 5 0	0 5 5	0 6 1	0 8 7	0 9 3	0 9 5	0 9 6	1 0 1	1 1 1	1 2 7	1 7 6	No. of Samples
IRL	A	C	T	T	A	A	A	T	G	G	G	T	T	G	T	A	C	T	A	T	A	A	A	G	T	A	G	G	A	T	G	G	G	A	A	C	A	22
E1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	1
E2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
E3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	G	.	.	.	.	A	.	.	A	.	A	.	G	.	.	4
E4	T	.	.	.	.	.	.	G	.	.	.	G	.	.	.	G	.	C	.	.	.	.	G	.	.	.	.	A	.	.	A	.	A	.	G	.	.	1
E5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	C	.	.	.	G	.	.	.	.	A	.	.	A	.	A	.	G	.	.	1
E6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	7
E7	T	.	.	.	T	G	.	G	A	.	.	A	G	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
E8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	1
E+N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	A	.	A	.	G	T	.	3
NI1	.	.	.	G	.	G	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.	A	A	.	.	.	.	.	1
S1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	G	.	G	7
SW+W+E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
SW1	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	A	.	.	.	C	.	.	C	.	.	C	.	.	A	.	.	.	.	1
SW2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
SW3	T	.	.	G	.	G	.	.	T	.	.	A	G	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	2
W10	-	T	C	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	16
W12	C	T	.	A	.	.	T	G	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
W2	.	.	.	G	.	G	.	G	T	.	.	A	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W3	T	.	.	G	.	G	.	G	.	.	.	A	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W4	.	.	.	G	.	G	.	G	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W5	.	.	.	G	.	G	.	.	.	.	.	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W6	T	.	.	.	.	G	.	G	.	.	.	.	.	A	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W7	T	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W8	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
W9	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	-	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	1
Wxa	.	.	.	C	.	.	.	A	.	.	.	A	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	G	.	A	.	A	.	G	T	.	1

Table 3.4. The 12 novel haplotypes amplified from the museum specimens. Mutation position, labelled with respect to the number of mutations after the primer, for each of the 37 polymorphic sites is shown. The two haplotypes which were found in the contemporary samples are also given as well as the museum ID's (see Chapter 2) for the samples in which each haplotype was found. English samples are indicated with *.

	0 0 8	0 0 9	0 1 0	0 1 1	0 1 2	0 1 3	0 1 4	0 1 5	0 1 6	0 1 7	0 1 9	0 2 0	0 2 2	0 2 3	0 2 4	0 2 5	0 2 6	0 3 1	0 3 2	0 3 4	0 3 5	0 3 6	0 3 8	0 4 5	0 4 7	0 4 8	0 5 0	0 5 5	0 6 1	0 8 7	0 9 3	0 9 5	0 9 6	1 0 1	1 1 1	1 2 7	1 7 6	Museum ID
IRL	A	C	T	T	A	A	A	T	G	G	G	T	T	G	T	A	C	T	A	T	A	A	A	G	T	A	G	G	A	T	G	G	G	A	A	C	A	1903.132.1
M1	T	.	C	.	.	G	.	.	.	.	.	A	G	.	C	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1911.129.1*
M2	T	.	C	.	T	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1897.6.1
M4	.	.	.	.	.	.	.	A	.	T	.	G	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1894.86.1
M5	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1897.6.2
M6	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1987.6.3
M7	C	.	.	C	G	G	G	A	.	T	.	A	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1897.6.4
M8	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1897.6.5
M10	.	.	.	.	G	G	.	.	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1913.53.1*
M12	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1913.53.2*
M17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1968.43.1
M24	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1907.152.1
SW+W+E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1966.8.86 + 1911.129.2*

Table 3.5. Haplotype frequencies in the 5 regions in Ireland. A graphical representation of these data is shown in Figure 3.2. Colours beside each haplotype name relate to pie chart colours in Figure 3.2.

	South(8)	Southwest(15)	North(2)	East(22)	West(40)
E1	0	0	0	0.046	0
E2	0	0	0	0.046	0
E3	0	0	0	0.182	0
E4	0	0	0	0.046	0
E5	0	0	0	0.046	0
E6	0	0	0	0.318	0
E7	0	0	0	0.046	0
E8	0	0	0	0.046	0
E+	0	0	0.5	0.091	0
IRL	0.125	0.6	0	0.046	0.25
N1	0	0	0.5	0	0
S1	0.875	0	0	0	0
SW+W+E	0	0.133	0	0.046	0.025
SW1	0	0.067	0	0	0
SW2	0	0.067	0	0	0
SW3	0	0.067	0	0	0
W1	0	0.067	0	0	0.025
W2	0	0	0	0	0.025
W3	0	0	0	0	0.025
W4	0	0	0	0	0.025
W5	0	0	0	0	0.025
W6	0	0	0	0	0.025
W7	0	0	0	0	0.025
W8	0	0	0	0	0.025
W9	0	0	0	0	0.025
W10	0	0	0	0	0.025
W11	0	0	0	0	0.425
W12	0	0	0	0	0.05
Wxa	0	0	0	0.046	0

Figure 3.2. Map showing relative frequencies of each of the 29 haplotypes in the five regions in Ireland. Colour legend and no. of samples collected from each region are given in Table 3.5

3.3.2. European phylogeography and historical demographics

After collapsing the sequences into haplotypes, H1 and H2 (Hale *et al.*, 2004), which differed by a single unknown base N, were combined into a new haplotype H99. Similarly H7, found in Britain, and H12, a Spanish haplotype, were designated as the single haplotype H712. Along the areas of sequence overlap between the amplifications in this study, and those carried out by Ogden *et al.* (2005) and Barratt *et al.* (1999), there was no variability between the two Welsh haplotypes ang1 and ang2, now a new haplotype Wang, or between wmz2 and ah163, labelled ZZ.

3.3.2.1. Population genetics

The AMOVA revealed varying genetic structure depending on the hierarchy of the analysis. In almost all cases, over 40% of the genetic variation was distributed among individuals within populations, and among populations within groups ($p < 0.0001$). The exception was the analysis comparing Irish regions to British regions and European countries (AMOVA 4 in Table 3.2), when only 12% of the variation occurred within groups. Significant division between groups occurred in three cases. When countries were partitioned into two groups (AMOVA 3 in Table 3.2); Ireland and the rest of Europe, a significant amount of variation occurred between them (14%; $p = 0.002$). Similar results was achieved when the data were split into Ireland, Britain and Europe (AMOVA 5 in Table 3.2), whereby 10.62% ($p = 0.002$) of the variation occurred between countries. However, the largest amount of between group variation was when the regions within Ireland and Britain were incorporated into the model (AMOVA 4 in Table 3.2), whereby 41.56% ($p = 0.035$) of the variation was partitioned between these groups. This suggests that not only is Ireland divergent from Britain and Europe, but regions within Ireland are divergent both from each other, as well as from regions in Britain.

The exact test of population differentiation supported these results. As would be predicted given the low number of haplotypes shared between counties, all counties were different ($p < 0.01$), with the exception of Britain and Sweden ($p = 0.068$), although this can be attributed to the rapid spread of a recently introduced Swedish haplotype into Britain, as discussed by Hale *et al.* (2004), and eastern Ireland and eastern Britain ($p = 0.055$), again, probably as a result of translocations. Within Ireland, differentiation only occurred at a temporal scale with a difference between the northern region and museum specimens ($p = 0.02$), possibly as no mtDNA data was available from museum specimens from Northern Ireland.

3.3.2.2. Phylogenetics

Phylogenetic relationships between haplotypes constructed with the parsimony, NJ and Bayesian tree construction methods are shown in Figures 3.3, 3.4 and 3.5 respectively.

Despite the large number of novel haplotypes identified in the Irish population, the phylogenetic trees did not support a partitioning of the Irish population from any other country from which haplotypes were available. As is typical for many intraspecific trees constructed from low divergent sequences, bootstrap support for the higher level clades was extremely low, or non-existent, in all comparisons. Also, as has been found in other studies of red squirrels in Europe (Barratt *et al.*, 1999; Hale *et al.*, 2004; Ogden *et al.*, 2005), the phylogenetic tree had little geographical structure, with no widespread clustering of haplotypes with respect to the country in which they were found.

However, although there was no clear division between Irish and European haplotypes, there was a grouping of some Irish haplotypes on one or more of the trees. E1 and E6 grouped together on both the parsimony and NJ trees (Figure 3.3: Clade Ia; Figure 3.4: Clade Ib) but not on the Bayesian tree. However, on the NJ tree, these haplotypes were also clustering with Italian, Dutch and British haplotypes (bootstrap support: 41%). On the Bayesian tree there was also a large clade (Figure 3.5: Clade IIa) which included 7 Irish haplotypes and a British museum haplotype. The Irish haplotype S1 grouped with H22 from Britain with high posterior support on the Bayesian tree (Figure 3.5: Clade IIIa).

Figure 3.3. Maximum parsimony strict consensus tree of Irish and other European haplotypes. Bootstrap values > 30% are shown. Clades shown are referred to in the text.

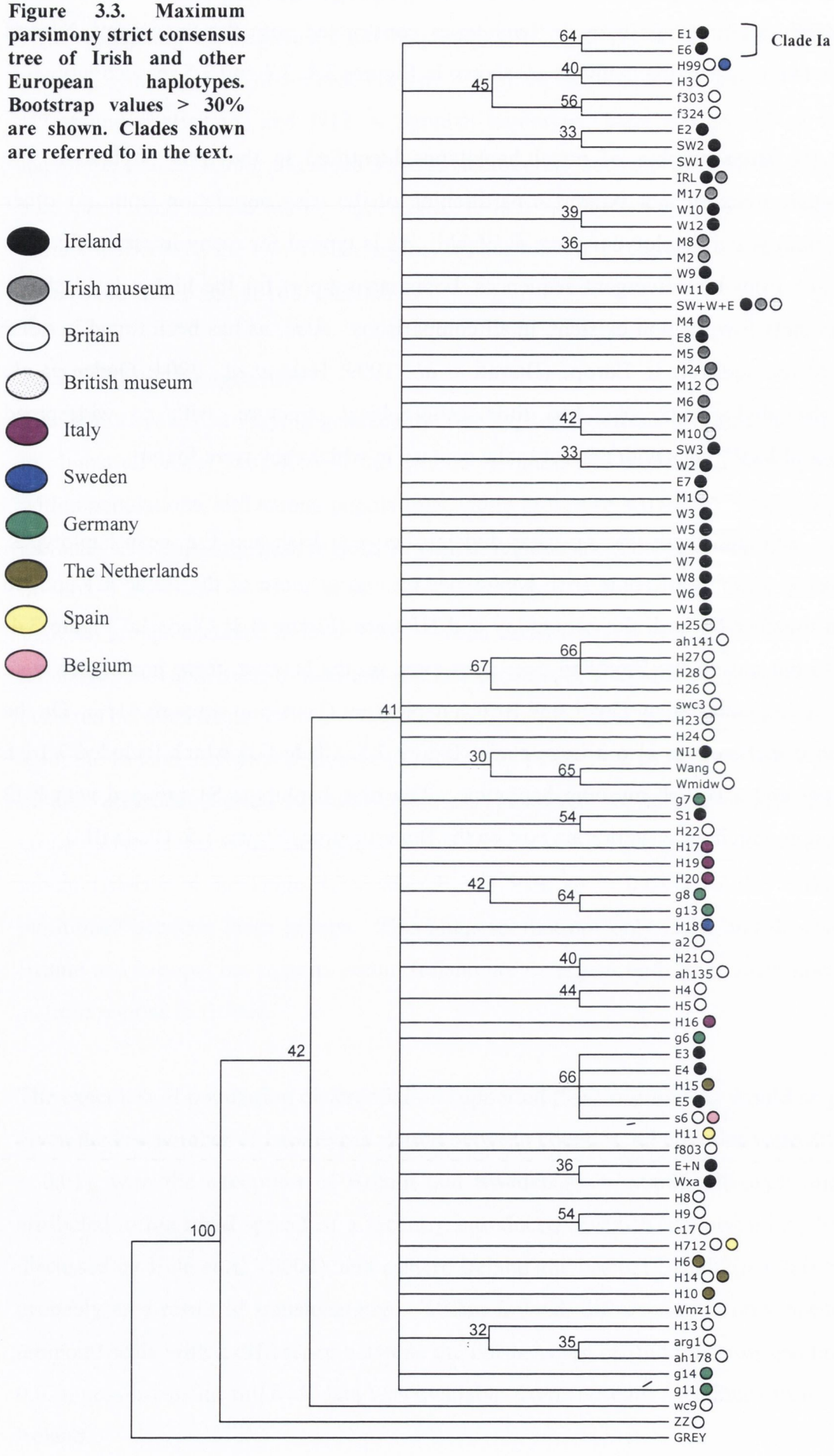
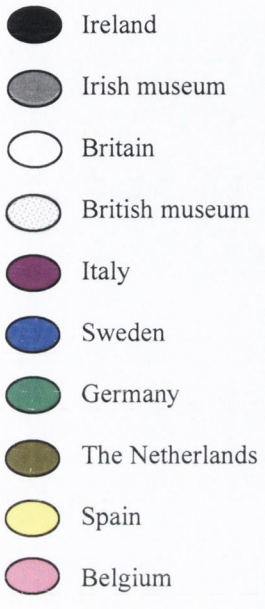


Figure 3.4. Neighbor-joining tree of *p* distances among Irish and other European haplotypes. Bootstrap values > 30% are shown. Clades shown are referred to in the text.



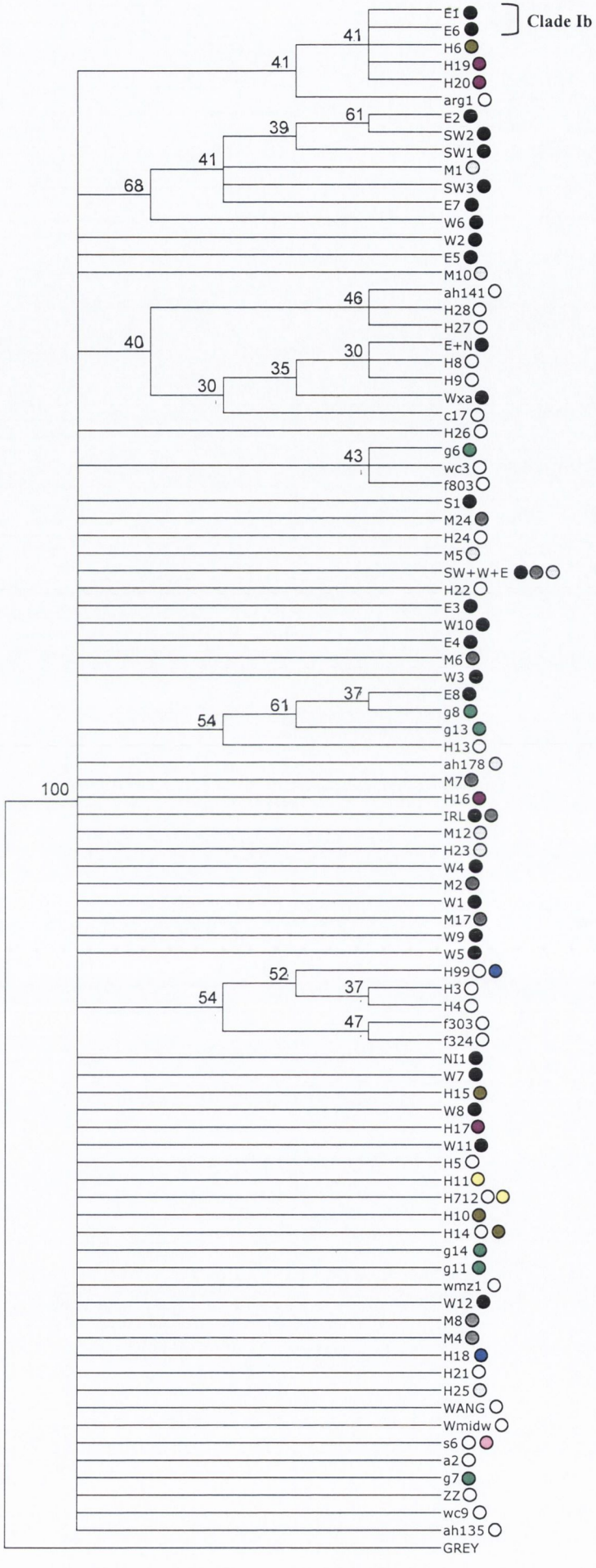
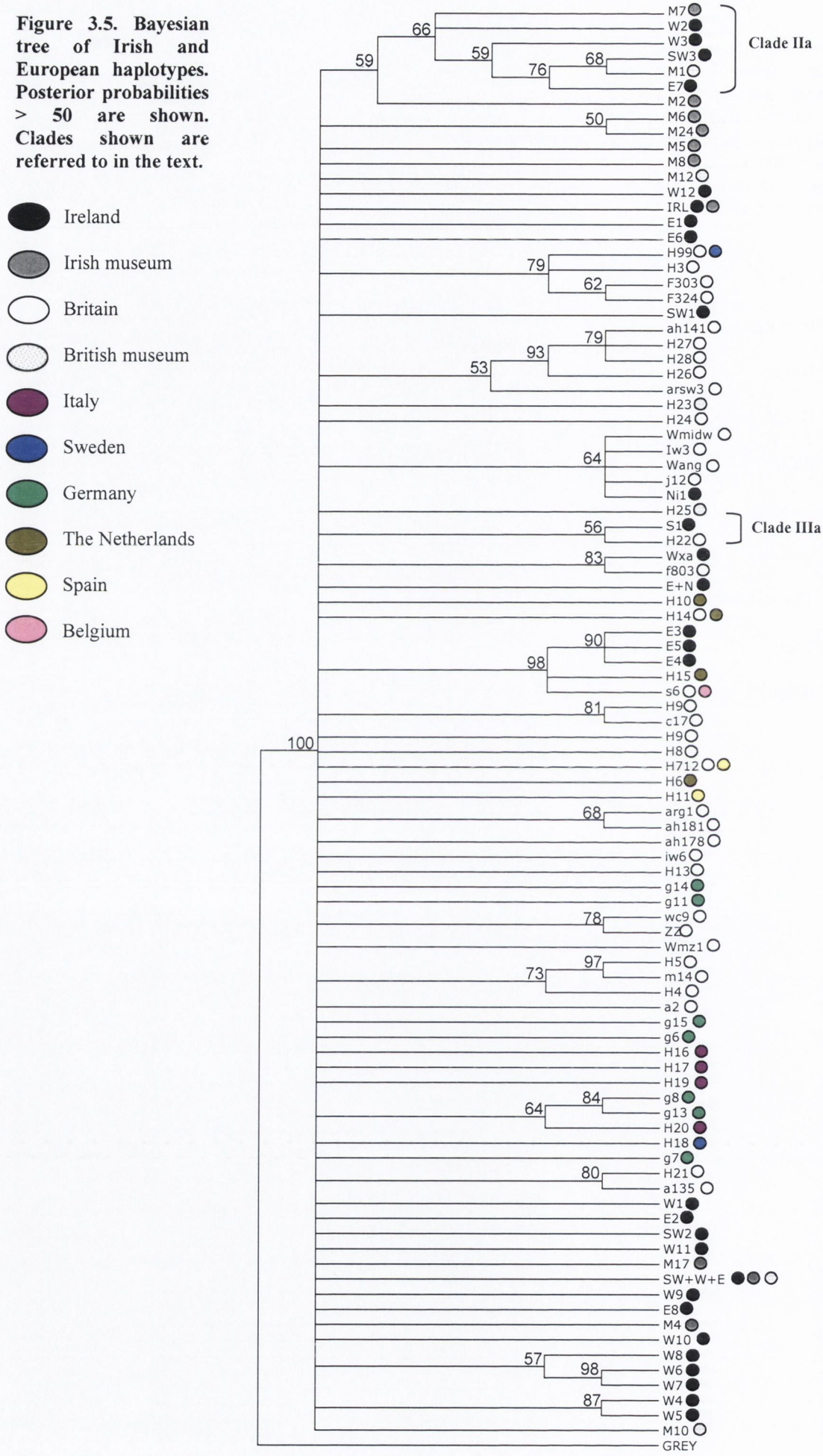


Figure 3.5. Bayesian tree of Irish and European haplotypes. Posterior probabilities > 50 are shown. Clades shown are referred to in the text.



The structure of the haplotype network immediately reveals why bootstrap support on the phylogenetic trees was so low (Figure 3.6). The star burst shape of the phylogeny, and the large number of reticulations within the network, explains both the lack of supported structure within, and the lack of agreement of haplotype relationships between, the haplotypes in the phylogenetic trees. Two haplotypes could not be joined to the network within the maximum number of 5 steps set by the 95% limit. These were f803, a British haplotype, and W12, an Irish haplotype. Although the haplotype H99 was designated as an outgroup root by the analysis, this was probably more a reflection on the large number of samples collected from Britain, and the large number of these which had that haplotype, rather than a this being the ancestral haplotype of the European phylogeny.

Overall the network results support the relationships found in the phylogenetic trees, with all haplotypes within the clades found on the trees occurring within a few mutations of each other on the network (Figure 3.6; Clade I, II and III). However, unlike what was found in the trees, there did seem to be some geographical partitioning of haplotypes between countries, although the large number of reticulations in the network complicates the relationships.

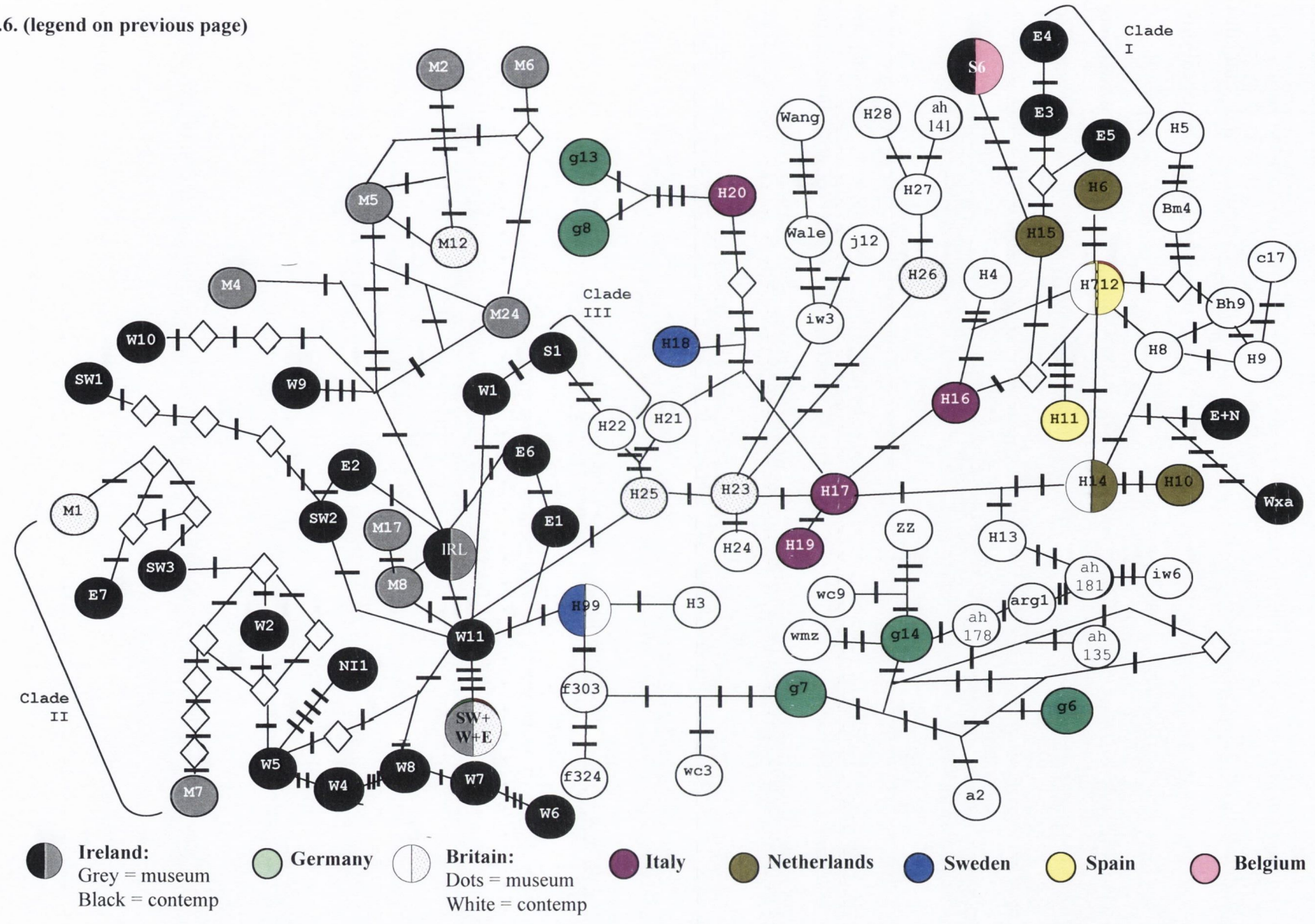
There was a clear clustering of the majority of Irish haplotypes together on the left hand side of the network, although the most common Irish haplotypes, IRL and W11, were only a maximum of 2 mutational steps from those found in Britain. Even those Irish haplotypes that appeared to be divergent from other contemporary European haplotypes were mutationally similar to British museum samples. On the extreme left of the network the haplotypes E7 and SW3 were quite similar to a haplotype found in a British museum specimen, M1. Similarly Irish museum haplotypes on the top left of the network were divergent from contemporary British haplotypes, but similar to M12, found in a British museum specimen.

There were, however, five Irish haplotypes that appeared extremely divergent from other Irish haplotypes. On the extreme right of the network, haplotypes E+N and Wxa were more similar to haplotypes from Britain, The Netherlands, and to some extent, Spain, than to other Irish haplotypes. Also, at the top right of the network, three eastern haplotypes in Clade I seem to be derived from an apparent Dutch introduction. With respect to other countries, there did not seem to be any affiliation between haplotype relationships and their country of origin.

The most divergent haplotypes in the network, besides the two which could not be inserted into the network under the maximum number of parsimonious steps, were three Irish haplotypes and two German. Wxa was a minimum of five mutational steps from any other haplotype in the network. SW1 was five steps from any other haplotype, and the network also suggested the existence of three intermediate and non-sampled haplotypes between that haplotype and the next nearest haplotype. Also, M7, an Irish museum haplotype was six mutational steps from any other haplotype.

Figure 3.6. (over) Statistical 95% parsimony network of all Irish and European haplotypes. Each line on a branch between haplotypes indicates one mutational change. Empty white circles are intermediate haplotypes that were not sampled. Clades I, II and III, corresponding to the clades on the phylogenetic trees, are indicated. Where haplotypes were found in more than one country, the colours within the node for that haplotype correspond to the countries as shown in the colour legend.

Figure 3.6. (legend on previous page)



3.3.2.3. Historical demographics and divergence

Population expansion

The different statistics measuring population expansion and population size showed varying results across the different regions (Table 3.6). The differences in the values of θ indicate that all populations, except northern Europe, are expanding, and that this expansion was most extreme in Britain, possibly influenced by the recent translocations. θ_f, reflecting current effective population size, was also largest in the British population, but also high in Ireland and western Europe. Conversely, a significant positive growth rate was only detected in Ireland (70.2 ± 13.22). Although the growth rate in Britain was also significant, even under the conservative 99% C.I., this was only slightly higher than zero. Similarly Fu's F_s also detected population growth only in Ireland.

Table 3.6. Various measures of population expansion and time, in generations, since expansion for the five European regions in the analysis. Significant deviations from Fu's selective neutrality are indicated in bold, as are deviations from the expected distribution of pairwise differences under sudden expansion in the mismatch analysis. Standard deviations for *g* and θ_f are also shown.

	τ	θ_o	θ_1	θ_f	g	Fu's F_s	Mismatch *p*
Ireland	7.79	0.00	7.54	0.19 ± 0.02	**70.20** ± 13.22	**-9.799**	0.90
Britain	6.14	0.00	43.91	0.37 ± 0.01	**9.77** ± 2.26	-6.029	**0.00**
West Europe	5.82	0.00	5.72	0.10 ± 0.02	19.20 ± 13.22	-0.689	**0.03**
South Europe	2.47	1.59	3.71	0.04 ± 0.01	-1.61 ± 12.83	0.839	0.09
North Europe	3.00	0.09	0.09	0.01 ± 0.003	-35.36 ± 19.68	2.299	**0.01**

The distribution of pairwise differences in the mismatch analysis conformed to the unimodal pattern expected of an expanding population in Britain, southern and western Europe (Figure 3.7a, b, c). The distribution of differences in Ireland did not show any peak in the number of differences between populations, with a gradual decrease across the graph (Figure 3.7d), albeit with a small peak at 7 differences between haplotypes. Statistically only the populations in Ireland and southern Europe were conforming to the sudden expansion model (p = 0.9 and 0.09 respectively).

The decreasing value of τ from Ireland to southern Europe suggests the southern region expanded relatively recently while countries in the west of Europe have had a longer more stable demographic history. However, the limited sampling in the southern region must be taken into account, and it is possible that this pattern would no longer be apparent with a larger sample size.

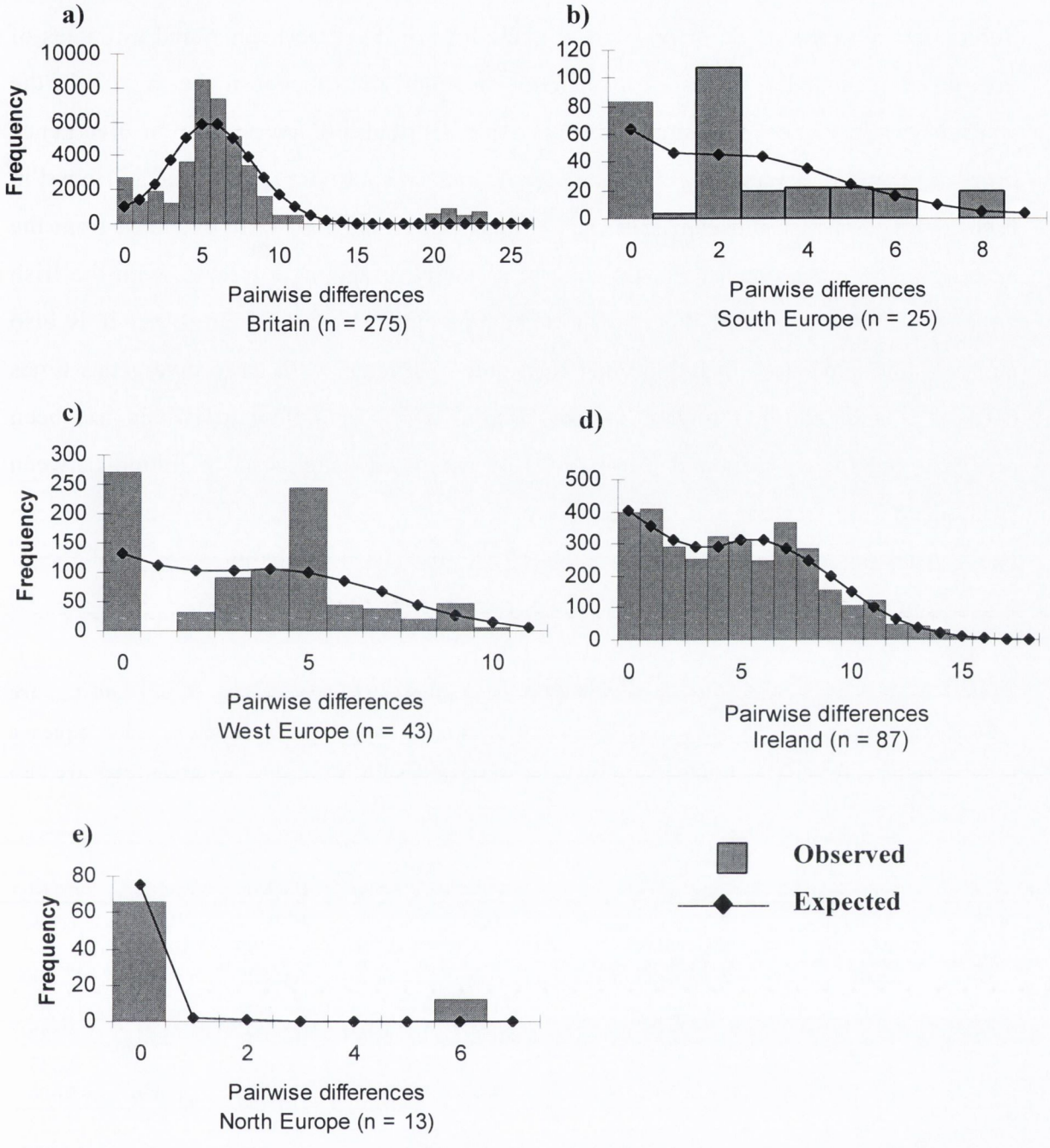


Figure 3.7. Mismatch distributions (observed and expected) for the five regions. The bars are the frequency distributions of pairwise differences between haplotypes within each region. Only distributions in Ireland (d) and southern Europe (b) did not deviate significantly from the expected distribution under the sudden expansion model.

Population divergence

Table 3.7 shows the mode of posterior probabilities of divergence time, and estimates of divergence time under a number of different mutation rates, between the regions in the analysis, while Figure 3.8a shows the posterior distributions for population divergence time. As SW+W+E was possibly a haplotype which was introduced into Ireland (found in both Irish and British museum specimens), this, as well as H99, were excluded from the analysis. The modes of t_{pop} do not support a glacial refugium in Ireland, with the Irish population diverging from the British population quite recently. However, it is also apparent that gene flow to Ireland has been quite restricted, with large divergence times between it and all other regions except Britain, while gene flow to Britain has been relatively high from mainland Europe. MDIV estimated patterns of migration between Britain and both western and northern Europe (mode = 1.3) were different to those seen between all other countries (mode = 0.3-0.7; Figure 3.8b), probably a result of recent translocations.

Table 3.7. MDIV estimates of divergence time (in generations) between regions. T_{MRCA} and t_{pop} are measured in units of $2N_e$ female generations and θ is $2N_e\mu$, where μ is the mutation rate per sequence per generation. Estimates of divergence time between regions for a range of mutation rates are also shown.

					T_{pop}			
		T_{MRCA}	t_{pop}	θ	0.5% Myr	1% Myr	5% Myr	20% Myr
IRL	Britain	3.210	0.32	11.07	298,666	149,333	29,860	7,460
	West	1.277	0.86	11.45	830,970	415,485	83,097	20,774
	South	1.177	0.56	12.58	600,000	300,000	60,000	15,000
	North	1.118	0.44	12.33	457,822	226,666	45,782	11,445
Britain	West	5.020	0.14	8.04	93,333	46,666	9,333	2,333
	South	5.304	0.32	7.40	133,333	66,666	13,333	3,333
	North	5.187	0.20	14.08	200,000	100,000	20,000	5,000
West	South	2.004	0.34	4.00	113,333	56,666	11,333	2,833
	North	2.241	1.32	3.81	400,000	200,000	40,000	10,000
South	North	3.321	1.68	2.24	266,666	133,333	26,666	6,666

The estimates of divergence time (t_{pop}) ranged from the most distant split between the northern and southern regions in Europe, and the most recent between Britain and western Europe, and between western and southern Europe. Divergence time between Ireland and Britain was dated towards the middle of the range of divergence times within Europe, with Ireland most divergent from western Europe.

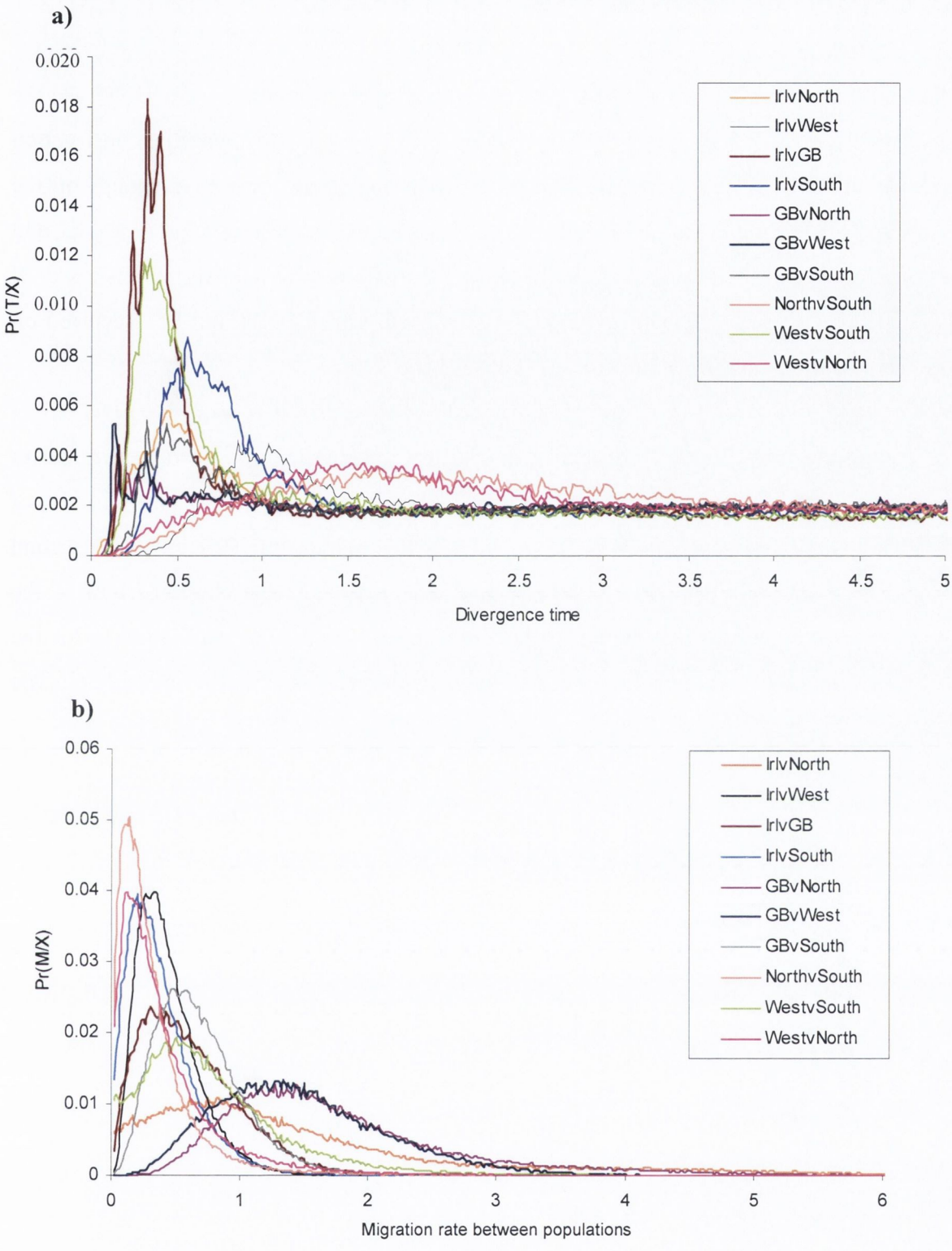


Figure 3.8. Posterior distributions of a) population divergence time (t_{pop}) and b) migration (M) for all possible comparisons of regions in Europe.

3.3.3. Irish phylogeography and historical demographics

3.3.3.1. Phylogenetics

When only the Irish haplotypes were used in the phylogenetic trees (Figures 3.9-3.11) the associations between haplotypes were more consistent across the different tree construction methods, and also reflected the division of the Irish haplotypes into three groups on the European parsimony network. Clade I, which consisted of two eastern haplotypes, E1 and E6, occurred on all the trees (Clade Ia, b, c), as did Clade II with the two haplotypes E+N and Wxa, while Clade III, with the three eastern haplotypes E3, E4 and E5, occurred on both the parsimony and Bayesian trees (Figure 3.9 and 3.11).

The parsimony tree (Figure 3.9) clearly divided the haplotypes into groups identical to those seen on the European haplotype network. One large group contained a mixture of Irish haplotypes and museum haplotypes, both from Ireland and Britain. The second group, Clade IIa, was the two haplotypes that had grouped at the extreme right of the European haplotype network, while in the third group, Clade IIIa, was made up of the haplotypes found to be more similar to Dutch haplotypes on the European haplotype network.

This pattern of distribution was not apparent on the other trees and there seemed to be no association between haplotypes and the region in which they were found.

Figure 3.9. Maximum parsimony strict consensus tree of Irish haplotypes. Bootstrap values > 30% are shown. Clades shown are referred to in the text.

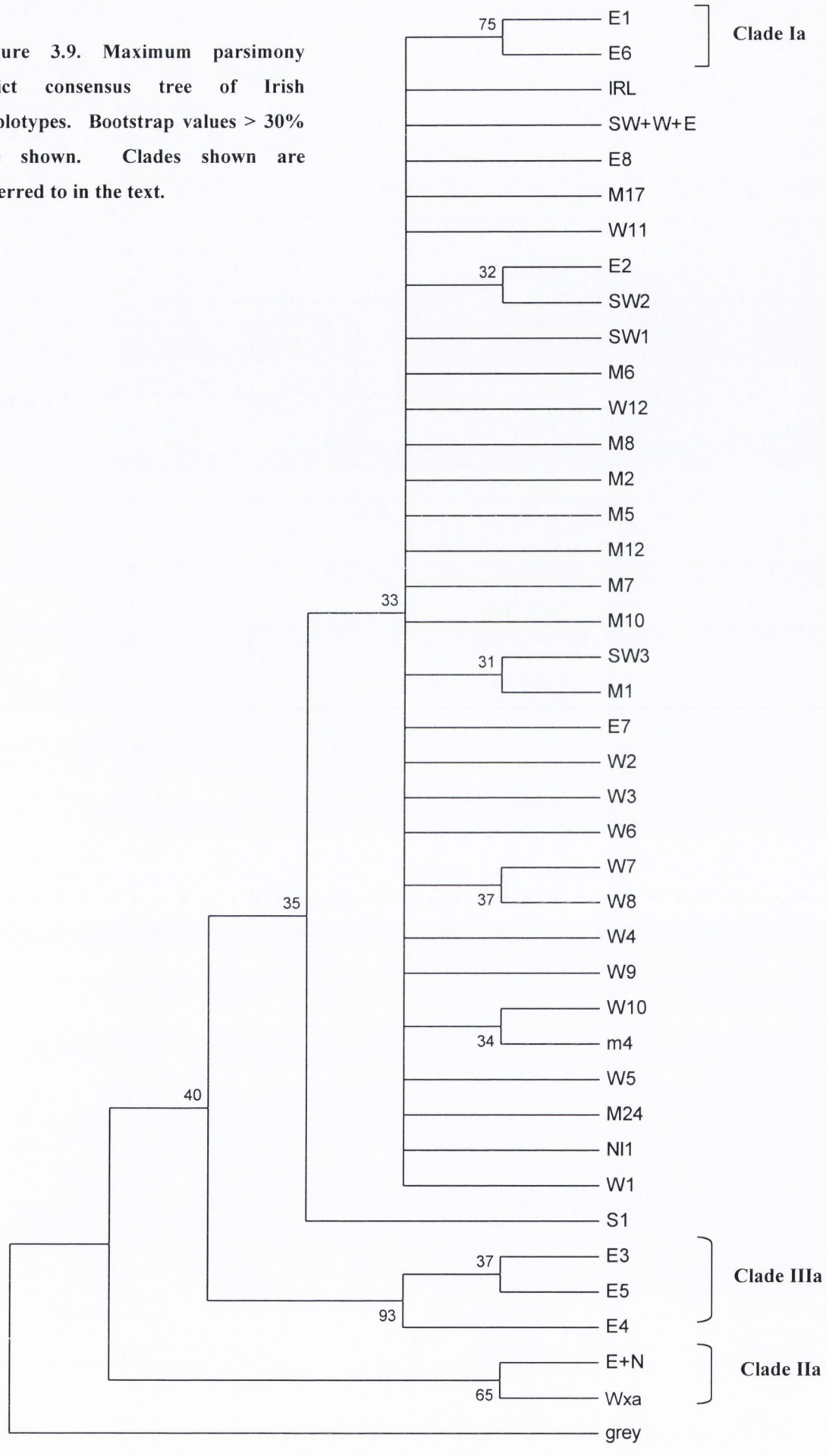


Figure 3.10. NJ tree of *p* distances among Irish haplotypes. Bootstrap values > 30% are shown. Clades shown are referred to in the text.

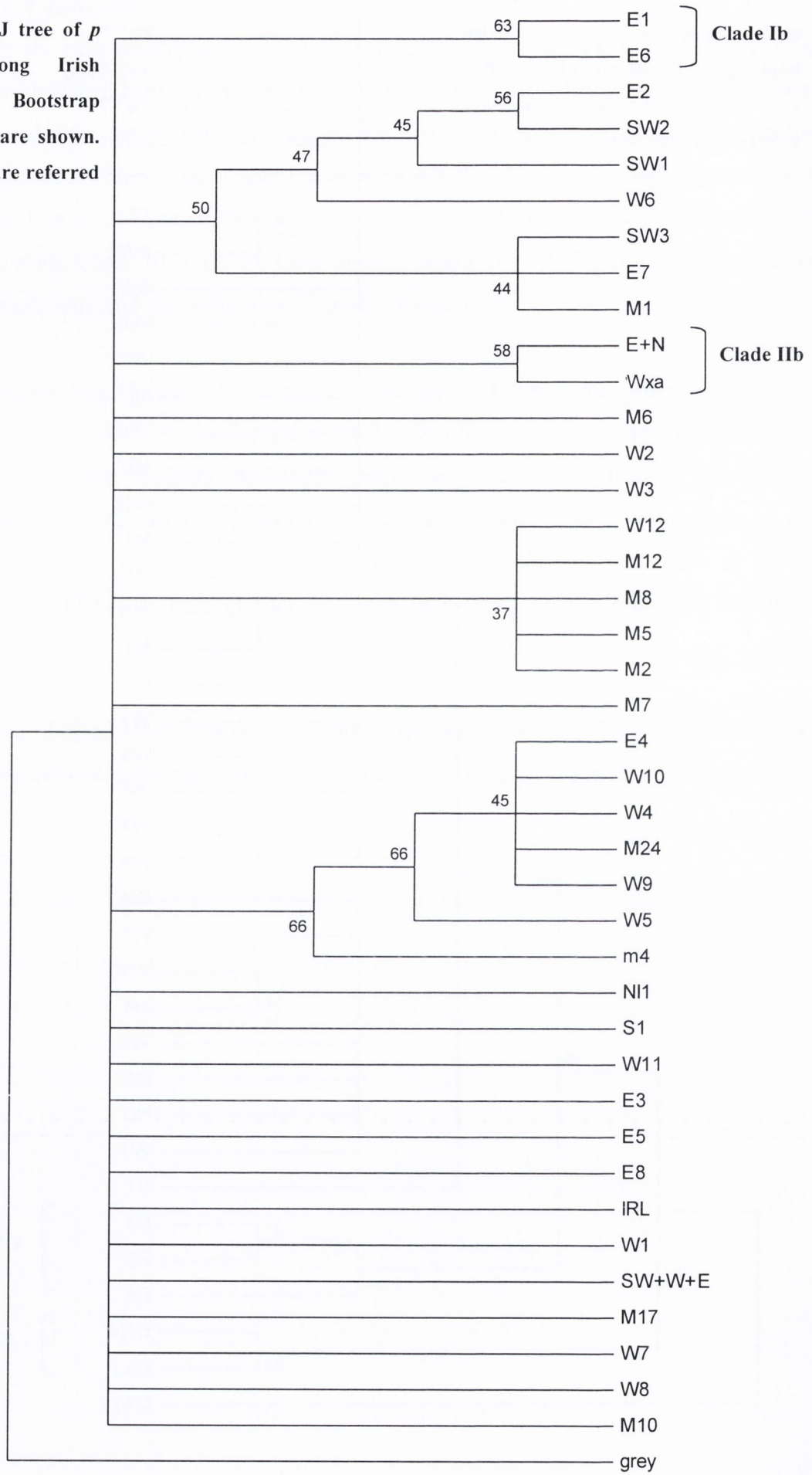
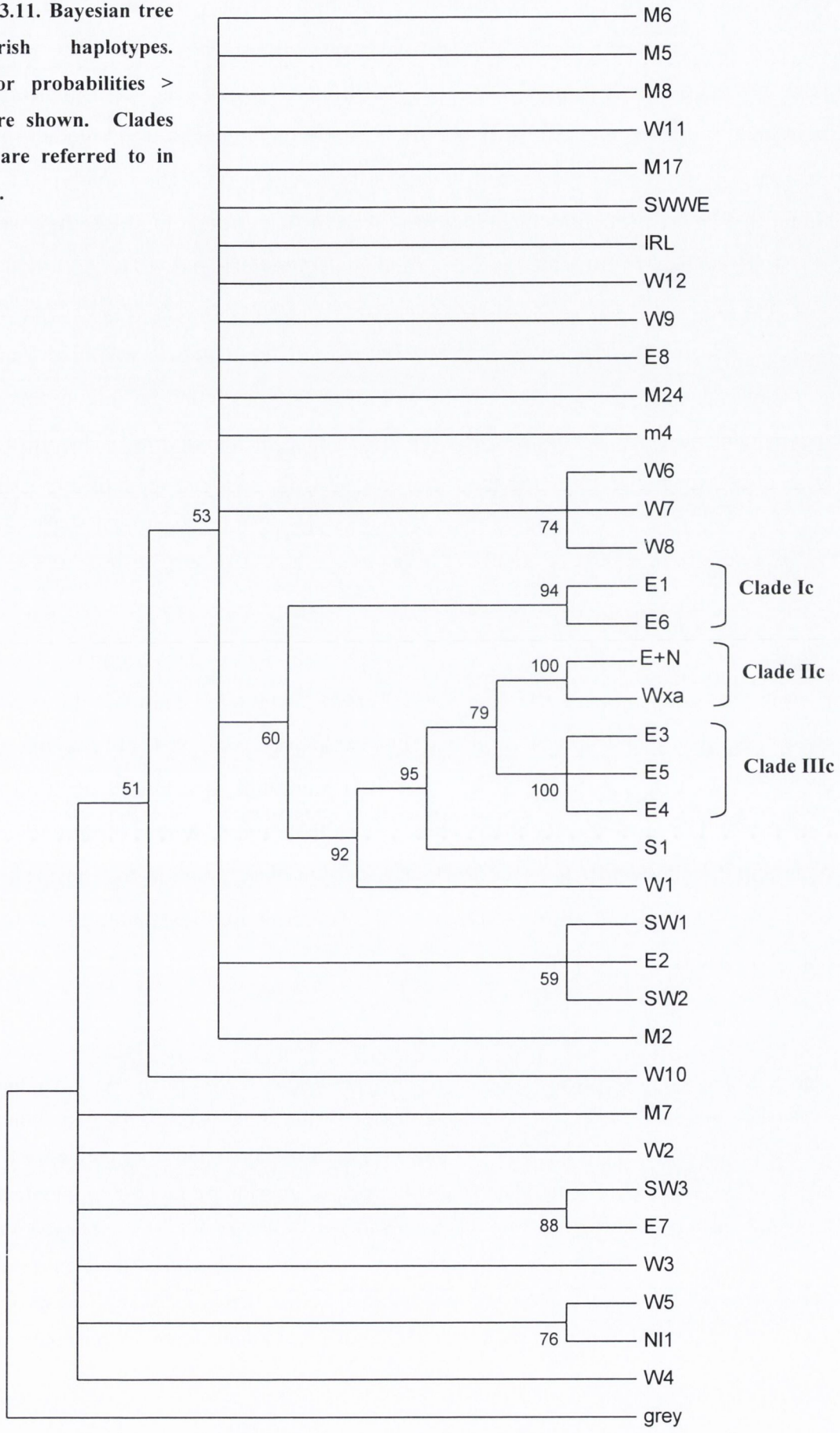


Figure 3.11. Bayesian tree of Irish haplotypes. Posterior probabilities > 50% are shown. Clades shown are referred to in the text.



The 95% parsimony network of Irish haplotypes (Figure 3.12) could not fit SW1, Wxa and M7 into the network within the maximum number of 4 steps defined by the parsimony criteria, although their position within the network, had they been included, is indicated. As with the European network, IRL and W11 were towards the centre of the network, with three groups deriving from them. The first group, shown in the top right corner of the network, groups the two clades II and III from the phylogenetic tree together, along with the most common haplotype found in the southern region, S1. The haplotypes E1 and E6, found in Clade I in the phylogenetic trees, were in this group, although they were only a few mutational steps from the most common haplotypes IRL and W11.

The second group of haplotypes, on the left side of the network, was also found on the European network, and consisted of the bulk of haplotypes found in Ireland, mixed in with the British museum haplotypes, and the Irish museum haplotypes. The third group of haplotypes, again also found on the European network, included most of the Irish museum haplotypes, a British museum sample and the haplotypes W9, W10 and W12 found in the west of Ireland.

Within this network, the haplotype W12, which could not be included under the parsimony rule on the European network, was now included towards the centre of the network, only two mutational steps from IRL. Divergent haplotypes were W9, NI and the group that contained E3, E4 and E5, all of which were a minimum of 3 mutations from any other haplotype. The most divergent haplotypes, with the largest number of mutations between them and the main body of the network, agreed with those found in the European network, i.e. SW1, Wxa and M7, all of which differed by a minimum of 5 mutations from any other haplotype within the network.

Figure 3.12. (over) Statistical 95% parsimony network of Irish haplotypes. Each line between haplotypes indicates one mutational change. Empty white circles are intermediate haplotypes that were not sampled. Haplotypes Wxa, M7 and SW1 could not be included on the network under the parsimony criteria but their position on the network, along with the number of mutations between them and the next nearest haplotype, is indicated by a dashed line. Where a haplotype was found in more than one region, the proportion of individuals with that haplotype in each region is shown. Node size reflects the number of individuals with each haplotype: IRL = 23, W11 = 16, E6 = 7, S1 = 7, SW+W+E = 6, W1 = 2, E+N = 3, W12 = 2, all others n = 1. Groups shown are referred to in the text.

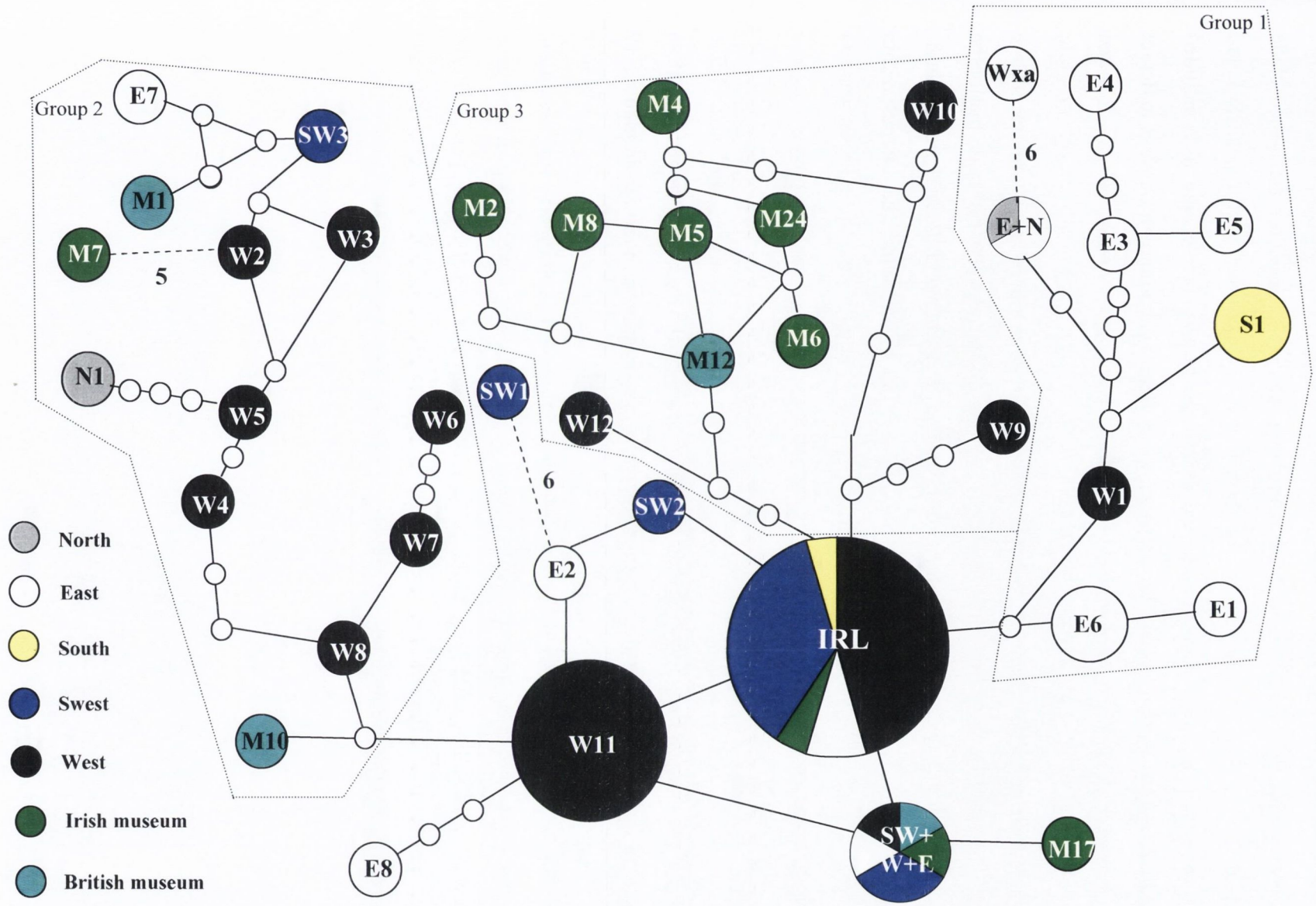


Figure 3.12. (legend on previous page)

3.3.3.2. Phylogeographic relationships

A haplotype network of all extant Irish haplotypes was constructed for the nested clade analysis (Figure 3.13). However, as the 95% parsimony excluded haplotypes SW1 and Wxa, the 93% parsimony network was used to infer the nested clade design, as it included all haplotypes. Also, there were two instances when there were a number of ways to depict the relationships between haplotypes. SW2 could be joined to either IRL or E2, and SW+W+E could be joined to IRL or W11. NCA was carried out using all eight combinations of these haplotype connections and, although only one set of results are presented, the results of the inference key did not differ between different haplotype associations. Figure 3.13 shows the nesting design constructed following the methods outlined by Templeton *et al.* (1992). The large number of unsampled haplotypes within the network made the nesting design far from definite, although when the analysis was run using different nesting designs, the results from the analysis remained the same.

The null hypothesis of no geographical association was rejected at all nesting levels (Table 3.8) including the entire cladogram. The inference key devised by Templeton (2004) suggested that the 1 step clades were produced by fragmentation, although in clade 1-19 this fragmentation occurred in the past, and could be combined with long distance dispersal, while within clade 1-21 this was wholly attributed to allopatric fragmentation. The 2 and 3 step clades showed genetic structure that was compatible with restricted gene flow within the red squirrel population, together with some long distance dispersal. In the total cladogram, distribution of haplotypes was consistent with restricted gene flow and isolation by distance (Table 3.8).

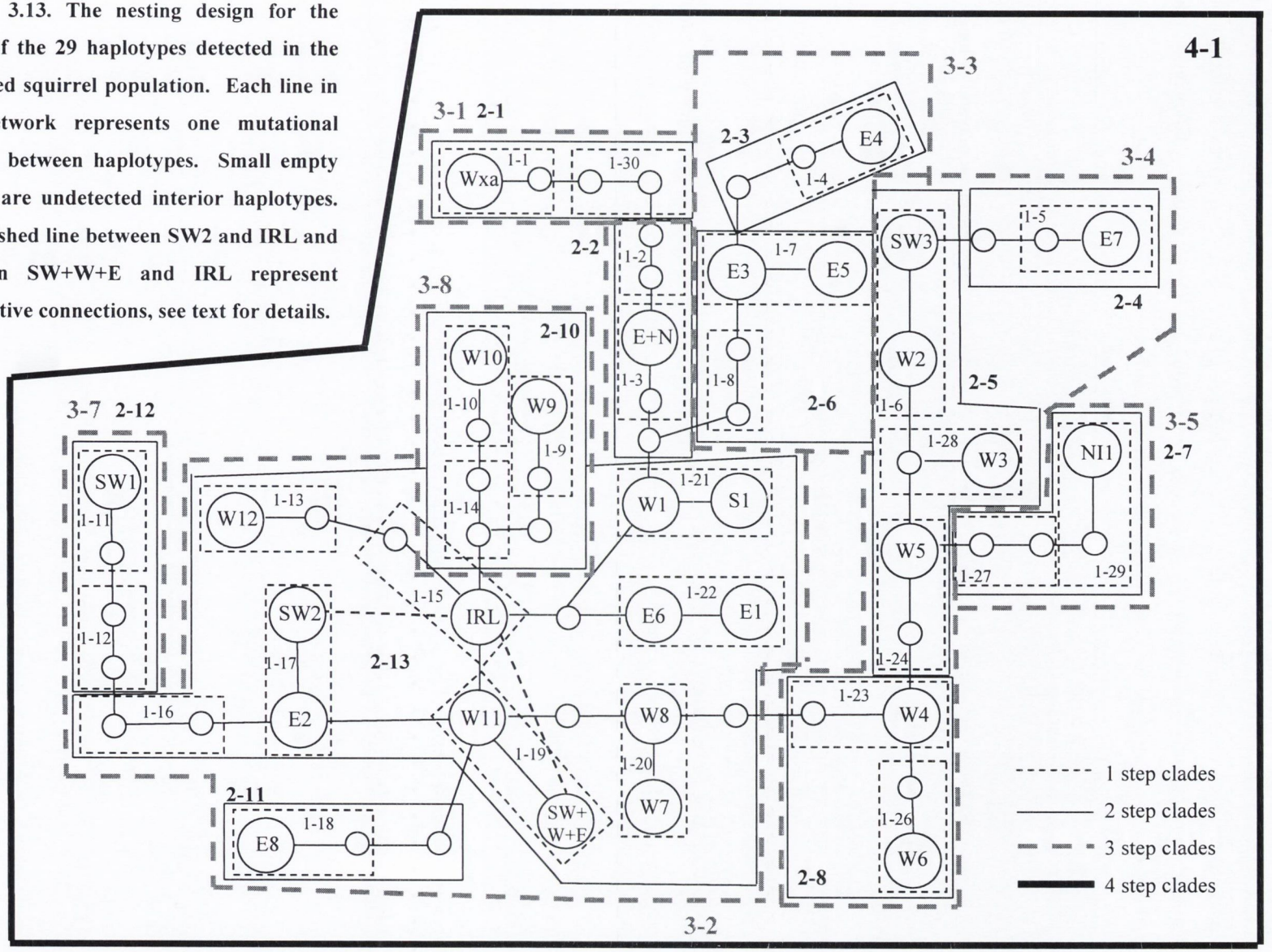


Figure 3.13. The nesting design for the NCA of the 29 haplotypes detected in the Irish red squirrel population. Each line in the network represents one mutational change between haplotypes. Small empty circles are undetected interior haplotypes. The dashed line between SW2 and IRL and between SW+W+E and IRL represent alternative connections, see text for details.

Table 3.8. Nested cladistic statistics of the relationship between haplotype distribution and geographical distance within the Irish red squirrel population. Only data from clades that had significant geographical or genetic variation are shown. Significant differences are indicated in bold. A superscript S means the distances were significantly smaller than expected at the 5% level, while L means differences were significantly larger than expected. The chain of questions leading to the hypotheses behind geographic association within each clade, following the updated inference key given by Templeton (2004), are also shown.

Clade		Nested clades	D_c	D_n	Chain of inference
1-19					
χ^2	**14.14**				
D_cI-D_cT	**-167.79^S**	W11	**4.56^S**	**37.63^S**	1,2,3,5,15, No = past fragmentation and/or long distance dispersal
D_nI-D_nt	**-79.29^S**	SW+W+E	**172.35^L**	**116.92^L**	
1-21					
χ^2	**9**				
D_cI-D_cT	61.47	W1	69.6	**81.3^L**	
D_nI-D_nt	**47.61^L**	S1	**8.13s**	**33.69^S**	1,19,20,2,3,4,9, No = allopatric fragmentation
2-13					
χ^2	41.27	1-13, 15, 16	121.3	**107.03^L**	
D_cI-D_cT	85.46	1-17, 19, 20	21.7	**149.83^L**	1,2,3,5,6,7, Yes = restricted gene flow with some long distance dispersal
D_nI-D_nt	5.73	1-21,22	**62.77^S**	**77.95^S**	
3-2					
χ^2	**94.15**				
D_cI-D_cT	**102.69^S**	2-14	**102.69^S**	112.81	1,2,3,5,6,7, Yes = restricted gene flow with some long distance dispersal
D_nI-D_nt	-20.78	2-2	94	**155.23^L**	
4-1					
χ^2	180.51				
D_cI-D_cT	**107.72^L**	3-3	**14.68^S**	120.88	1,2,3,4, No = restricted gene flow with isolation by distance
D_nI-D_nt	-6.64	3-5	0	**198.08^L**	

3.3.3.3. Irish historical demographics and divergence

Population expansion

The differences in the values of θ (Table 3.9) indicate that all regions except the southwest are undergoing expansion. However Fu's F_s only detected expansion in the western region and in museum samples, while significant growth (*g*) was only found in the latter. The largest effective population size was in the western region and the museum samples, reflecting both the large number of haplotypes collected in the western region, and the fact that no two samples had the same haplotype in the museum samples. However, in the mismatch distributions (Figure 3.14) none of the regions had the unimodal distribution characteristic of population growth. The two peaks in the southwestern and eastern regions suggest two separate colonisation events of those areas (Figure 3.14b and d).

Table 3.9. Various measures of population expansion and time since expansion for the five regions in Ireland plus samples collected before 1915. Significant deviations from Fu's selective neutrality are indicated in bold, as are deviations from the expected distribution of pairwise differences under sudden expansion in the mismatch analysis. τ , θ_o, θ_1 and analysis of mismatch distributions could not be calculated for the northern region and the Irish museum samples, the former due to the small sample size (n = 2) and the latter because the analysis could not converge did 1800 steps. Likewise θ_f and g could not be calculated for the north due to the small sample size. Standard deviations of *g* and θ_f are also shown.

	τ	θ_o	θ_1	θ_f	*g*	Fu's F_s	Mismatch *p*
South	3.0	0.00	0.186	0.03 ± 0.09	-14.9 ± 13.63	1.946	**0.85**
Southwest	6.0	1.65	1.653	0.12 ± 0.003	-2.03 ± 4.03	-0.075	**0.55**
West	7.6	0.00	3.405	0.28 ± 0.068	10.76 ± 4.90	**-3.973**	**0.70**
East	8.6	0.00	13.405	0.045 ± 0.068	**15.3** ± 2.86	-1.274	**0.60**
<1915				0.84 ± 0.38	**85.37** ± 16.20	**-4.090**	
North						2.197	

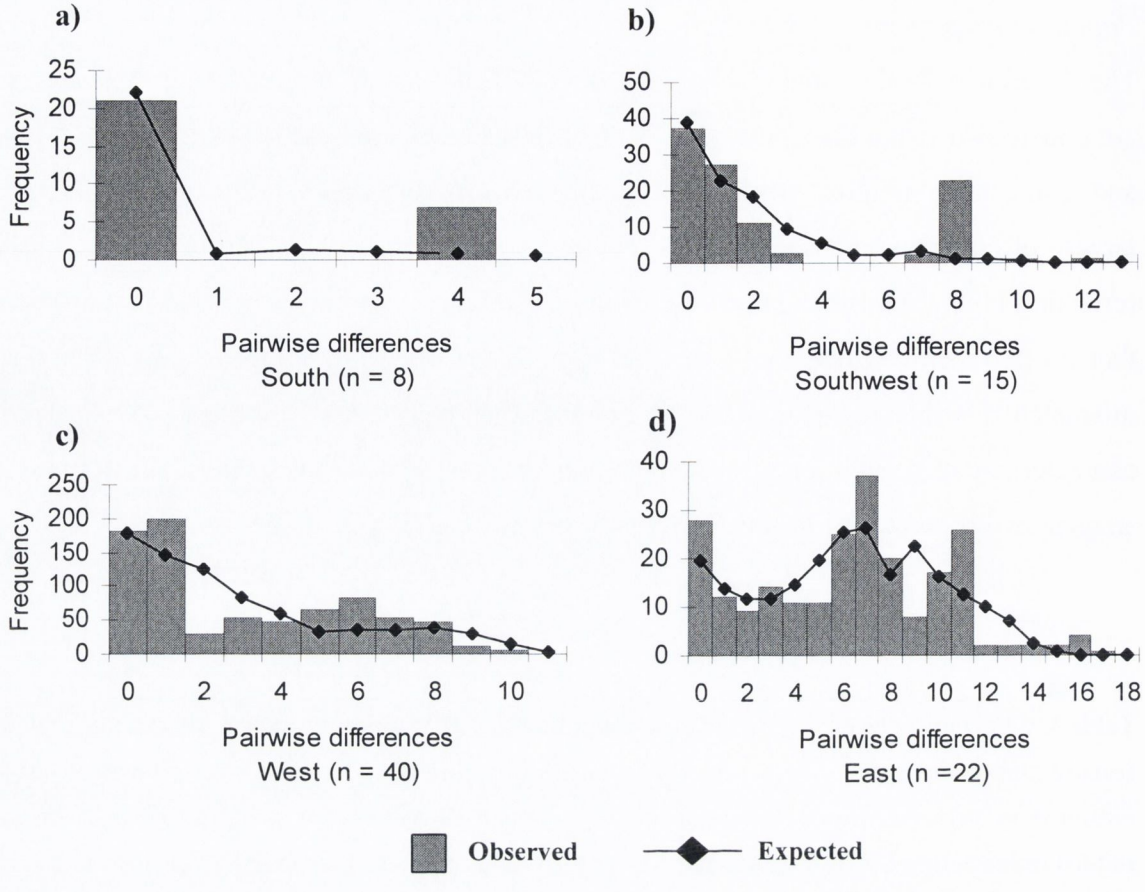


Figure 3.14. Mismatch distributions (observed and expected) for the four regions in Ireland. The north could not be analysed due to the small sample size. No region had the unimodal distribution described for population growth although the bimodal distributions in the east and southwest suggest two colonisation events of those areas.

Population divergence

The analysis of divergence within Ireland (Table 3.10) revealed some differentiation between regions. The most divergent regions were the west and north (t_{pop} = 10), and the west and the east (t_{pop} = 1.14), while the southwest and west, and the southwest and south were most similar (t_{pop} = 0.004; 0.020 respectively). However, the plots of posterior probabilities of t_{pop} (not shown) revealed no peak in the distribution of values, indicating that the populations have not diverged significantly from each other. Generally the linearised F_{ST} values did reflect the results obtained from the MDIV analysis, with the largest F_{ST} not between the south and southwest, but instead between the north and south

.

Table 3.10. MDIV estimates of divergence time (in generations) between Irish regions. T_{MRCA} and t_{pop} are measured in units of $2N_e$ female generations and θ is $2N_e\mu$, where μ is the mutation rate per sequence per generation. Slatkin's linearised F_{ST} between regions are also shown.

		T_{MRCA}	t_{pop}	θ	F_{ST}
South	Southwest	4.010	0.020	2.430	1.27
	West	2.246	0.200	3.244	1.21
	East	2.800	0.400	5.460	0.29
	North	3.566	0.760	3.070	1.59
Southwest	West	2.112	0.004	4.744	0.08
	East	2.325	1.140	6.070	0.21
	North	2.496	0.660	4.730	0.94
West	East	1.982	0.460	6.990	0.35
	North	1.834	10.000	6.060	0.91
East	North	2.137	0.940	7.220	0.09

The plot of Slatkin's linearised F_{ST} between all regions in Ireland and all regions in Britain (Figure 3.15) did not reveal a clear picture of the origin of the Irish red squirrel population. The northern Irish region (North Irl) was more similar to Anglia in eastern England than to other Irish regions, suggesting this may be a possible reintroduction source for that region. However, eastern and southern Ireland (East and South Irl) grouped together and appear to be divergent from all regions in Britain. The nearest British region to these Irish regions was East UK.

The most unusual result is the extreme closeness of Southwest, West and museum (Irl < 1915) specimens from Ireland to the museum specimens from Britain (UK < 1914; Figure 3.15, inset). Despite being separated by almost 100 years, the southwestern and western regions were more similar to the museum specimens than to other regions within Ireland.

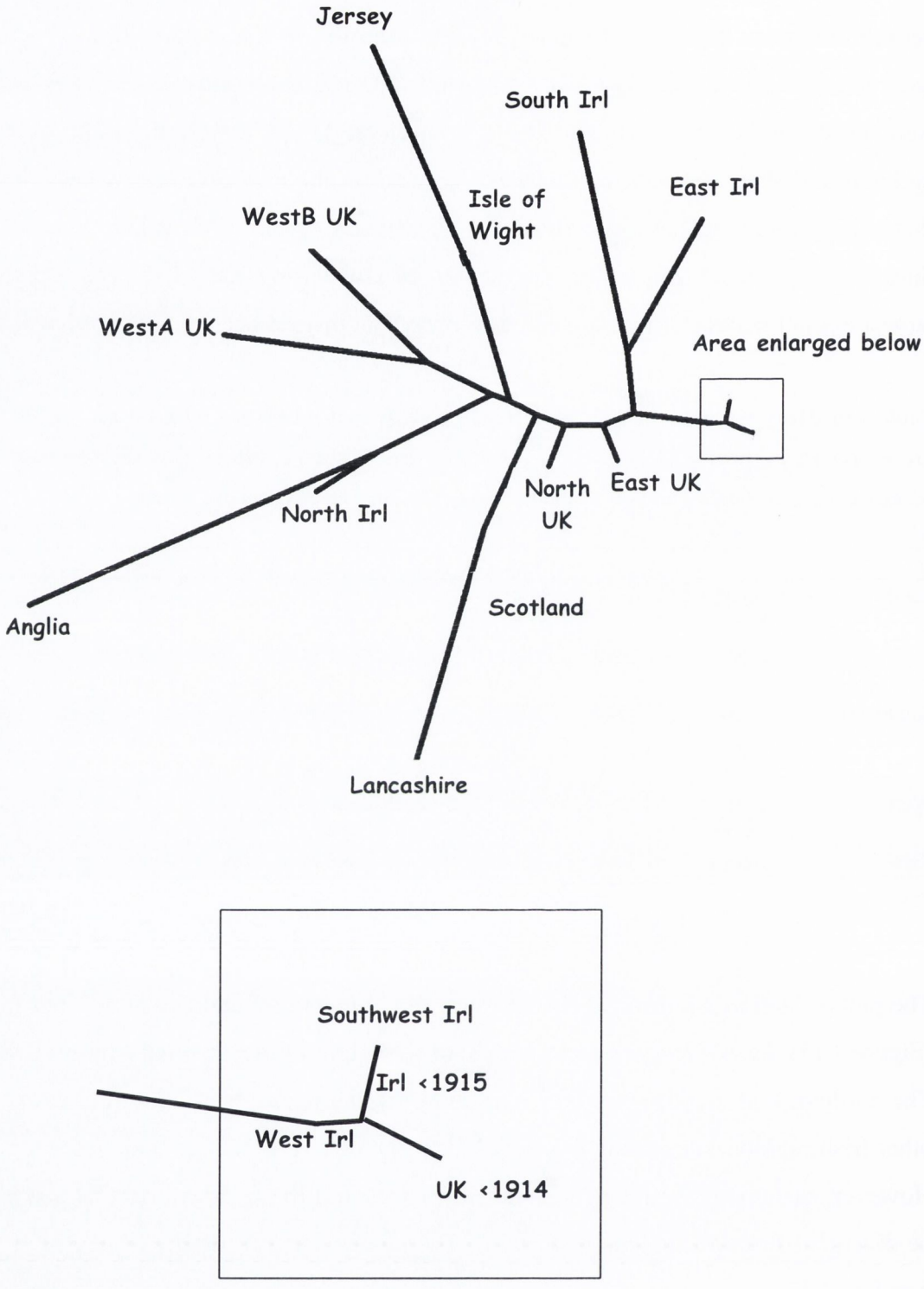


Figure 3.15. Bootstrapped unrooted NJ tree of Slatkin's linearised distance. All bootstrap values for all branches were 100%.

3.4. Discussion

The large number of haplotypes (n = 29) amplified from the samples in this study is not unusual for a red squirrels and is, in fact, lower than the number found by Trizio *et al.* (2005) in a more restricted geographical area in Italy (n = 23). However, it is higher than the 22 haplotypes which have been described in the British population (Barratt *et al.*, 1999; Hale *et al.*, 2004) and the possibility of errors within the data set from this study in Ireland must be acknowledged. Amplification of mtDNA from degraded samples, like museum or hair samples, may result in erroneous base pairs or deletions in the sequence. To avoid this amplification and sequencing of internal overlapping fragments, and checking for nuclear copies, is recommended (Martínková & Searle, 2006). Unfortunately the limited funds available for this study meant internal sequencing was not possible. Nevertheless an attempt was made to minimise errors by sequencing, and comparing, amplified fragments in both directions, and running amplification products on an agarose gel to check for nuclear copies. However, even given these precautions, the possibility of errors within the sequences used in this analysis must be acknowledged, although their influence on the comparisons carried out here was probably minimal due to a) the only variable sites detected in the Irish samples were also variable in research described from other countries (Barratt *et al.*, 1999; Hale *et al.*, 2004; Ogden *et al.*, 2005), and b) in no case were there ambiguities (i.e. unknown bases on one of the sequences) at those variable sites in the sequences obtained in this study.

3.4.1. European phylogeography

The phylogenetic trees constructed here reflected the pattern seen in other trees constructed from red squirrel data (Barratt *et al.*, 1999, etc.), with low bootstrap support, and little geographical division. However, the construction of a haplotype network revealed patterns of distribution and association hitherto undetected in red squirrel populations. Despite the European network (Figure 3.6) being skewed by the large number of individuals collected from Ireland and Britain, and limited by the large unsampled areas within Europe, e.g. France, its structure suggests the patterns of postglacial colonization of Europe from Mediterranean refugia recorded in other studies (e.g. Brito *et al.*, 2005; Michaux *et al.*, 2003; Piertney *et al.*, 2005).

Traditionally three glacial refugia have been suggested in Europe; the Iberian peninsula, the Balkans and Italy (Hewitt, 1999; Taberlet *et al.*, 1998). As already reported by Hale *et al.* (2004), the low number of haplotypes in the red squirrel population in Spain is unusual for a refuge population, but this could be attributed to genetic drift or, more likely, the limited samples available from that country. The high haplotype diversity in Italy, found by both Hale *et al.* (2004) and Trizio *et al.* (2005), is more indicative of a glacial refugium; however, again, further sampling in Italy would be needed to fully confirm this.

Also, the similarities between the Italian and Swedish, plus some of the German, haplotypes suggest a northwesterly spread from Italy to western Europe. The low number of mutations between the Dutch and Spanish haplotypes suggests that the Iberian Peninsula is the main source of haplotypes in Holland and Belgium. By combining these results, it seems that western European red squirrels diverged from a mixture of individuals from the Iberian and Italian refugia. Although the low number of samples resulted in a pooling of data from the Iberian Peninsula and Italy, the MDIV results of t_{pop} supported this stepwise spread from south to north, with the most recent split between western and southern Europe, the most distant between northern and southern Europe, and an intermediate split between western and northern Europe.

The results from this study imply that the spread of the red squirrel throughout Europe may follow the 'hedgehog' pattern of postglacial spread (Hewitt, 1999) although, further sampling from both Italy and Spain, and more eastern parts of Europe, is required to confirm this. This paradigm of postglacial spread was also described for white oak (Dumoulin-Lapègue *et al.*, 1997), with spread from two, but possibly all three, glacial refugia, and no evidence that the Italian Alps presented a significant barrier to dispersal. Unlike the spread of Norway spruce, *Picea abies*, there is no evidence of colonisation of the red squirrel of Scandinavia from a refugium in Russia (Lagercrantz & Ryman, 1990).

Although red squirrel spread seems to reflect white oak spread, the species is not a tree specialist, and it is likely that its current distribution within Europe reflects a mixture of the colonisation histories of different tree species. The only possible constraints, which may have resulted in a more definite association between red squirrel spread and a particular tree species, is if there is indeed some adaptive association between a particular tree species, or habitat type, in the red squirrel population. Ecologically, the red squirrel seems to be equally adapted to survival in forests of most tree species (e.g. Holm, 1990; Lurz,

1995; Lurz *et al.*, 2005; Reilly, 1997), but there may be some morphological or genetic adaptations in red squirrel populations in response to different habitat types, and these will be investigated further in Chapters 4 and 5.

Nevertheless, even though the data suggested a spread from south to north, the close relationships between geographically diverse haplotypes must be taken into account. Could red squirrels have survived in other refugia in Europe as well, with these close relationships between haplotypes a result of introgression between a number of different refugia populations? Refugia have been suggested, based on both pollen records and faunal remains, in Belgium, Slovakia, southwest Ireland and England, northwest Scotland and even as far north as the western coast of Norway (Stewart & Lister, 2001 and references therein, but see Deffontaine *et al.*, 2005). Evidence suggests that not only small, possibly tundra-adapted small mammals survived in these refugia (Bilton *et al.*, 1998; Brunhoff *et al.*, 2003; Jaarola & Searle, 2002), but also that conditions may have been suitable for more temperate, forest species.

Willis *et al.* (2000) carbon dated charcoal residues from Hungary in central Europe, an area which was unglaciated during the last full ice age (Denton *et al.*, 1971 in Willis *et al.*, 2000) and found evidence that *Pinus sylvestris*, *P. cembra* and a number of other tree species were present in the area during the last full glacial maximum 32,500 -16,500 years B.P. In fact, temperate species were detected in all of the cryptic refugia mentioned above (Stewart & Lister, 2001 and references therein). Recent genetic work by Deffontaine *et al.* (2005) and Kotlík *et al.* (2006) found evidence for a temperate mammal surviving in a central European refugium. From their results, they hypothesized that the bank vole, *Clethrionomys glareolus*, survived in the unglaciated, forested river systems in the Alps or Carpathian mountains. Like the bank vole, the current distribution of red squirrels includes a wide range of latitudes and it too can survive in cold environments where suitable habitat is available (Lurz *et al.*, 2005). It is therefore possible that the red squirrel also survived in these hypothesised central refugia, or even more northerly refugia, and postglacially radiated outwards.

Although neither contemporary samples nor archaeological remains have been collected from central refugial areas to support the through-glacial persistence of red squirrels in these areas, archaeological samples have been recovered from the suggested northerly refugium. These fossil remains of *Sciurus vulgaris*, which dated to 11,510 – 10,360 years

BP, were found in a cave in western Norway (Larsen *et al.*, 1987), and indicate firstly that these smaller cryptic refugia were suitable to support a red squirrel population, but also, more significantly, as previously suggested, that the pattern of distribution of red squirrel mitochondrial relationships may be a result of introgression between individuals from a number of different refugia. Likewise, the pattern of divergence time previously described, which was attributed to a spread from south to north, could equally be explained by colonisation from north to south.

The demographic history of the different regions was difficult to interpret owing to the influence of indistinguishable historical and recent events on the populations, and should be treated with caution. However, the different measures of growth indicated that the northern populations were not expanding and, in fact, seemed to be declining, while south Europe seemed relatively stable and the data for western Europe suggested that it was expanding. As previously discussed by Hale *et al.* (2004), the northern region was dominated by a single haplotype, which was found over both a widespread geographical and temporal range in Sweden. This implies that the red squirrel population in the refugium in Norway did not persist, and the strong relationship between haplotypes in Sweden and mainland Europe suggests that colonisation was from south to north rather than vice versa. The widespread distribution of haplotype H99 in Sweden could be a result of a severe bottleneck as a result of a founder event, or due to this haplotype being associated with a particular adaptive trait, resulting in its dominance in Sweden. This will be discussed further in Chapter 5. Whether the red squirrel survived in central European refugia will only be determined after further sampling.

If interpretation of the phylogeographical results on mainland Europe was difficult, the results for Britain are even less clear. The British population has been subjected to numerous introductions from mainland Europe (Harvie-Brown 1880; Lowe & Gardiner, 1983), and the haplotype network indicates these introductions could have been from any, or all, of the mainland European countries sampled in this study. The sharing of haplotypes between Britain and Sweden, and Britain and Spain, indicates translocations of individuals between those countries at some stage in the past. Hale *et al.* (2004) have already shown that, in the first case, the translocation was from Sweden to Britain, and not vice versa, however, too little data are available to determine the direction of the translocation in the latter case. Certainly some British individuals appear more related to Dutch and Spanish haplotypes than to other British haplotypes, and there was another

group of British samples that were more similar to two sequences from Germany, but there was no regional pattern to this distribution. Depending on when in time the translocations took place, and the extent of forest fragmentation afterwards, and presuming that individuals were not translocated all over the country at the same time, some regional pattern of distribution of mainland European haplotypes within Britain would be expected. Therefore, it is possible that these associations between European and British haplotypes indicate the source of individuals involved in the postglacial colonisation of Britain.

The MDIV results, however, refuted this. Both in the analysis of t_{pop}, and when actual divergence times were calculated from the range of mutational rates, divergence time between Britain and any of the mainland European populations was dated as being quite recent, more recent in fact than the divergence between Ireland and Britain, which is known to have occurred approximately 150 years ago (Barrington, 1880). Therefore, the similarities between Britain and mainland Europe seem to be a result of postglacially colonising, or introduced, individuals mixing with more recent translocations; the translocations occurring after the translocations into Ireland, and the original colonisation at some stage before then - nested clade analysis of British haplotypes might confirm this.

3.4.2. Is the Irish red squirrel Irish?

The nested clade analysis performed here (Figure 3.13; Table 3.8) concluded that the genetic structure in Ireland was not a result of postglacial colonisation from either a single colonisation from Europe or Britain, or from a refugium in the southwest of the country, but rather was a result of fragmentation combined with some long distance dispersal, probably due to the translocations from Britain in the 1800's. Also, the population expansion analysis (Table 3.6) found the red squirrel population in Ireland is undergoing recent expansion from a population bottleneck. These results were not unexpected, given the history of the red squirrel in Ireland where, even if remnant red squirrel populations had survived in the country, those populations would have been reduced to low numbers, leading to the presumption that the species was extinct in Ireland (Barrington, 1880). Therefore, population expansion would be expected in Ireland no matter whether the population consists of solely reintroduced individuals, or a mixture of reintroduced and native individuals.

What was curious was the division of Irish haplotypes into three groups on the network. Understandably, the largest group was associated with the British haplotypes; however, there was one group of haplotypes that seemed to be derived from Dutch introductions, while a second group was associated with Dutch and Spanish haplotypes. Although the proximity between Irish and Dutch haplotypes may suggest that colonisation of Ireland was stepwise in nature (western Europe to Britain to Ireland), the fact that there were no intermediate British haplotypes between Holland and Ireland implies that this Irish/Dutch association is not a result of natural colonisation, but possibly due to human translocations, particularly as the association was only between haplotypes found on the east coast of Ireland. There are no records of squirrels being translocated from Holland to Ireland, but there is the possibility that squirrels which had been brought from Holland as pets, escaped, and these are the source of the Irish/Dutch haplotypes, although why these haplotypes were found over such a broad geographical area (north and east) is unclear.

Regarding the similarities between Irish and Spanish haplotypes, although the relationship on the network was tenuous, the associations were supported by the MDIV analysis with the Irish and southern European population diverging more recent than Irish and western European, suggesting colonisation of Ireland may have been from Iberia, rather than stepwise from western Europe. This so-called 'Lusitanian element' (Corbett, 1961) has long been recorded for numerous plant and invertebrate species (see Yalden, 1982 for a complete list), but, it was only recently, through the use of molecular techniques, that evidence for 'Lusitanian' mammals was found. Davison *et al.* (2001) found that the source of the Irish pine marten, *Martes martes*, population was not Britain, as would be expected if colonisation had occurred via a landbridge, but instead was southwest Europe. A study of the pygmy shrew, *Sorex minutus* (Mascheretti *et al.*, 2003), revealed similar results. However, the restricted nature of sampling from some areas in both of these studies may mean these results are not conclusive. In fact, recent reanalysis of pygmy shrew phylogeography within Europe has revealed the species also colonised Ireland via Britain (J. Searle, pers. comm.). Clearly further sampling of southern Europe, especially France (from which no data were available) and Spain, is required to confirm whether some of the divergent lineages found in Ireland are indeed of Lusitanian origin.

When mutation rates were taken into account, the split between Ireland and western Europe occurred *before* that between Ireland and Iberia. Therefore, if is presumed that the 'Lusitanian' squirrels arrived in or around the same time as human colonisation of Ireland,

then the Dutch haplotypes could not be recent translocations, and may, in fact, represent evidence that squirrels may also have arrived in Ireland from western Europe after the last glacial maximum, *via* a landbridge from Britain. If this is the case, since this time, either the intermediate British haplotypes must have become extinct, or they were simply unsampled in the previous studies, although, given the extensive sampling range within Britain, the latter is probably unlikely.

Whether red squirrels also survived through the last glacial maximum in an Irish refugium is unclear. Three haplotypes found in Ireland (SW1, W10 and M7) seem to be more divergent from both British and European haplotypes than might be expected from solely postglacial colonisation, suggesting that they may be native Irish in origin. M7 was amplified from a museum specimen collected in 1897 from Kilkenny in the centre of Ireland; it was a minimum of six mutational steps from any of the other haplotypes in the network. Although there were two translocations near this area (Barrington, 1880), the large number of differences between this and all other haplotypes suggests that it may be a native Irish haplotype. However, M7 was not amplified in any of the contemporary specimens and it is likely that it is now extinct. The second divergent haplotype was found in the west of Ireland; W10 was a minimum of 4 mutations from other haplotypes. Only one translocation occurred west of the river Shannon, slightly north of where these samples were collected (Figure 3.1; Barrington, 1880) and this involved the introduction of eight squirrels i.e. a maximum of eight maternal lineages. It is unlikely that in the restricted time frame of less than 200 years that enough mutational differences could have occurred to classify this haplotype as introduced. The final haplotype is SW1, found only in the southwest of Ireland, where the glacial refugium is likely to have been located. This haplotype was 5 mutations from all other haplotypes and, taking into account the nearest reintroduction point was over 100 km away (Figure 3.1; Barrington, 1880), and the fact that it was found in no other population or region, suggests that this too is a remnant Irish haplotype. The fact that these haplotypes, SW1 and W11, were only found in old forests (areas which had been continuously forested since at least 1850, see section 2.2.1 for more detail), which were located in areas of Ireland where large tracts of forests survived through the tree felling over the industrial revolution (McCracken, 1971), also supports their remnant status.

However, clearly the majority of the haplotypes in Ireland were British in origin (Figure 3.2 and 3.5). Hypothesised introduction points of each haplotype and probable subsequent

movement patterns are shown in Figure 3.16. W11 was probably introduced with the eight individuals brought into Galway, explaining its restriction in the Irish population west of the River Shannon. IRL is widespread throughout the Irish population, found in 22 of the 87 individuals sampled and it is likely that it was a common English haplotype that has since become extinct in England, but was widespread in the translocated population. Its existence west of the River Shannon is explained by its presence in the sample collected from that area in 1903; therefore W11 and IRL seem to both have been introduced at the same point in time and space. Likewise, haplotype SW+W+E was also reintroduced at that point, and possibly in one of the central introductions, as it was amplified from a sample from Tipperary in 1964, and was found in almost equal frequencies in eastern and southwestern regions. SW+W+E is quite divergent from extant British haplotypes and represents genetic diversity from southern England, which may since been lost from the British population due to the spread of the grey squirrel.

It also appears that haplotypes E7 and SW3 were derived from the same reintroduction as each other. They group with M1 on the haplotype network, a haplotype that was amplified from an individual from Sussex from 1911. It is possible that these individuals originated from the translocation into Offaly, where individuals are reported as being from Sussex (Barrington, 1880), and spread both east and southwest from that point. Overall, there seems to be no pattern of association between distinct translocation points and haplotype distributions, with red squirrel range expanding from translocation points and then converging with populations derived from other translocation points (Figure 3.16).

The nested clade statistics supported this, with genetic structure attributed to fragmentation and long distance dispersal, the latter probably referring to the presence of IRL and SW+W+E west of the Shannon as a result of translocations. However, clearly the introduced individuals are outcompeting and replacing those haplotypes that were found in Ireland prior to the translocations. Why this replacement may be occurring will be assessed by investigation of possible associations between haplotypes and adaptive traits in Chapter 5.

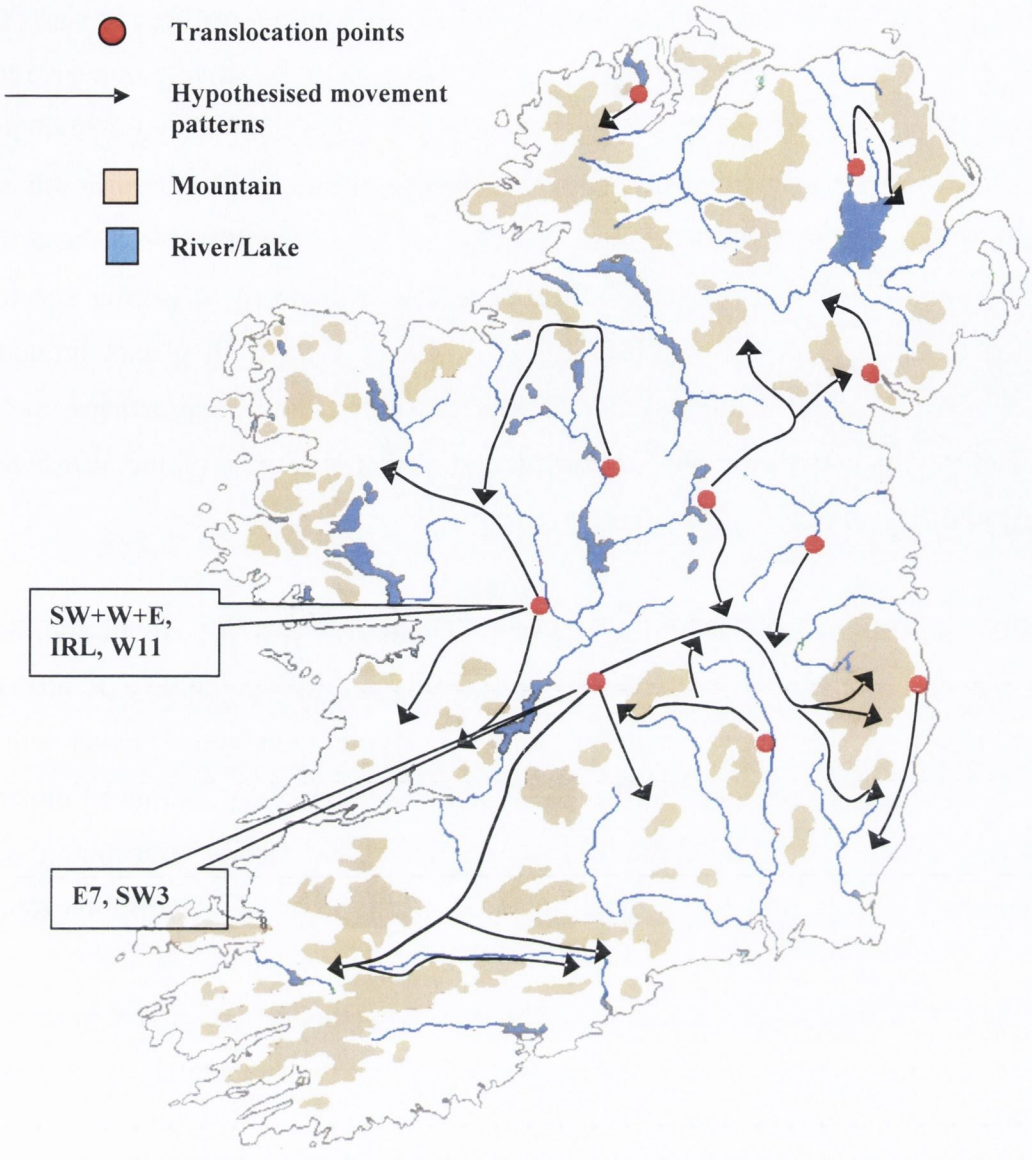


Figure 3.16. Map showing translocation points and major topographical barriers to dispersal (mountains and rivers). Hypothesised movement patterns from the introduction points, constructed from the data in this study, are also shown. Data suggested SW+W+E, IRL and W11 were introduced at the translocation site west of the River Shannon, while E7 and SW3, although found in geographically divergent areas, were mutationally similar, and therefore possible originated from a central introduction point. Due to the widespread distribution of IRL and SW+W+E it is likely that those were introduced, as well as west of the Shannon, at a number of central translocation points. It is clear why the eastern region had a high number of haplotypes, with numerous colonisation opportunities from a number of translocation points.

3.4.3. Conclusions

In summary, phylogeographic relationships between red squirrels in Europe suggested a pattern of postglacial range expansion from at least two of the Mediterranean refugia. However, the presence of a central European refugium may explain the close phylogenetic relationships within the widespread geographical range of the samples used in this study, and further sampling in central Europe would be needed to confirm this. These results suggest the red squirrel can not only be used to map the spread of conifer species, as suggested by Hale *et al.* (2004), but also broadleaf species, from glacial refugia. A detailed phylogeographical study of the red squirrel within Europe, and perhaps into Asia, is required to fully understand the effect of the last glacial maximum on the distribution of diversity within the Eurasian red squirrel.

The haplotype relationships within Ireland supported all four possible colonisation events of the island; 1) before the last ice age with persistence in a refugium, 2) postglacial colonisation *via* a landbridge from western Europe to Britain to Ireland, 3) either deliberate or accidental translocations in prehistoric times from southern and/or western Europe; and 4) the well documented translocations from Britain in the 1800's (Barrington, 1880). Nevertheless, although the results agreed with all of the proposed colonisation routes of Ireland, it seems that, given the complicated history of the red squirrel in Ireland, and the dominance of the British haplotypes within the current population, the phylogenetics of the Irish red squirrel population will, unfortunately, not be fundamental in increasing our understanding of the faunal history of Ireland.

From a conservation point of view, the Irish red squirrel population represents an interesting conservation phenomenon. The eastern region, which is most threatened from the spread of the grey squirrel (Ó Teangana *et al.*, 2000), contains haplotypes that may reflect the natural colonisation of Ireland by fauna postglacially, while the western region contains individuals that may have a deeper ancestral relationship with mainland Europe. However, both of these important lineages are being replaced by what appear to be extinct lineages in Britain which, from a British point of view, are also important for conservation, especially in the face of the rapid spread of an introduced Swedish haplotype across Britain (Hale *et al.*, 2004).

On the whole, the predominance of introduced British haplotypes, and the scarcity of possible native haplotypes, in Ireland, means that mtDNA diversity and distribution in the

samples in this study reflect recent human interference, rather than historical biogeography and postglacial spread. It is therefore likely that the mtDNA diversity within Ireland, and its distribution, will reveal little of the effect of barriers to dispersal, and environmental change, on insularity, and subsequent possible microevolution, in the Eurasian red squirrel, but this will be investigated further in the next chapter.

Chapter 4: The influence of habitat and landscape on the genetic structure, diversity and insularity of the red squirrel.

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4.1. Introduction

Distribution and differentiation of genetic diversity within, and between, populations, regions and countries, is affected by a number of dynamic and interrelated factors such as geographic location (Lesica & Allendorf, 1995), landscape structure and insularity of populations (Caughley, 1994, Taylor *et al.*, 1993) and local adaptations (Fink *et al.*, 2004). An understanding of these factors is essential in interpreting the ecology and distribution of species and the effectiveness, if needed, of conservation measures (Moritz, 1994a).

On a broad scale, the location of the population of the species, with respect to other populations of the same species, can greatly affect the amount of gene flow into, and through, the population; resulting in patterns of diversity and structure not seen elsewhere. This phenomenon has been recorded in comparisons of populations at the edge of a species range, both distributionally and ecologically (so called marginal or peripheral populations) to populations located nearer the range core. Conventionally the location of peripheral populations at the edge of a range, and the resulting restriction of gene flow is described as having similar genetic effects on the population as habitat fragmentation (Young *et al.*, 1996); i.e. reduced genetic diversity as a result of bottlenecks, founder effects or genetic drift, and also increased genetic structure, due to reduced gene flow (Durka, 1999; Lesica & Allendorf, 1995). However, some research has revealed an increase in genetic diversity in peripheral populations (Durka, 1999; Eckstein *et al.*, 2006) and it has been suggested that this increased diversity is a result of natural selection on adaptively significant variation on a local scale (García-Ramos & Kirkpatrick, 1997), and eventually, could possibly lead to speciation (Lesica & Allendorf, 1995). Consequentially, peripheral populations have been suggested as important for conservation, as they can represent large amounts of genetic variation within the species (Eckstein *et* al, 2006; Lesica & Allendorf, 1995) and also, their adaptation to range margins, possibly more variable, extreme or sub optimal habitat, may be important to conserve in the species in the face of global habitat change (Safriel *et al.*, 1994 in Eckstein *et al.*, 2006).

However, geographic location does not only affect genetic structure and variation on a species at the peripheral/core scale, but also on a more local scale. Isolation by distance (IBD) is the increasing genetic differentiation, with increasing geographical distance, between populations. It is apparent first in small amounts, at a low geographical range, but increases sharply as geographical distance increases (Slatkin, 1993). Although this pattern

has been recorded in a wide range of species (e.g. pocket gophers, Alvarez-Castañeda & Patton, 2004; otters, Dallas *et al.*, 2002; alpine marmots, Goossens *et al.*, 2001), it is quite a simple explanation for the complex dispersal patterns which can occur between populations, and has been shown to vary in its significance with respect to scale (Bockelmann *et al.*, 2003, Jones *et al.*, 2004; Rousset, 1997, Trizio *et* al, 2005), and the influence that barriers to dispersal can have on species distribution (Lugon-Moulin *et al.*, 1999; Riginos & Nachman, 2001).

Logically IBD would be expected to occur at a more significant level in highly dispersive and/or generalist species, while genetic structure of habitat specialists and/or low distance disperser species may be more affected by a combination of both geographic distance and barriers to dispersal. The analysis of genetic structure and diversity with respect to both geographic distance and landscape connectivity and homogeneity is referred to as 'landscape genetics' (Manel *et al.*, 2003). Landscape genetics is similar to phylogenetics, but is usually carried out at more local and finer scale, allowing investigations of microevolution within a population or species. Although increasingly being used to interpret patterns of genetic structure (Bockelmann *et al.*, 2003; Keyghobadi *et al.*, 1999; Riginos & Nachman, 2001 and references therein), landscape genetics is still in its infancy, and few investigations have been carried out on mammals (e.g. deer, Coulon *et al.*, 2006; wolves, Geffen *et al.*, 2004; voles, Lugon-Moulin *et al.*, 1999).

The Eurasian red squirrel, *Sciurus vulgaris*, is an ideal species in which to study the effects of peripheral/core differences, and the influences of landscape, on genetic diversity and structure. It is the most widespread of the *Sciurus* species, its range extending across the Palaearctic (Gurnell, 1987; Lee & Fukuda, 1999 in Lurz *et al.*, 2005). It is a habitat specialist (Gurnell, 1987), favouring large tracts of mature forests, and research suggests a preference for forests with a high percentage of pine species (Lurz *et al.*, 1995; Wauters & Lens, 1995). The population in Ireland is of particular interest as it is the most westerly peripheral population of this widespread species and is also an island population. Island populations of terrestrial and/or habitat specialist species, whether inhabiting actual physical islands or habitat islands, have been suggested as especially suitable for landscape genetics as they are discrete (Petren *et al.*, 2005), and separation of island populations from the mainland removes the possible confusing effect of continual gene flow into the populations; therefore clarifying the contribution that localised gene flow, barriers to

dispersal etc. have on the diversity and structure of the study population (Hinten *et al.*, 2003).

Within Ireland, the red squirrel is widespread, although it has disappeared from the centre of the country due to the spread of the grey squirrel, and inhabits conifer, mixed conifer and broadleaf, and broadleaf forests in the country (Ó Teangana *et* al, 2000; Reilly, 1997). However, although considered native to Ireland, it was translocated into the country over the 19th century (Barrington, 1880), and the population in Ireland may be a mixture of native and reintroduced stock. However, the locations and dates of these translocations were well documented (Barrington, 1880) and can be incorporated into the interpretation of results. Even taking into account the translocations, the Irish red squirrel population is one which has been isolated from mainland European populations for at least 100 years, and with the generation time of red squirrels being approximately 3 years (Gurnell, 1987), there has been ample time for the biogeography of Ireland to have effected genetic structure and variation, possibly more strongly with respect to microsatellite variation as opposed to mtDNA with its slower mutation rate (Cruzan & Templeton, 2000), so as to allow an investigation of the effect of landscape on genetics of the red squirrel to be carried out.

Previous research on red squirrels has shown that discontinuous habitat represents a significant barrier to dispersal in the species (Hale *et al.*, 2001a) and Trizio *et al.* (2005) hypothesised that discontinuous habitat, mountains and rivers, were shaping the genetic structure of the red squirrel in the Alps. However, no research to date has investigated the effect of, and interaction between, possible barriers to dispersal on gene flow, nor have studies addressed the possibility of adaptive divergence, in specific habitat types, of the species, over a broad scale, both of which are need to understand the distribution of, and the distribution of diversity within, the red squirrel as a species. Also, as research has been carried out on the species in a number of different countries nearer to the core of red squirrel range (Barrett *et al.*, 1999; Hale *et al.*, 2001a, b, 2004; Todd, 2000a; Trizio *et al.*, 2005), the investigation of diversity and structure within the Irish population, combined with other published results, facilitates a comparison between diversity and structure, on a peripheral-core gradient, within this widespread species.

In this part of the study two molecular markers, 16 microsatellites and mtDNA, were used to investigate the genetics of the Irish red squirrel population. By collecting samples from

Ireland over a widespread distribution, and different habitat types, and incorporating historical and contemporary effects on gene flow within the sample population, it was aimed to increase the understanding of patterns of gene flow in red squirrels, and subsequentially, the factors which determine insularity of populations, and influence the distribution of the species as a whole. There were 3 main aims to this part of the study:

- To investigate population level/regional differences in diversity and structure of red squirrel in Ireland.
- To assess the influence of landscape connectivity and habitat type on the genetic insularity of the red squirrel.
- To compare diversity and structure of a peripheral population to that of more central populations of a species.

4.2. Materials and Methods

4.2.1. Sample collection

Samples were collected, as outlined in Chapter 2, from live trapped and roadkilled squirrels from around Ireland. 14 populations were sampled with a total sample size of 100 individual squirrels (Figure 4.1). These 14 populations were allocated into 3 regions – south/southwest, east/north and west (Table 4.1) using results from genetic analysis (see section 4.2.5). The forests from which the populations were collected represented two different habitat types, conifer and mixed conifer/broadleaf (mixed), and two age classes of forest, old and new. Old forests were defined as continuously forested since at least 1850, while new forests were planted around the mid 20th century. The number of samples collected from each population was generally low, with only 6 populations represented by more than 2 individuals (Table 4.1). Also, despite the large distance between populations sampled in the north of Ireland (Figure 4.1), as the entire north of Ireland was only represented by a two individuals, it was decided to pool these two samples and treat them as one population in all analyses.

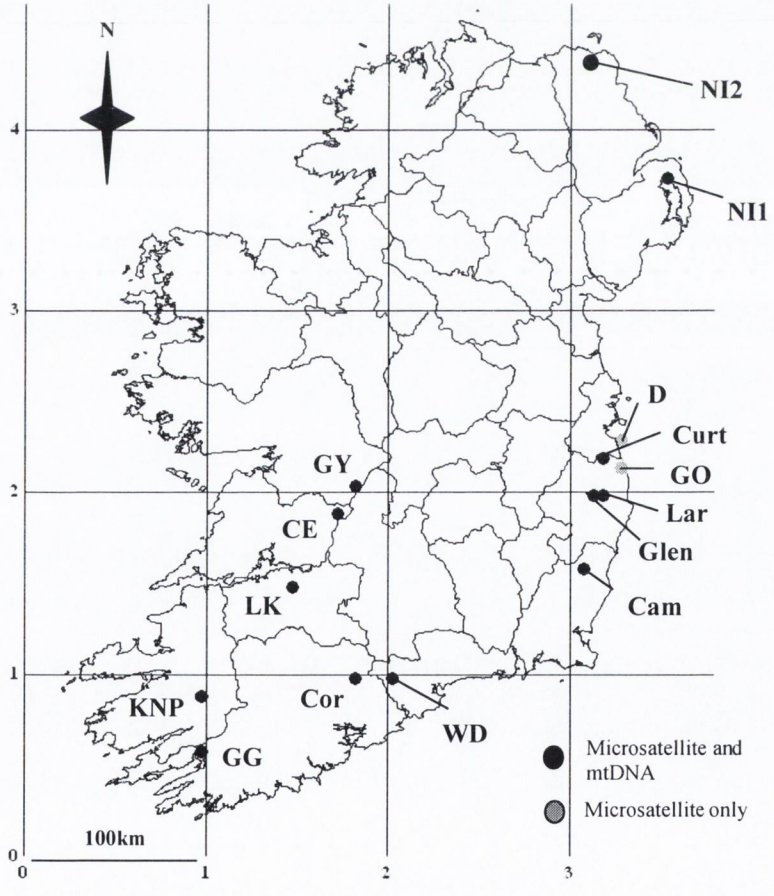


Figure 4.1. Locations of 14 sites from which red squirrel samples were collected. Sites where only microsatellite DNA was analysed are indicated. Population codes are explained in Table 4.1

Table 4.1. The 14 populations from which samples were collected in this study. The region into which each population was assigned and the number of samples collected from each population are indicated. Codes correspond to those on Figure 4.1. Codes beside the sample size refer to the habitat type of the forests where those samples were collected from. Old: continuously forested since 1850, New: planted since 1950, Mix: mixed broadleaf and conifer, Con: conifer forest.

Region	Code	Population	N	Old/New	Mix/Con
South/ Southwest	GG	Glengarriff, Co. Cork	2	Old	Mix
	KNP	KNP, Co. Kerry	13	Old	Mix
	LK	Adare, Co. Limerick	1	New	Con
	Cor	Corrin, Co. Cork	8	New	Con
	WD	Lismore, Co. Waterford	1	Old	Mix
East/north	Cam	Camolin, Co. Wexford	2	Old	Mix
	Curt	Curtlestown, Co. Wicklow	8	New	Con
	D	Killiney, Co. Dublin	1	New	Con
	Glen	Glendalough, Co. Wicklow	8	Old	Mix
	GO	Glen of the Downs, Co. Wicklow	1	Old	Mix
	Lar	Laragh, Co. Wicklow	6	New	Con
	NI1	Glenhesk, Co. Antrim	1	Old	Mix
	N12	Newtownards, Co. Down	1	New	Con
West	CE	Lakeside, Co. Clare	2	Old	Mix
	GY	Portumna, Co. Galway	44	Old	Con

4.2.2. DNA extraction

DNA was extracted from tissue and hair following the methods outlined in Chapter 3.

4.2.3. MtDNA amplification and sequencing

MtDNA was amplified and sequenced from 87 red squirrels as outlined in Chapter 3. The locations of the populations from which mtDNA was obtained are indicated in Figure 4.1.

4.2.4. Microsatellite amplification and visualisation

DNA was amplified at 16 polymorphic microsatellites developed by Todd (2000b; Table 4.2) and Hale *et al.* (2001b; Table 4.3) from individuals from all 14 populations. DNA amplification reactions were as described by the authors (Table 4.4a, b) with either 5 µl of hair or 0.5 µl of tissue DNA amplified in 15 µl or 12.5 µl reactions for the Hale *et al.* (2001b) and Todd (2000b) primers respectively. Amplifications using the primers described by Hale *et al.* (2001b) were carried out under the published conditions: an initial step of 95 °C for 12 minutes, 10 cycles of 94 °C for 15 seconds, annealing temperature (T_a, °C) for 15 seconds (see Table 3.2), 72 °C for 15 seconds and then 30 cycles (35 for hair) of 89 °C for 15 seconds, T_a (°C) for 15 seconds and 72 °C for 15 seconds followed by a final step of 72 °C for 10 minutes.

Due to a high amount of unspecific binding in the PCR reactions carried out using Todd's (2000b) primers, the annealing temperature was adjusted in the PCR reaction using a gradient PCR from 52 °C to 62 °C. As a result of this, annealing temperature of primers Ru1, 3, 4 and 6 was increased to 62 °C while, as the gradient PCR of Ru5 did not increase the quality of the PCR product, it was decided to retain the annealing temperature at 52 °C, as the unspecific products did not lie within the allele size range described for that primer. Using the adjusted annealing temperatures, amplification was carried out under the published conditions: 94 °C for 3 minutes, 30 cycles (35 for hair) of 94 °C for 1 minute, T_a (°C) for 1 minute and 72 °C for 90 seconds, followed by a final extension step of 72 °C for 5 minutes.

All microsatellite amplifications were carried out in a Biometra® T1 Thermocycler, with the exception of the gradient PCR which was facilitated by the use of a Thermo Hybaid® PCR Express Thermal Cycler. Positive (red squirrel DNA provided by M. Hale) and negative (dH_20) controls were used in all PCR reactions.

Microsatellite products were visualised by electrophoresis on Spreadex EL 500 Wide Mini S-2x25 (Elchrom Scientific) gels and subsequent staining with Syber Gold. They were sized manually by comparison to two separate size standards; 10 bp DNA ladder (Invitrogen) and M3 Marker (VHBio ltd). In order to validate the results obtained the products were sized independently three times 'blind' without knowing the expected size range of the allele or the sizes obtained in other studies (Hale *et al.*, 2001a,b; Todd, 2000b). Furthermore a subset of samples (approx n = 20) were amplified twice as they

were used as positive controls in later amplifications and this allowed further validation of the obtained allele sizes.

Table 4.2. Forward and reverse sequences, annealing temperature (T_a), expected product size and number of recorded alleles of the five primers used in this study which were developed by Todd (2000b). All Todd primers are referred to as Ru x in the text; where x = 1, primer = R Sμ1, x = 3, primer = RSμ3 etc.

Locus	Repeat Structure	No. of Alleles	Primer Sequence (5' - 3')	Ta (°C)	Product size (bp)
RSμ1	$[GGAT]_{13}$	7	F: CTGGGTTCACTGACTTCTCC R: CACTCTCAGAGGCCAAAGTC	62	172-196
RSμ3	$[GA]_9[GACA]_9$	7	F: GCCAAAATCTAGCCCAAGAAG R: CTCAGGTGTGGGAAAGAAGC	62	161-173
RSμ4	$[ATCC]_{12}$	8	F: CAATCCTCCCATCCTGCTGC R: TAGGCAGTCAGATAGGTGGG	62	256-284
RSμ5	$[GT]_{10}$	7	F: CCCAGTCTACATTAAAGGGC R: GCCTATACACTATAATTGACT	54	123-143
RSμ6	$[GTT]_{10}$	4	F: GGCATAGGGCACGTGAAG R: GGGCCAATCTCATACCAAG	62	122-131

Table 4.3. Forward and reverse sequences, annealing temperature (T_a), expected product size and number of recorded alleles of the 11 primers used in this study which were developed by Hale *et al.* (2001b). All primers are referred to as their numbers in the text, i.e. Scv1 = 1, Scv3 = 3 etc.

Locus	Repeat Structure	No. of Alleles	Primer Sequence (5' - 3')	Ta (°C)	Product size (bp)
Scv1	$[CA]_{28}$	8	F: CTCCTCTTCCAAGGGTGACA R: GATGGCCTCTGTTTCTCTGC	54	134-183
Scv3	$[GA]_{26}$	12	F: TTGGCTCATGGTTTCAGAGA R: CCCCTCACTTCCTCCATTTC	52	128-208
Scv4	$[GT]_{23}$	6	F: CTGGAGATGGAGTGAGTGAGG R: CCAGGAATCCTCTTGAATGC	52	199-215
Scv6	$[TG]_{22}$	5	F: GCAATCCTTGTCCTTGCATT R: TGAGTCATTGGATGAAAAACC	52	185-201
Scv10	$[AG]_{19}$	6	F: AGAGCTGACATTGCCAAACA R: AGGGGAATCCTGGGACTTT	54	72-86
Scv13	$[GA]_{15}$	3	F: CTGGGTTCAATCCCAATGTC R: ATAGATGCACCCTGAAGCTG	54	167-181
Scv14	$[AC]_{14}$	3	F: AGGTGCACTGATATGCATCG R: GGTCAGCTTTGTGGCTTTGT	54	192-206
Scv18	$[CA]_{11}$	2	F: AGGCATTGTCCAGTGATTCC R: ACATGGTTGGGCTTTGAGTC	52	238-255
Scv19	$[TG]_{11}$	4	F: ATGACTTGGGACCAGTTTGC R: CTGATTCCCAGGGATTGAAA	54	182-212
Scv23	$[GT]_{10}$	8	F: AAACACCTGAGACAGGCAAC R: GTGTTTGGCAATGTCAGCTC	52	151-167
Scv31	$[AG]_{29}$	10	F: CCAAGTTCCAGACCAACCTC R: TCGGGTCTCTAAGGAGATGG	52	179-201

Table 4.4. Reaction mix used to amplify the 16 primers (a) as devised by Hale *et al.* (2001b) and (b) Todd (2000b). The volumes of each reagent used and their final concentrations in a 15 µl (a) or 12.5 µl (b) reaction mix are given.

a)

Reagent	Stock Conc	Volume in Reaction	Conc in Reaction
PCR Buffer	10 x	2.5 µl	1 x
dNTP mix	10 mM	0.2 µl	0.08 mM
Primer FWD	10 µM	0.5 µl	0.2 µM
Primer REV	10 µM	0.5 µl	0.2 µM
$MgCl_2$	50 mM	1 µl	2 µM
Taq	5 unit/µl	0.2 µl	1 unit
Sterile Distilled Water		15.1 µl	
Template:			
Tissue		0.5µl (+ 4.5 µl dH_20)	
Hair		5µl	
Total		25 µl	

b)

		Ru 1		Ru 3		Ru 4		Ru 5		Ru 6	
Reagent	**Stock Conc**	**Volume (µl)**	**Conc in Reaction**	**Volume (µl)**	**Conc in Reaction**	**Volume (µl)**	**Conc in Reaction**	**Volume (µl)**	**Conc in Reaction**	**Volume (µl)**	**Conc in Reaction**
PCR Buffer	10 x	1.25 µl	1x	1.25 µl	1x	1.25 µl	1x	1.25 µl	1x	1.25 µl	1x
dNTP mix	10 mM	0.18 µl	0.15 mM	0.125 µl	0.1mM	0.25 µl	0.2 mM	0.075 µl	0.05 mM	0.075 µl	0.05 mM
Primer FWD	10 µM	0.5 µl	0.4 µM	0.5 µl	0.4 µM	1 µl	0.8 µM	1 µl	0.8 µM	1 µl	0.8 µM
Primer REV	10µM	0.5 µl	0.4 µM	0.5 µl	0.4 µM	1 µl	0.8 µM	1 µl	0.8 µM	1 µl	0.8 µM
$MgCl_2$	50 mM	0.75 µl	1.5 mM	0.5 µl	1 mM	0.5 µl	1 mM	0.5 µl	1 mM	0.5 µl	1 mM
Taq	5 unit/µl	0.2 µl	1 U	0.2 µl	1 U	0.2 µl	1 U	0.2 µl	1 U	0.2 µl	1 U
Sterile Water		4.12 µl		4.425 µl		3.3 µl		3.475 µl		3.475 µl	
Template:											
Tissue					0.5µl (+ 4.5 µl dH_20)						
Hair					5µl						
Total					12.5 µl						

4.2.5. Data analysis

Haplotypes were identified as outlined in Chapter 3. Microsatellite results were checked for typing errors, allelic dropout, null alleles, stutter peaks and scoring errors using the MICROSATELLITE TOOLKIT for M.S. EXCEL (Stephen Park, UCD) and MICRO-CHECKER 2.2.3. (van Oosterhout *et al.*, 2004).

The number of 'regions' represented by the data set was determined using the program STRUCTURE (Pritchard *et al.*, 2000). This program determines the number (K) of genetically distinct populations in sample data. The program is run for a number of K and the probabilities of the data fitting each K (Pr(X/K)) is calculated – the highest probability being assigned to the value of K which most likely describes the number of 'populations' in the data. Analysis was run with K ranging from 1-14, using a MCMC chain length of 100,000 iterations and a burn in of 10,000 iterations and with the admixture model, with alleles assumed to be correlated and independent (2 separate analyses). After repeated analysis PrX/K did not plateau, nevertheless, the program consistently allocated individuals to one of 5 'populations', a south/southwest population, an east/north population and three western populations. The three western populations, as they were essentially from the same study site (GY) were combined into a single 'population', resulting in a total of three 'populations', and these 'populations' were used as regions in the regional analysis.

4.2.5.1. Genetic diversity

MtDNA diversity measurements; gene diversity, number of polymorphic sites, haplotype frequency, unique haplotypes and nucleotide diversity for each population and region were calculated in ARLEQUIN 3.01 (Schneider *et al.*, 2000). Allelic richness (AR), observed heterozygosity (Ho), and gene diversity (He), of microsatellite data were calculated in FSTAT 2.93 (Goudet, 2001). Exact tests of deviations from Hardy-Weinberg (HW) equilibrium and heterozygosity deficiency were carried out using a Markov chain exact probability test (Guo & Thompson, 1992) of 10,000 steps in GENEPOP 3.4 (Raymond & Rousset, 1995b). Fisher exact tests of linkage disequilibrium (LD), the non random association of alleles at loci, were carried out in ARLEQUIN 3.01 (Schneider *et al.*, 2000).

Evidence for population bottlenecks was assessed with the Wilcoxon signed ranks test (all 6 populations with n >2), Sign tests (where n > 10; GY and KNP), and the allele

frequency distribution test in the program BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996). The occurrence of a genetic bottleneck results in a faster reduction of allelic diversity than heterozygosity (Nei *et al.*, 1975), resulting in a larger than expected heterozygosity in the population until mutation drift equilibrium is restored (Cornuet & Luikart, 1996). It also results in a loss of alleles which occur at a low frequency in a population, causing a mode shift from the nonbottlenecked L shaped distribution of allele frequency (Luikart *et al.*, 1998; Nei & Li, 1976). As there is no strict consensus as to the model with best describes microsatellite mutation (Balloux & Lugon-Moulin, 2002), three modes of mutation were used in the analysis; the stepwise mutation model (SMM), infinite allele model (IAM) and the two-phased mutation model (TPM), the latter a mixture of one step and multistep changes. TPM was run with a mix of 90:10% SMM: IAM, and with a variance of 10%.

Comparisons between diversity in populations and regions were carried out in SPSS 12.0.1. for Windows. All data were tested for normality prior to analysis with Levene's test of homogeneity (Dytham, 2003). Ho, the proportion of heterozygosity in a sample, was arcsine square root transformed and multiplied by 57.295; the standard transformation for proportional data (Dytham, 2003), prior to analysis. The relationship between the various diversity measurements and sample size were assessed with Pearson (r) and Spearman ranked (r_s) correlations where appropriate. Diversity comparisons between countries, populations and regions were carried out with 2 sample t tests, one way ANOVAs, Mann-Whitney U Tests and Kruskal-Wallis tests where appropriate. Where differences were detected in ANOVA or Kruskal-Wallis analysis, subsequent LSD post-hoc or multiple Mann-Whitney U tests were carried out on the data respectively. Where multiple Mann-Whitney U tests were performed on data α was retained at < 0.05 by sequential Bonferroni corrections (Rice, 1989).

The relationships between diversity and forest size, latitude, longitude and isolation (the distance in km from the forest where the population was resident to the next nearest forest) was investigated with simple and multiple linear regression analysis. Possible relationships between diversity and habitat type (conifer/mixed, old/new) were assessed with 2 way ANOVA.

All tests were 2 tailed and a p value of < 0.05 was accepted as significant, except in the cases of multiple testing where $p < {}^{0.05}/_{\text{no. of tests}}$ was significant.

4.2.5.2. Genetic structure

Population structure was assessed using a number of different methods. Differentiation between populations was investigating by examining allele and haplotype frequencies between populations and comparing haplotype and allele frequencies with a χ^2 test, the latter comparison facilitated by the program CHIFISH (Ryman, 2006). An AMOVA was carried out and pairwise F_{ST} (Reynolds *et* al, 1983) were calculated, based on allele numbers or haplotype frequencies, to determine at which level structure exists (population level, regional etc) in ARLEQUIN 3.01 (Schneider *et al.*, 2000), significance being assessed with 1000 permutations. D_S (Nei, 1972) between populations, from both allele and haplotype frequency data, was also calculated using GENDIST in PHYLIP version 3.5c (Felsenstein, 2004) and a graphical representation of the distance matrix, with 100 bootstrap replicates, was constructed with SEQBOOT, GENDIST, NEIGHBOR and CONSENSE in PHYLIP version 3.5c (Felsenstein, 2004) using the Neighbor-Joining (NJ) method. Resulting trees were visualised in TREEVIEW version 1.1.6. (Page, 1996). F_{ST} and D_S distance measures were chosen as they have been used extensively in population genetic studies using microsatellites and mtDNA, including those on red squirrels (Hale *et al.*, 2001a, 2004; Trizio *et al.*, 2005), allowing comparisons to be made. F_{ST} assumes differences between populations are solely a result of genetic drift (Reynolds *et al.*, 1983), the loss of genetic variation through the process of only a small number of individuals contributing to the next generation. Conversely, D_S assumes mutation drift equilibrium within the populations (Nei, 1972), which is a balance between loss of diversity due to drift and increase in diversity due to mutations.

Differentiation based on microsatellite data, on both a population and regional basis, was also assessed using an assignment test (Paetkau *et al.*, 1995). The assignment test determines the probability, using permutation, of each individual genotype occurring in each population/region and assigns each individual to the population/region with the highest probability. Assignment tests were carried out using the assignment calculator program (J. Brzustowski, http://www.biology.ualberta.ca/jbrzusto/Doh.html). Graphical representations of regional assignment were constructed from log likelihood values of individual membership in each region calculated with the genotype assignment test in ARLEQUIN 3.01 (Schneider *et al.*, 2000).

Three methods were used to assess the possible effects of landscape and genetic diversity on the genetic structure of the Irish red squirrel population calculated from both

microsatellite and mtDNA data. Firstly, the influence of geographic distance, between populations and regions, on genetic structure (IBD), was investigated by comparing the matrix of genetic distance (F_{ST} and D_S) with the natural logarithm of geographic distance using a Mantel test (Mantel, 1967) with 1000 permutations in the program IBD (Bohonak, 2002). Comparisons were made with the entire data set and the 6 populations with $n > 2$.

Secondly, the relationship between genetic distance and spatial parameters was investigated using distance based redundancy analysis (dbRDA, Legendre & Anderson, 1999; McArdle & Anderson, 2001) with the program DISTLM (Anderson, 2003; McArdle & Anderson, 2001). DbRDA uses principal coordinate analysis (Gower, 1966 in McArdle & Anderson, 2001) to compare distance matrices and other variables in a regression analysis. Like Mantel tests the *p* value is obtained through permutation – allowing analysis to be performed on data which is not normally distributed.

The analysis was performed on both the full set of data and on the 6 populations with sample size > 2. Predictor variables and their codes are given in Table 4.5. Three predictor variables were related to habitat or biogeographic information, while 7 (only 5 of which could be calculated in the populations with sample size ≤ 2), were measurements of genetic variability within the populations. The relationship between each variable, and each group of variables, and genetic distance was investigated both with (partial tests), and without (marginal tests), geographic distance (GEO) as a covariate. By inputting geographic distance as a covariate the extent, if any, of the ability of the predictor variables to explain genetic structure, over and above that explained by geographic distance, could be investigated.

The analysis was also run on the relationship between habitat variables and genetic distance using measures of genetic variation as covariates (GEN), again, with and without geographic distance (GEN*GEO) – assessing to what extent genetic structure is explained by landscape features, above and beyond that explained by differences in genetic variation between populations. Another analysis was run on the genetic measures with landscape features (LAND), and landscape features and geographic distance (LAND*GEO), as covariates. All *p* values for this analysis were obtained from 999 permutations of the genetic distance matrix.

Lastly, 5 predictor barriers to gene flow between populations (all 14 populations and 6 populations whose n > 2) were expressed as matrices between populations. The predictor variables were based on geographical distance and barriers to dispersal (rivers, roads, mountains, discontinuous habitat) and interactions between these barriers and between barriers and geographic distance (Table 4.6). Barriers to dispersal were determined by examination of Ordnance survey maps (Discovery series, 2nd edition, 1:50,0000cm) whereby a straight line, as the crow flies, was drawn between populations and the number and/or extent of barriers to dispersal along that line calculated. Although this method overly simplified dispersal between populations, it was the only feasible method of comparison over such a broad geographical area, and has been used in other studies of small mammal genetic structure (Lugon-Moulin *et al.*, 1999). Matrices were compared using Mantel tests (Mantel, 1967) and partial Mantel tests (Smouse *et al.*, 1986) using FSTAT 2.93 (Goudet, 2001). 5000 matrix randomizations were used for each test and α retained at < 0.05 using sequential Bonferroni corrections (Rice, 1989), as previously described.

Table 4.5. Predictor variables used in the dbRDA analysis. Variables are referred to as their codes in the results.

Code	Variable
GEO	Geographic distance between populations (km)
LAND	All landscape variables described below
REG	The region the population was located in: south/southwest (1), west (2), east/north (3)
AGE	Habitat either old (1), continuously forested since 1850 or new (0), planted since 1950
HAB	Habitat either conifer (1) or mixed (mixed conifer and broadleaf)(0)
GEN	14 population comparison: all variables marked with *, 6 population comparison: all variables described below
AR	Allelic richness for each population
NUC	Nucleotide diversity
GENED*	Gene diversity calculated from microsatellite data
POLY	Mean number of polymorphic sites between haplotypes in each population
PAIR	Mean number of pairwise differences between populations
Ho*	Mean heterozygosity in the population
MGENED	Gene diversity calculated from the mtDNA data.

Table 4.6. Variables used in the Mantel and partial mantel tests. Variables are referred to as their codes in the results.

Code	Variable
GEO	Geographic distance between populations (km)
RIV	Number or rivers between populations (the River Shannon, from its estuary to Portumna was weighted as 10)
DISCON	The maximum distance of unforested land between populations
ROAD	The number of N class roads between populations
MOUNT	The number of unforested mountains/mountain ranges, between populations

4.2.5.3. European comparisons

The differences between genetic diversity and structure in peripheral and core populations were investigated through comparison of the results from this study with research on red squirrels in other countries in Europe (Hale *et* al, 2001a, b, 2004; Ogden *et al.*, 2005; Todd, 2000a, b; Trizio *et* al, 2005). However, statistical tests could only be carried out on the microsatellite data, and comparisons were made between Ireland (this study), Britain (Hale *et al.*, 2001a, b), Italy (Trizio *et al.*, 2005) and Belgium and Germany (Todd, 2000a, b).

As before, data were checked for normality using the Levene's test, and proportional data (Ho) were arcsine square root transformed (Dytham, 2003). The mean number of alleles (MNA) was compared between populations in Britain and Ireland, and between Ireland, Belgium and Germany, with 2 sample t tests and one way ANOVA respectively. Only loci which were common to all countries in the comparison were used in the analysis. AR in Ireland and Britain was compared with a 2 sample t test, heterozygosity was compared between Ireland, Britain and Italy with one way ANOVA, and He differences between Ireland and Italy were investigated with a Mann Whitney U test. However, raw, loci by loci, data were not available for these comparisons therefore, comparisons were being made between different loci, as well as between different countries. Raw data were available for the study carried out in Belgium and Germany, and Ho, AR and He, were compared between those countries and Ireland with one way ANOVA and LSD post hoc tests using only the loci which were common to all three countries.

Differences between genetic differentiation, and whether increased genetic differentiation occurred over shorter differences in peripheral populations, was assessed by comparing F_{ST} and F_{ST}/geographic distance (km) between countries with Kruskal-Wallis, Mann Whitney U tests, 2 sample t tests and one way ANOVA where appropriate. Data on D_S in Ireland and Britain were also available, and these, and D_S corrected for geographic distance, were compared with Mann Whitney U tests. All tests were 2 tailed and a $p < 0.05$ was accepted as significant in all cases.

4.3. Results

4.3.1. Genetic variation

Microsatellite variation

Complete microsatellite results are given in Appendix 8.3. Table 4.7 shows the measures of diversity across the 16 loci. Polymorphism ranged from 3 to 13 alleles (mean 6.875, SEM 0.66), the proportion of heterozygosity was quite high at all loci (mean 0.39, SEM 0.03), and gene diversity was relatively constant across the loci (mean 0.63, SEM 0.02). The regions amplified by microsatellite 3 and 31 were the most polymorphic of the 16 microsatellite regions, with the highest number of alleles (n = 13) and the highest gene diversity (0.624, 0.803 respectively) although levels of heterozygosity at these loci was low (0.318, 0.304 respectively). Primer 23 had the highest proportion of heterozygous alleles (0.803). Although Ru6 had the lowest number of alleles (n = 3), Ru1 was the least polymorphic microsatellite across the three diversity measures (5 alleles, Ho 0.177, He 0.554). No LD was found between any of the 120 loci pair comparisons ($p > 0.1$ in all cases).

Table 4.7. Number of alleles, Ho and He found across all 16 microsatellites in the samples collected from this study.

Locus	3	4	5	18	23	31	1	10
No. of Alleles	13	6	5	6	7	13	8	6
Ho	0.318	0.252	0.527	0.575	0.593	0.304	0.374	0.434
He	0.624	0.571	0.645	0.663	0.753	0.803	0.743	0.59

Locus	13	14	19	Ru1	Ru3	Ru4	Ru5	Ru6
No. of Alleles	7	7	6	5	5	6	7	3
Ho	0.484	0.444	0.42	0.177	0.326	0.323	0.373	0.421
He	0.624	0.668	0.538	0.554	0.502	0.527	0.745	0.544

MtDNA variation

Haplotype sequences are given in section 3.3.1. As described in Chapter 3, 29 haplotypes were identified from the mtDNA of the 87 red squirrel samples amplified. Divergence between haplotypes was low with 35 polymorphic sites found within the 395 bp region amplified. Mean gene diversity within the Irish population was high (0.89 ± 0.02), as was the mean number of pairwise differences between haplotypes (4.75 ± 2.34) however nucleotide diversity was low at 0.063 ± 0.01.

4.3.1.1. Irish population variation

Microsatellite variation

Red squirrels over the 14 study sites showed high levels of variability at the 16 microsatellite loci, with the number of alleles found at each locus ranging from three to 11 per locus (mean 2.67). As the sample sizes for LK, D, WD, Cam, CE, GG and NI were low (≤ 2), and gene diversity, level of heterozygosity and number of alleles in these populations were lower than those from which larger samples were obtained (He: $U_{(160)}$ = 2475, p = 0.037; Ho: $U_{(160)}$ = 2206, p = 0.002; No. of alleles: $U_{(224)*}$ = 669, $p < 0.001$ (*df varies between comparisons as diversity and Ho could not calculated from the populations whose sample size was 1)), those eight populations were excluded from the population level comparison of He, Ho and number of alleles. The only comparison these populations were used in was in the comparison of AR, which is a measure of diversity calculated independent of sample size. Although there was a difference between AR in sites which had a sample size ≤ 2 and those which had > 2 samples collected from them ($U_{(224)}$ = 5197, p = 0.047), it was decided that, as the difference was just below the 95% level of significance, AR would be investigated in two groups; all 14 populations and populations with n > 2. All other comparisons were made between GY, Cor, Lar, Glen, Curt and KNP (6 populations with n > 2).

Null alleles were detected in all of the 6 populations. The proportion of null alleles was highest in KNP, Curt and GY (0.16, 0.18 and 0.18 respectively), while CK, Lar and Glen had few null alleles detected (0.04, 0.02 and 0.04 respectively). The observed alleles and genotype frequencies were adjusted in each population to account for these null alleles using the method outlined by Chakraborty *et al.* (1992) in MICRO-CHECKER (van Oosterhoult *et al.*, 2004); recoding null alleles as missing data. Diversity comparisons were then carried out in two sets 1) original data set and 2) the null allele corrected data set and diversity measurements from both sets of data are summarized in Table 4.8.

There was no relationship between the number of samples collected from each population and the mean number of alleles (MNA), AR, He or Ho in each site ($p > 0.05$ in all cases) in either data set. Over the six sites MNA at all the loci was high at 4 alleles, however the MNA differed significantly between the sites ($F_{(5,90)}$ = 8.475, p = 0.001) and GY had a higher number of alleles across all 16 loci than the other five populations (LSD post hoc: $p < 0.05$ in all cases). Ho in the original data also differed across the sites ($F_{(5,90)}$ = 443.6, p = 0.028). LSD post hoc tests revealed a higher proportion of heterozygosity in Cor, Lar

and Glen than in Curt (p = 0.029, 0.009, 0.005 respectively) and a lower heterozygosity in GY when compared to Glen (p = 0.034). However, when the data was adjusted for null alleles, there was no longer a difference in the proportion of heterozygosity between populations ($F_{(5,89)}$ = 0.744, p = 0.593).

There was no difference in He in either the original ($F_{(5,90)}$ = 1.85, p = 0.111) or null allele corrected ($F_{(5,90)}$ = 1.34, p = 0.256) data between the six sites. AR did not vary across the 6 populations with sample size > 2 in either sets of data (Original: $F_{(5,90)}$ = 1.45, p = 0.213, Null corrected: $F_{(5,90)}$ = 1.314, p = 0.265). However, although the original data found no difference in AR across all 14 populations ($\chi^2_{(13, 95)}$ = 21, p = 0.07), there was a difference in AR in the null corrected data ($\chi^2_{(13, 95)}$= 26.6, p = 0.014). Subsequent Mann-Whitney U tests, with Bonferroni corrections for multiple comparisons, revealed that AR in NI was significantly lower then that in Lar ($U_{(32)}$ = 52, p = 0.003) and Curt ($U_{(32)}$ = 40, p = 0.001), while Curt also had significantly higher AR than CE ($U_{(32)}$ = 42.5, p = 0.001; Figure 4.2).

Table 4.8. Mean measures of microsatellite diversity across the 6 sites. Null corrected data are in parenthesis.

	KNP	Cor	Lar	Curt	Glen	GY
N	13	8	6	9	8	44
MNA	3.88	3.06	3.88	3.88	4.00	5.31
AR	3.3(2.4)	2.9(2.25)	3.9(3.6)	3.5(2.6)	3.7(2.5)	3.6(2.5)
Ho	0.36(0.45)	0.48(0.52)	0.47(0.49)	0.28(0.53)	0.5(0.5)	0.32(0.31)
He	0.62(0.65)	0.58(0.61)	0.73(0.71)	0.67(0.65)	0.69(0.69)	0.62(0.62)

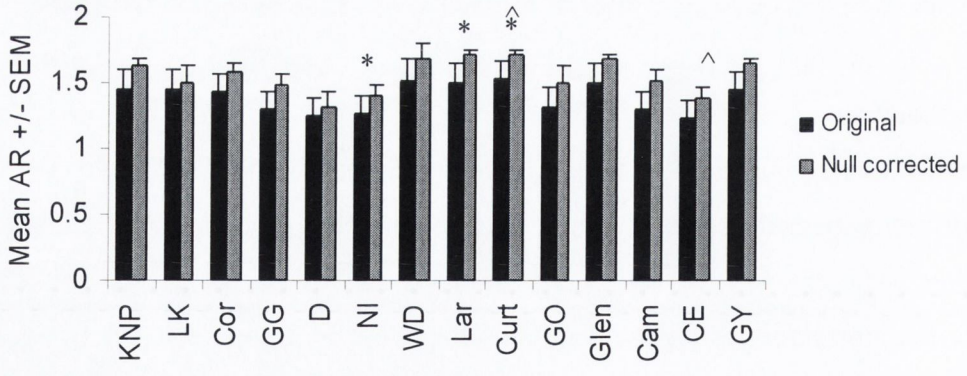


Figure 4.2. AR ± SEM for both original and null corrected data in all 14 study sites. Significant differences are indicated by * and ^. As AR is calculated based on the smallest number of samples in a population the AR measures from the 6 populations with > 2 samples were lower when all 14 populations were compared.

No LD was found among the locus pair/study site comparisons in either data set (n = 691 comparisons, $p > 0.0004$ in all cases). Significant HW disequilibrium was found in all six sites both before and after the Bonferroni correction for multiple comparisons ($p < 0.0005$ in all cases) in the original data set, but in only KNP, Lar and GY after Bonferroni correction in the corrected data set ($p < 0.008$ in all cases). Further investigation revealed significant levels of heterozygosity deficiency at loci 31, 14, Ru1, Ru4 and Ru5 in four or more of the six populations ($p < 0.05$ in all cases) in both sets of data. The test for Hardy Weinberg equilibrium was re run with those loci excluded and no evidence of Hardy Weinberg disequilibrium was found in Cor, Lar and Glen (p = 0.56, 0.06, 0.08 respectively) in the original data and in all populations *except* GY in the corrected data ($p <$ 0.0001).

Both sets of data revealed evidence of recent population bottlenecks in Cor, under both the IAM and SMM mutation models ($p < 0.05$ in all cases). Significant heterozygosity excess was also detected in Curtlestown, GY, KNP and Lar under the IAM model ($p < 0.003$ in all cases), but not under the SMM model ($p > 0.1$ in all cases). No evidence of a bottleneck was detected in Glen with either the original, or the null corrected, data ($\alpha = 0.003$; IAM Original: $p = 0.006$, Null corrected $p = 0.006$; SMM Original: $p = 0.13$, Null corrected $p =$ 0.1). The large sample sizes from KNP and GY (> 10 individuals) also allowed heterozygosity excess to be assessed using the Sign test. Results from this analysis agreed with that of the Wilcoxon tests in GY; heterozygosity excess under IAM, and no evidence of a bottleneck under the SMM mutation model. However, no evidence of a bottleneck was detected in KNP under the SMM model in either the original data set (IAM; GY $p =$ 0.001, KNP $p = 0.007$, SMM; GY $p = 0.06$, KNP $p = 0.147$), or the null allele corrected data (IAM; GY $p = 0.001$, KNP $p = 0.007$, SMM; GY $p = 0.06$, KNP $p = 0.058$). When the data were analysed under the TPM mutation model there were significantly deficient levels of heterozygosity in Cor, both in the original and null corrected data sets, indicating a recent population bottleneck ($\alpha = 0.003$, $p < 0.003$ in all cases). None of the other five populations showed evidence of a bottleneck under the TPM model.

In the original data, Cor, Curt and Glen had allele frequency distributions which had shifted from the expected L-shaped distribution found in a nonbottlenecked population (Nei and Li, 1976), while only GY displayed the L shaped distribution of allele frequencies in the null allele corrected data set (Figure 4.3).

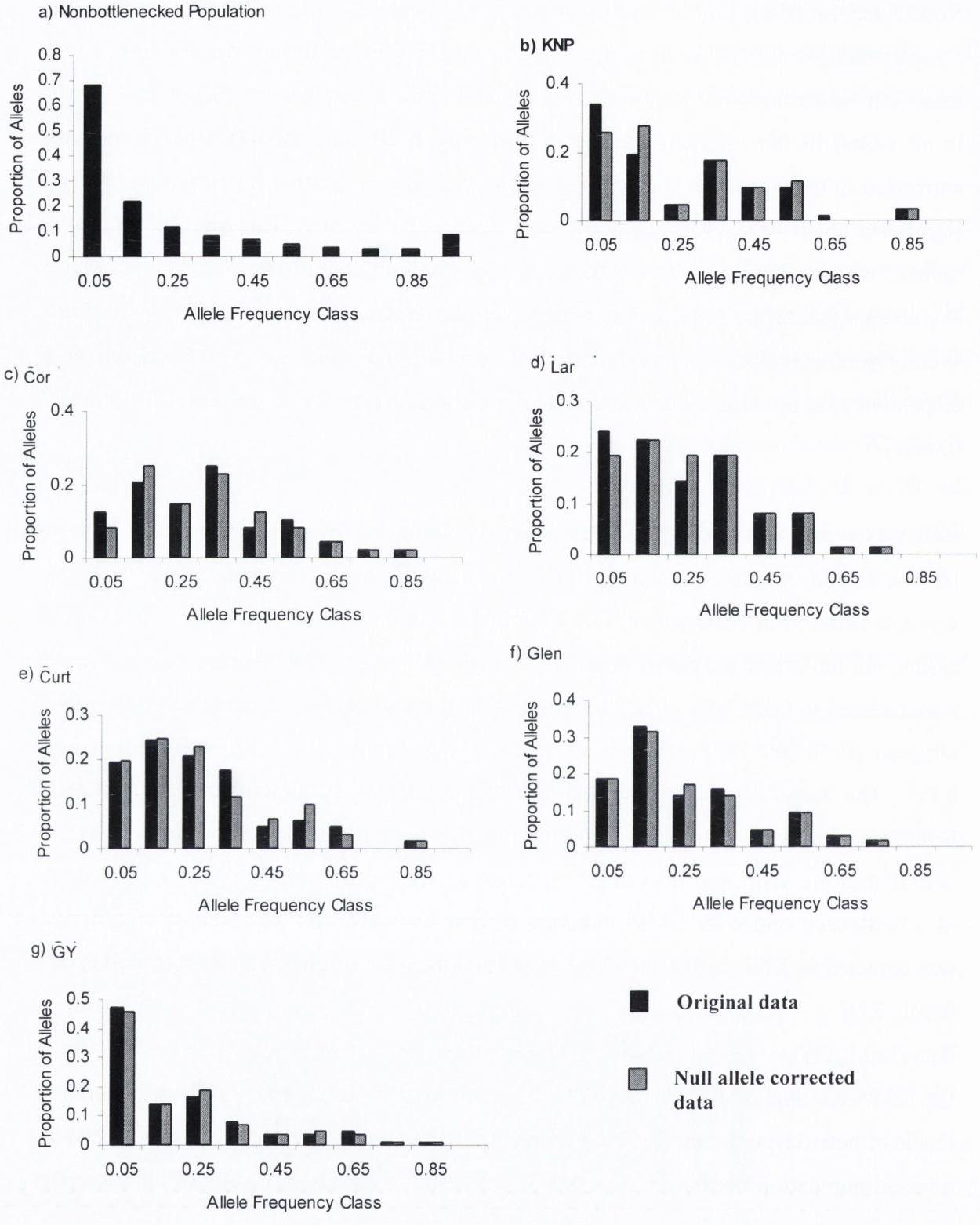


Figure 4.3. Distribution of allele frequencies a) expected in a nonbottlenecked population (Nei & Li, 1976) and (b-g) observed in original and null corrected data in the six populations whose sample size was > 2. In the original data set a) KNP and d) Lar had the L shape distributions expected of a nonbottlenecked population. Only one population, g) GY had an L shaped distribution in both the original and corrected data and GY was the only population which had an L shaped distribution in the null allele corrected data set (b-g).

MtDNA variation

Of the six populations where sample size was > 2 the GY population had the largest number of haplotypes (n = 14) while Cor had the lowest number at 2. Cor was also the only population which had no private haplotypes (Table 4.9). However, the number of haplotypes and number of private haplotypes found in a population was significantly related to the number of individuals sampled from the population (No. of haplotypes: $r_{(6)}$ = 0.962, p = 0.002, No. of private haplotypes: $r_{(6)}$ = 0.841, p = 0.036). After these were corrected for sample size, no significant correlation occurred (p > 0.4 in both cases), and the populations from Wicklow; Lar, Glen, Curt had the highest proportion of haplotypes per individual (0.5 - 0.8). The highest proportions of private haplotypes were found in KNP, Curt and GY (0.6 - 0.78; Table 4.9).

Nucleotide and gene diversity, mean pairwise differences, and the number of polymorphic sites found within each population had no relationship with sample size (p > 0.25 in all cases). MtDNA diversity was lowest in Cor, as this had the lowest number of polymorphic sites, pairwise differences, proportion of haplotypes, proportion of unique haplotypes and nucleotide and gene diversity (Table 4.9). Lar had a low number of polymorphic sites, but both its nucleotide diversity and gene diversity were similar to that found in other populations. When Cor was excluded from the comparison there was little variation between both nucleotide diversity and gene diversity across the five sites (Table 4.9). The mean number of pairwise differences between haplotypes found within a population was low in Cor, KNP, GY and Lar (1.142 - 3.4) and high in Curt and GY (6.392 and 5.57 respectively).

Table 4.9. Measures of mtDNA diversity across the six sites. Sample size corrected number of haplotypes and private haplotypes are given in parentheses.

	KNP	Cor	Lar	Curt	Glen	GY
Sample Size	12	7	5	8	8	38
No. of haplotypes	5 (0.4)	2(0.3)	4(0.8)	6(0.75)	4(0.5)	14(0.36)
No. of private haplotypes	3 (0.6)	0	1(0.25)	4(0.66)	1(0.25)	11(0.78)
No. of polymorphic sites	16	4	8	16	19	19
Nucleotide diversity	0.04	0.02	0.05	0.09	0.07	0.04
Gene diversity	0.67	0.28	0.9	0.89	0.64	0.78
Mean pairwise	2.95	1.142	3.4	6.392	5.57	3.15

4.3.1.2. Regional variation

Microsatellite variation

Measures of regional diversity across the 16 microsatellite markers are given in Table 4.10. There was no relationship between the number of samples collected from each region and any of the four diversity measurements used in the analysis in either the original or null corrected data set ($p > 0.05$ in all cases). The number of alleles varied between regions (Log_{10} No. of alleles $F_{(2,221)} = 3.097$, $p = 0.047$) and post hoc tests found a higher number of alleles in the west than the south/southwest ($p = 0.014$).

There was no difference in diversity between regions as measured with AR, Ho or He (Log_{10}AR: $F_{(2,221)} = 0.21$, $p = 0.812$; He: $F_{(2,157)} = 0.921$, $p = 0.4$; Ho: $F_{(2,138)} = 1.37$, $p = 0.26$) in the original data, neither were there any differences in the corrected data set ($p > 0.05$ in all cases).

Table 4.10. Mean values for each diversity measurement calculated from microsatellite data for each region. Null allele corrected data are in parentheses.

Region	N	Ho	He	AR	MNA
South/Southwest	25	0.417 (0.494)	0.594 (0.652)	1.582 (1.596)	2.396
East/North	29	0.446 (0.467)	0.561 (0.714)	1.904 (1.489)	2.292
West	46	0.329 (0.329)	0.544 (0.544)	1.500 (1.515)	3.531

MtDNA variation

Regional diversity indices are given in Table 4.11. Again, the number of haplotypes and private haplotypes were corrected for sample size, and there was no relationship between either of these variables and either sample size (Proportion haplotypes: $r_{s(3)} = -0.216$, $p = 0.861$; Proportion private haplotypes: $r_{s(3)} = 0.97$, $p = 0.156$), or the number of populations in each region (Proportion haplotypes: $r_{s(3)} = 0.62$, $p = 0.58$; Proportion private haplotypes: $r_{s(3)} = -0.77$, $p = 0.44$).

From Table 4.11 it is clear that there is cline of increasing diversity from the south/southwest to the west to the east/north, with the exception of the data concerning private haplotypes whereby the highest overall number of private haplotypes, and number of private haplotypes corrected for sample size, was highest in the western region.

Table 4.11. Measures of mtDNA diversity across the three regions. Sample size corrected number of haplotypes and private haplotypes are given in parentheses.

Regions	South/Southwest	East/North	West
Sample Size	23	25	39
No. of haplotypes	8(0.3)	14(0.56)	14(0.35)
No. of unique haplotypes	5(0.2)	10(0.4)	11(0.78)
No. of polymorphic sites	14	24	19
Nucleotide diversity	0.024	0.1	0.04
Gene diversity	0.45	0.94	0.77
Mean pairwise	1.82	7.58	3.09

4.3.1.3. Biogeography, landscape and genetic variation

There was no relationship between most of the microsatellite diversity measurements calculated in a population and the latitude or longitude within Ireland where that population was located ($p > 0.05$ in all cases; Table 4.12), with the exception of He calculated from the null allele corrected data, which increased with increasing longitude ($r_s = 0.829$, $p = 0.042$; Figure 4.4a). In the mtDNA diversity measurements there was a significant relationship between nucleotide diversity and longitude ($r_s = 0.886$, $p = 0.019$), and the mean number of pairwise distances between haplotypes in a population and longitude ($r_s = 0.886$, $p = 0.019$), with both increasing from west to east (Figure 4.4b). No relationship between the numbers of polymorphic sites, number of haplotypes, number of haplotypes corrected for sample size, and gene diversity with longitude or latitude was found (Table 4.12).

AR, in both the original data and the corrected data, in a population, was influenced by the degree of isolation of the forest where the population was found (Original: $R^2_{adj(13)} = 0.29$, $F = 6.7$, $p = 0.022$; Null corrected: $R^2_{adj(13)} = 0.355$, $F = 8.6$, $p = 0.011$, Figure 4.5). No other relationships between diversity and distance to nearest forest were found (Table 4.12). Although there was a relationship between AR in the null corrected data and the degree of forest isolation and forest size in the multiple regression ($p = 0.046$), only isolation was contributing significantly to the model ($p < 0.05$). The number of haplotypes in a population was related to the size of the forest where the population was resident ($R^2_{adj(5)} = 0.771$, $F = 17.8$, $p = 0.013$), but this relationship was no longer apparent when the number of haplotypes was corrected for the number of individuals sampled from the population ($R^2_{adj(5)} = -0.194$, $F = 0.187$, $p = 0.688$; Figure 4.6). No other relationships between diversity in a population and the size of the forest where the population was resident were found (Table 4.12).

Table 4.12. r_s (correlations) and R^2_{adj} (linear regressions) between the different diversity measurements in each population and landscape variable. Significant relationships are indicated in bold.

		r_s		R^2_{adj}		
		Longitude	Latitude	Size	Dist	Size*Dist
Microsatellite						
Original Data	AR	-0.09	-0.285	-0.042	**0.29**	0.243
	He	0.771	0.429	-0.123	-0.165	0.33
	Ho	-0.25	-0.486	-0.045	0.066	0.172
Null corrected	AR	-0.064	-0.274	-0.072	**0.355**	**0.301**
	He	**0.829**	0.486	-0.087	-0.247	-0.449
	Ho	-0.371	-0.029	0.318	0.029	0.314
MtDNA	Nucleotide diversity	**0.886**	0.771	-0.159	-0.215	-0.469
	Gene diversity	0.657	0.429	-0.228	-0.228	-0.597
	Pairwise differences	**0.886**	0.771	-0.186	-0.17	-0.438
	Polymorphic sites	0.088	0.5	-0.154	-0.074	-0.353
	No. of Haplotypes	0.174	0.58	**0.771**	-0.236	0.698
	No. of Haplotypes/n	0.771	0.314	-0.194	-0.086	-0.4

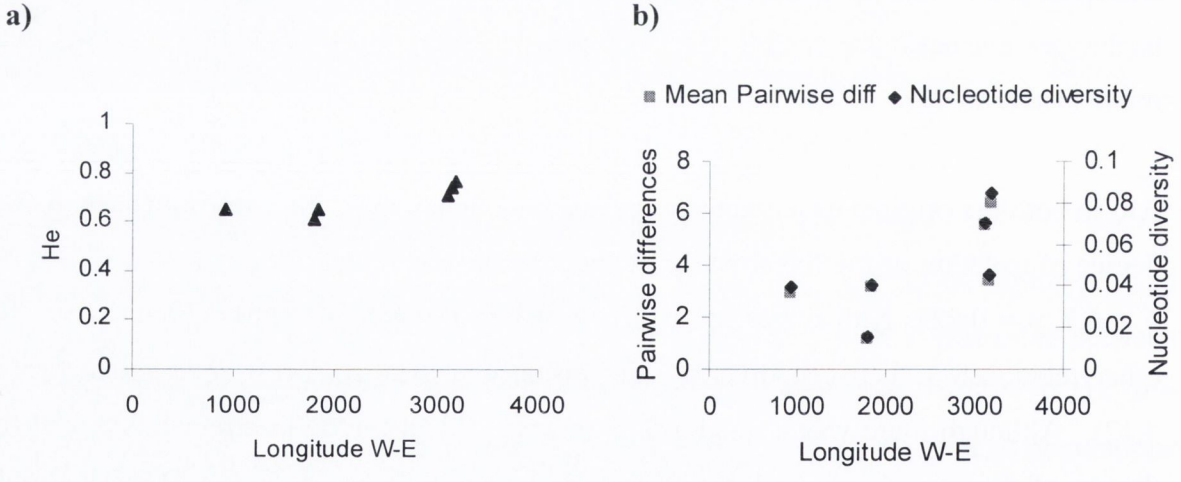


Figure 4.4. a) Relationship between He in each population calculated from the null corrected data and longitude. Gene diversity increased from west to east. b) Relationship between the number of pairwise differences and nucleotide diversity between and within haplotypes in each population, and the longitude at which the population was located. Both diversity measurements increased with increasing longitude.

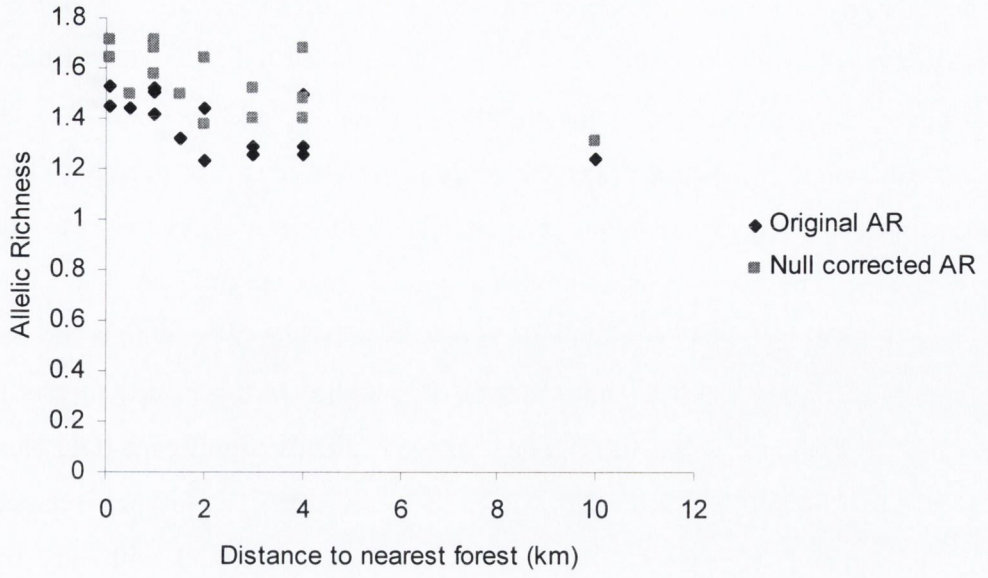


Figure 4.5. Relationship between AR in each population and forest isolation. Formulas for the regression ± SEM.: Original data: y = (-0.026 ± 0.01) (Isolation (km)) + (1.444 ± 0.035); Null corrected y=(-0.033 ± 0.011)(Isolation (km)) + (1.625 ± 0.039).

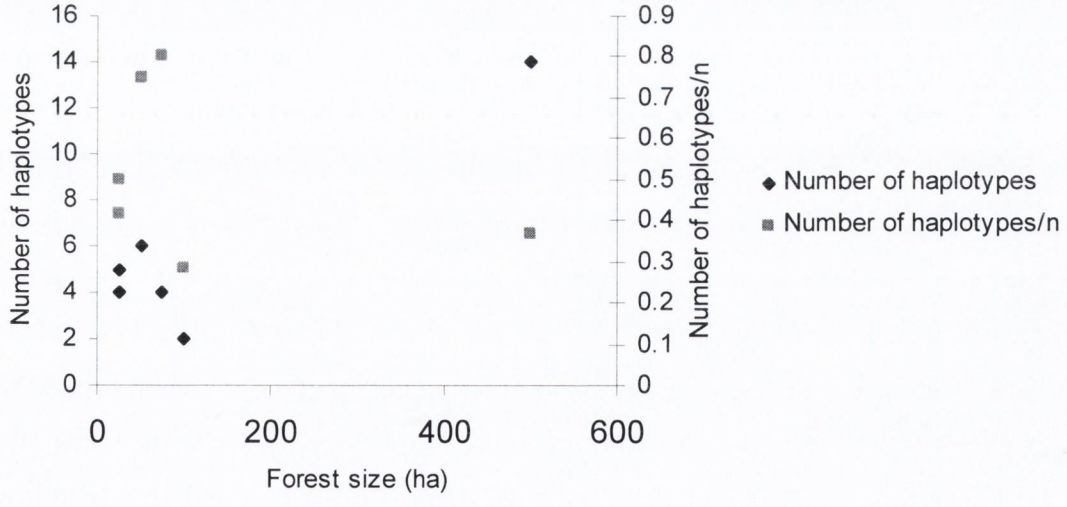


Figure 4.6. Relationship between the number of haplotypes, and the number of haplotypes corrected for sample size, and the size of the forest where the population was resident. There was a relationship between the number of haplotypes and site size (Formula for the regression ± SEM: y = (0.021 ± 0.005)(Forest Size) + (3.159 ± 1.039). After the data was corrected for the number of samples collected from each population a significant relationship no longer occurred (p = 0.481).

Although mean Ho calculated from the null corrected data in new forests was slightly higher than that in old forests, the difference was not significant ($U_{(141)} = 2164$, $p = 0.844$). Likewise, mean AR in the old sites was higher than that in the new sites in the original data, but again the difference was not significant ($t_{(112)} = -0.82$, $p = 0.222$). No other differences were evident between either the composition of the site or the age of the site, in either the original or corrected data set ($p > 0.1$ in all cases). Neither was there any difference between diversity measurements calculated from the mtDNA data set in either habitat classification ($p > 0.0.5$), although levels of polymorphism within the old forest sites was slightly higher than the new forests, and higher in the mixed forests than the conifer forests but, again, neither difference was statistically significant (Old/New: $t_{(4)} = 2.364$, $p = 0.077$; Conifer/Mixed: $t_{(4)}$ -1.087, $p = 0.388$). Haplotypes seemed to be regionally partitioned (see section 4.3.2.2.) rather than associated with any particular habitat type or habitat age.

4.3.2. Genetic structure

In the microsatellite data only the null corrected data set was used in the analysis.

4.3.2.1. Population and regional genetic structure

Allele frequency results are summarised in Table 4.13. 15 population private alleles were identified in the microsatellite data set. Three in Lar, one in Cam, one in Glen, one in Cor and 8 in GY. Most of these alleles occurred at a low frequency within the population ($\leq$ 10%), however, one of the alleles in Lar was found at a frequency of 17% within the population, and the allele in Glen also occurred at a high frequency (12.5%). The single private allele found in Cam was amplified in 25% of the samples, although, may have been skewed by the small sample size from that population (n = 2). In the population from GY only one of the alleles occurred at a frequency greater than 5% (allele 179: 19%). Overall allele frequencies varied at all loci between populations ($\chi^2_{(1222)} = 3008$, $p < 0.00001$) and regions ($\chi^2_{(188)} = 864$, $p < 0.00001$), however frequency differences at locus 19 were not significant ($\alpha = 0.003$, Population: $p = 0.01$; Region: $p = 0.06$), and were also low at locus 23 in the population comparison but not significantly so ($p = 0.007$).

Although the majority of dominant alleles were common throughout all 14 populations, showing no regional affiliation, 'rare' alleles, amplified in 3 or less populations, had some regional structure; particularly between the populations in the east and those in the rest of the country. Loci 3, 4, 6, 14, 18, Ru 1 and 23 had rare alleles amplified at them which

were region specific to the east/north region. Locus 31 had rare alleles which were unique to the south/southwestern region while genetic similarities were also detected at this locus between the east and west.

Of the three regions the east/north seems to be the most divergent with a highest frequency of rare alleles (2.6%), and a higher frequency of these alleles than in the south/southwest, and an almost equal ratio of these alleles to the west (Table 4.13). However, in the regional comparisons there was very little association between the rare alleles found in the east/north and those found in the west, with any rare alleles which were found in both regions occurring at a much higher frequency in the western region (8.2:1; Table 4.13).

Table 4.13. Summary of allele frequencies in the three regions. The number of unique alleles in each region in given in parenthesis after the region name. On the diagonal, in bold, is the ratio of rare alleles to the total number of alleles amplified in that region (and the percentage frequency of those rare alleles within that region). Below the diagonal are the number of shared 'rare' alleles between those two regions, with the number of alleles which were found only in both of those regions in parenthesis. Above the diagonal is the ratio of the frequency of the shared alleles between those regions (x/y; a value > 1 indicates there was a higher frequency of those alleles in the region on the x axis).

	S/SW(1)	E/N(10)	W(8)
South/Southwest	**0.16 (1.2)**	1.63	0.9
East/North	10(6)	**0.18 (2.6)**	8.2
West	9(3)	7(5)	**0.24 (0.8)**

Haplotypes frequencies varied both among populations ($\chi^2_{(13)} = 536$, $p < 0.0001$) and regions ($\chi^2_{(3)} = 864.8$, $p < 0.0001$). One haplotype, IRL, was found in 23 of the 87 samples amplified, and was found in all regions, and in over 50% of the samples from the south/southwest (Table 4.14). The south/southwest had 6 unique haplotypes, and the most frequent haplotype in the south/southwest was also found in the east/north and west. No haplotype was dominant in all regions, in fact no haplotype was dominant in more than one region. The dominant haplotype in the west was W11 (41%) and was only found in the west. As already mentioned, IRL was dominant in the south/southwest (42%) but also found in other regions, while the east/north was dominated by E6 (30%) a haplotype found only in that region. At a population level haplotype dominance only varied in the east with E6 dominant in Lar and Glen, E3 dominant in Curt, WxA in Cam, and IRL in GO, but only one sample was collected from the latter two populations.

Table 4.14. Haplotype frequencies in the 13 populations. Sample size for each population given in parenthesis. Dominant haplotypes in each population are in bold, while those dominant in a region are italicised.

	South/Southwest					East/North					West		
	GG(2)	KNP(12)	LK(1)	Cor(7)	WD(1)	Curt(8)	Glen(8)	GO(1)	Lar(5)	Cam (1)	NI(2)	CE(1)	GY(38)
S1	0	0	0	***0.857***	1	0	0	0	0	0	0	0	0
IRL	**1**	***0.583***	0	0.143	0	0	0	**1**	0.2	0	0	0	0.263
SW1	0	0.0833	0	0	0	0	0	0	0	0	0	0	0
SW2	0	0.0833	0	0	0	0	0	0	0	0	0	0	0
SW3	0	0.0833	0	0	0	0	0	0	0	0	0	0	0
SW+W+E	0	0.167	0	0	0	0.125	0	0	0	0	0	0	0.0263
W1	0	0	**1**	0	0	0	0	0	0	0	0	0	0.0263
E+N	0	0	0	0	0	0	0.125	0	0.2	0	**0.5**	0	0
N1	0	0	0	0	0	0	0	0	0	0	**0.5**	0	0
E1	0	0	0	0	0	0	0	0	0.2	0	0	0	0
E6	0	0	0	0	0	0	***0.625***	0	***0.4***	0	0	0	0
E3	0	0	0	0	0	***0.375***	0.125	0	0	0	0	0	0
E7	0	0	0	0	0	0	0.125	0	0	0	0	0	0
E4	0	0	0	0	0	0.125	0	0	0	0	0	0	0
E2	0	0	0	0	0	0.125	0	0	0	0	0	0	0
E5	0	0	0	0	0	0.125	0	0	0	0	0	0	0
E8	0	0	0	0	0	0.125	0	0	0	0	0	0	0
WXA	0	0	0	0	0	0	0	0	0	**1**	0	0	0
W2	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W3	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W4	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W5	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W6	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W7	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W8	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W9	0	0	0	0	0	0	0	0	0	0	0	0	0.0263
W10	0	0	0	0	0	0	0	0	0	0	0	**1**	***0.395***
W11	0	0	0	0	0	0	0	0	0	0	0	0	0.0526
W12	0	0	0	0	0	0	0	0	0	0	0	0	0.0263

Population pairwise F_{ST} of microsatellite data revealed a number of significant genetic distances between populations (Table 4.15a). F_{ST} values were significant between Cor and all populations *except* GO, LK, WD, Cam, CE and D (F_{ST} = -0.02 - 0.48, $p < 0.03$ in all cases) and between those same populations as well as the north and Cor, and GY (F_{ST} = 0.06-0.9, $p < 0.03$ in all cases). The largest significant distance was between CE and GY (F_{ST} = 0.6, $p < 0.00001$). Similar patterns were seen with the mtDNA data (Table 4.15b). Again Cor and GY were significantly different from the largest number of populations (Cor: n = 7, GY, n = 5; Table 4.15b) and also from each other (F_{ST} = 0.35, $p < 0.0001$), however, Curt also had a high amount of differentiation from the other populations sampled. The largest F_{ST} value was between Cor and GG (F_{ST} = 0.72, $p < 0.0001$).

Within regions there was little differentiation. In the southwest KNP and GG were different (F_{ST} = 0.3, p = 0.018), while in the east, Curt was different to Cam (F_{ST} = 0.27, p = 0.018). In the mtDNA data set the only differentiation within regions occurred between Curt and Lar (F_{ST} = 0.1, p = 0.04), and Curt and Glen (F_{ST} = 0.19, p = 0.018).

Despite the significant differentiation between some populations, the hierarchal AMOVA revealed that the highest percentage of variation in both sets of data was within populations (Microsatellite: 75.1%, $p < 0.0001$; MtDNA: 75.8%, $p < 0.0001$). Both sets of data detected differentiation between populations within regions, but not between regions as significant (Microsatellite; Populations within regions: 22.71%, $p < 0.0001$; Regions: 0.31%, p = 0.65, MtDNA; Populations within regions: 19.33%, $p < 0.0001$; Regions: 4.87%, p = 0.06).

This difference in genetic relationships between populations based on the microsatellite and mtDNA data was supported by the neighbour joining trees constructed from D_s. On the microsatellite tree there was some regional clustering with Glen, GO and Lar all grouping together, although bootstrap support was poor (Figure 4.7a), however some populations clustered strongly with populations from different regions (Cam with CE, D with GG). When the tree was constructed from only the 6 populations with n > 2, a clear east-west/south division became apparent, with all of the 3 eastern populations clustering together, and the west and south/southwestern populations grouping together (Figure 4.7b).

Table 4.15. Population pairwise F_{ST} (above diagonal) and D_S (below diagonal) for (a) microsatellite and (b) mtDNA data. Significant differences measured from the F_{ST} data are indicated in bold, p <0.05, *p <0.001.

a)

	South/ Southwest				East/North							West	
	GG	KNP	LK	Cor	WD	Curt	D	Glen	GO	Lar	Cam	NI	CE	GY
GG		**0.303**	0.489	**0.502**	0.273	**0.275**	0.209	-0.600	0.489	0.111	-0.333	0.000	0.250	**0.537***
KNP	0.341		0.066	**0.274***	-0.156	-0.065	0.225	-0.042	-0.123	-0.046	**0.303**	0.019	**0.253**	**0.147***
LK	0.546	0.487		0.586	-0.200	0.043	0.333	0.003	0.000	0.002	0.489	-0.081	0.529	0.068
Cor	0.597	0.383	0.713		0.212	**0.306***	0.458	**0.277***	0.289	**0.209**	**0.502**	**0.434***	-0.020	**0.598***
WD	0.699	0.666	0.525	0.696		-0.103	0.000	-0.237	-0.500	-0.263	0.273	-0.263	0.111	0.220
Curt	0.557	0.513	0.777	0.556	0.826		0.184	0.021	0.019	-0.040	**0.275**	0.009	0.257	**0.23***
D	0.478	0.692	1.201	0.969	1.155	0.612		0.246	0.333	-0.028	-0.600	-0.212	0.111	0.508
Glen	0.672	0.406	0.721	0.485	0.689	0.257	0.874		-0.272	0.000	0.330	0.050	**0.251**	**0.142***
GO	0.791	0.800	1.017	0.809	0.860	0.639	1.040	0.430		-0.094	0.489	0.024	0.291	0.076
Lar	0.639	0.569	0.608	0.419	0.559	0.291	0.992	0.261	0.517		0.111	-0.103	0.092	**0.239***
Cam	0.515	0.747	0.750	0.610	1.062	0.474	0.909	0.730	0.905	0.494		0.000	0.250	0.538
NI	0.646	1.188	1.039	0.836	0.876	0.832	1.450	0.844	0.863	0.596	1.073		**0.246**	0.192
CE	0.715	0.823	0.666	0.696	1.005	0.555	1.338	0.693	1.104	0.496	0.426	1.050		**0.607***
GY	0.546	0.299	0.477	0.454	0.637	0.302	0.809	0.333	0.710	0.453	0.487	0.922	0.664	

b)

	South/Southwest					East/North					West	
	GG	KNP	LK	Cor	WD	Curt	Glen	Lar	Cam	NI	CE	GY
GG		0.123	1.000	**0.728**	1.000	**0.318**	**0.490**	0.200	1.000	0.500	1.000	0.167
KNP	0.067		0.333	**0.446***	0.333	**0.213***	**0.343***	0.135	0.333	0.258	0.333	**0.129***
LK	1.000	1.000		0.715	1.000	0.107	0.350	0.100	1.000	0.000	1.000	0.193
Cor	1.804	1.871	1.000		-1.000	**0.397***	**0.526***	**0.428**	0.714	**0.564**	0.714	**0.356***
WD	1.000	1.000	1.000	0.014		-0.103	0.350	0.100	1.000	0.000	1.000	0.214
Curt	1.000	2.637	1.000	1.000	1.000		**0.190***	**0.103**	0.107	0.077	0.107	**0.168***
Glen	1.000	1.000	1.000	1.000	1.000	1.887		-0.050	0.350	0.217	0.350	**0.260***
Lar	0.973	1.040	1.000	2.777	1.000	0.973	0.241		0.100	0.000	0.100	0.126
Cam	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000	1.000	0.214
NI	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.973	1.000		0.000	0.167
CE	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		-0.297
GY	0.613	0.651	2.915	2.417	1.000	4.235	1.000	1.586	1.000	0.613	0.206	

The tree constructed from mtDNA data had a stronger regional structure (Figure 4.8). The two populations from the west grouped together in 100% of trees, as did those from the south, and two populations from the southwest. The only non-regional grouping occurred with Curt and NI clustering nearer to WD and Cor in the south/southwestern region, than to other east/north populations, supporting the significant F_{ST} found between Curt and other eastern populations, and the fact that Curt shared a haplotype with the north. When only the 6 populations whose n > 2 were used in the tree construction, Curt grouped with Cor in 100% of the trees, the other two eastern populations grouped together, while GY and KNP also grouped together in all trees (Figure 4.8b)

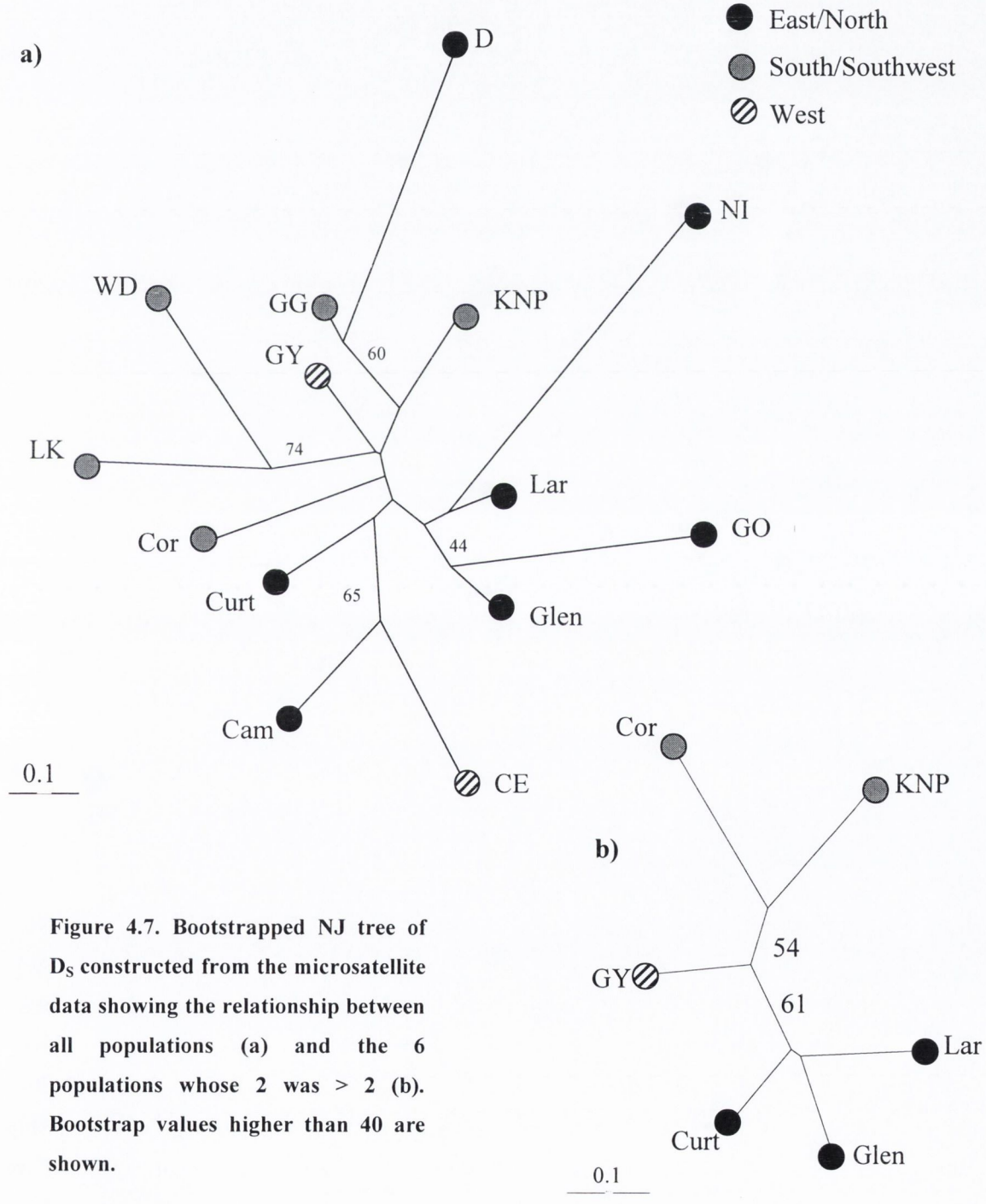


Figure 4.7. Bootstrapped NJ tree of D_S constructed from the microsatellite data showing the relationship between all populations (a) and the 6 populations whose 2 was > 2 (b). Bootstrap values higher than 40 are shown.

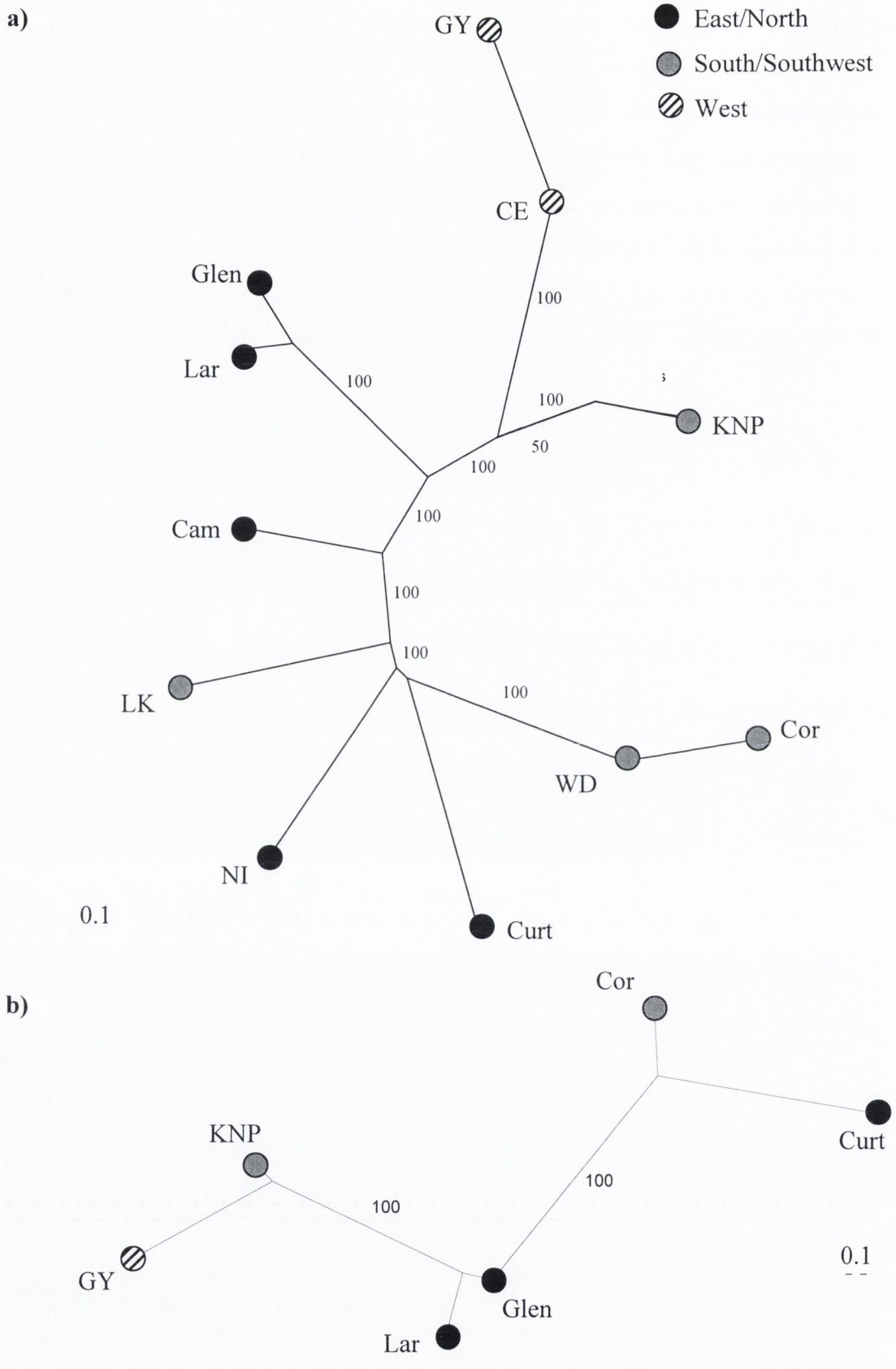


Figure 4.8. Bootstrapped NJ tree of D_S constructed from the mtDNA data showing the relationship between all populations (a) and the 6 populations whose sample size was > 2 (b). Bootstrap values are shown.

The population level assignment tests revealed some genetic structure in Ireland, with the majority of the samples assigned to the correct population, or another population within the same region (Figure 4.9; Table 4.16). The exception was Lar where out of six individuals one was assigned to Waterford, one to Lar, two to Glen, one to the GO and one to CE ($p <$ 0.001 in all cases; Table 4.16). The scatterplots of population membership (Figure 4.9) did show differentiation between regions, however, differentiation between the south/southwest and west was the most marked, with no individuals in either of those regions falling on or across the line marking differentiation between regions.

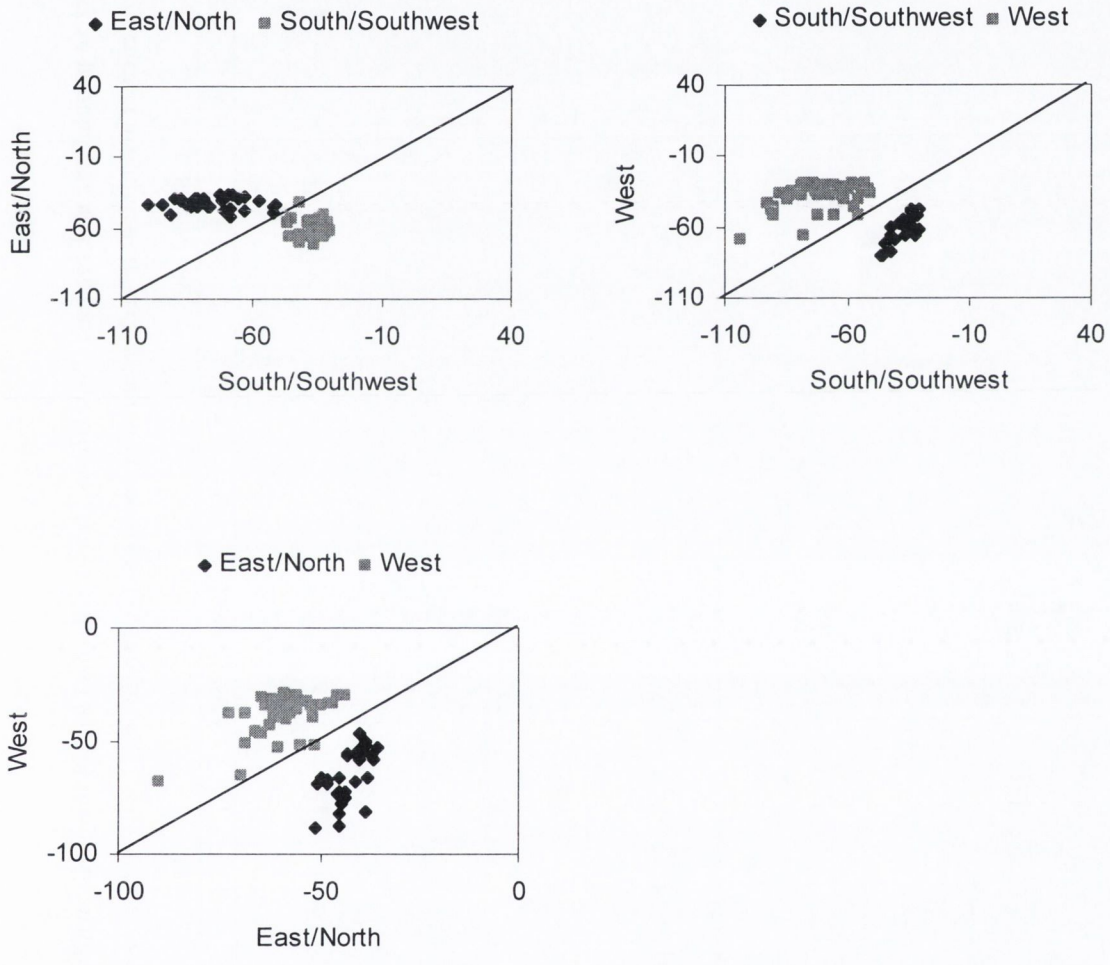


Figure 4.9. Scatter plots of log likelihoods of population membership in each possible pair of regions. Squares and diamonds represent individuals in each population, coded on the basis of which region that population was in. Individuals that fall above, or the same side of, the diagonal line in each graph, as the region they were collected from, were correctly assigned.

Table 4.16. Population assignment into respective populations and regions. Populations (and sample size) are on the y axis, while the populations and regions they were assigned to are on the x axis. The percentage of correct assignment in each population and region is also given.

From \ To	South/Southwest					East/North							West		% Correct Assignment	
	GG	KNP	LK	Cor	WD	Curt	D	Glen	GO	Lar	Cam	NI	CE	GY	Pop	Region
GG(2)	1	-	-	-	-	1	-	-	-	-	-	-	-	-	50	50
KNP(13)	-	12	-	-	-	-	-	1	-	-	-	-	-	-	92.3	92.3
LK(1)	-	-	1	-	-	-	-	-	-	-	-	-	-	-	100	100
Cor(8)	-	-	-	8	-	-	-	-	-	-	-	-	-	-	100	100
WD(1)	-	-	-	-	1	-	-	-	-	-	-	-	-	-	100	100
Curt(9)	-	-	-	-	-	4	-	2	-	3	-	-	-	-	44.4	100
D (1)	-	-	-	-	-	-	1	-	-	-	-	-	-	-	100	100
Glen (8)	-	-	-	-	-	1	-	3	1	3	-	-	-	-	37.5	100
GO(1)	-	-	-	-	-	-	-	-	1	-	-	-	-	-	100	100
Lar(6)	-	-	-	-	1	2	-	1	-	1	-	-	1	-	16.6	66
Cam(2)	1	-	-	-	-	1	-	-	-	-	-	-	-	-	0	50
NI(2)	-	-	-	-	-	-	-	-	-	-	-	2	-	-	100	100
CE(2)	-	-	-	-	-	-	-	-	-	-	-	-	2	-	100	100
GY(44)	-	2	-	-	1	-	-	1	-	-	1	-	-	39	88.6	88.6

4.3.2.3. Influences behind genetic structure

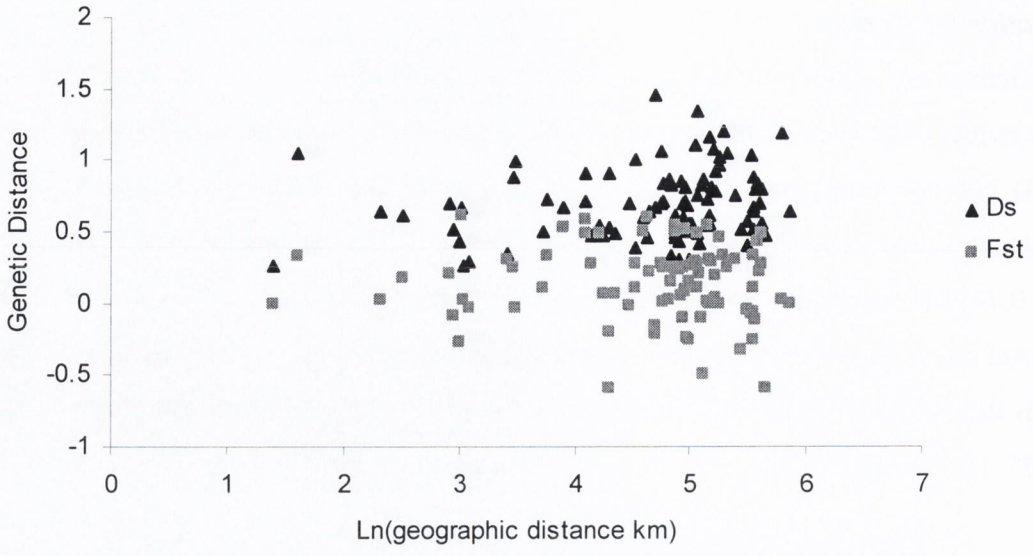
Isolation by distance

The matrix of geographic distances between populations is given in appendix 8.4. IBD was evident between populations with F_{ST} calculated from the mtDNA ($r_{(66)}$ = 0.2, p = 0.04) but not from microsatellite ($r_{(91)}$ = 0.-0.06, p = 0.694) data, and with only the microsatellite data when D_S was used as a genetic distance measure (MtDNA: $r_{(66)}$ = -0.08, p = 0.74; Microsatellite: $r_{(91)}$ = 0.22, p = 0.02; Figure 4.10a and b). A similar relationship existed when all but the populations whose sample size was < 2 individuals were excluded from the analysis (F_{ST}: MtDNA: $r_{(15)}$ = 0.54, p = 0.027; Microsatellite: $r_{(15)}$ = 0.22, p = 0.257, D_S: MtDNA: $r_{(15)}$ = 0.29, p = 0.13; Microsatellite: $r_{(15)}$ = 0.41, p = 0.03).

When populations were pooled into regions there was no relationship between genetic distance between regions and either the minimum, or mean, geographic distance between regions and either genetic distance measure (p > 0.2 in all cases).

IBD was also investigated within the south/southwestern and north/eastern regions as these regions had samples from 3 or more populations collected from them. There was no evidence of IBD in the south/southwest (p > 0.1 in all cases), however, there was IBD within the eastern region with F_{ST} from the mtDNA data ($r_{(6)}$ = 0.62, p = 0.001). This correlation was no longer significant once Cam, the population which shared no haplotypes with other north/eastern populations, was removed from the analysis.

a)



b)

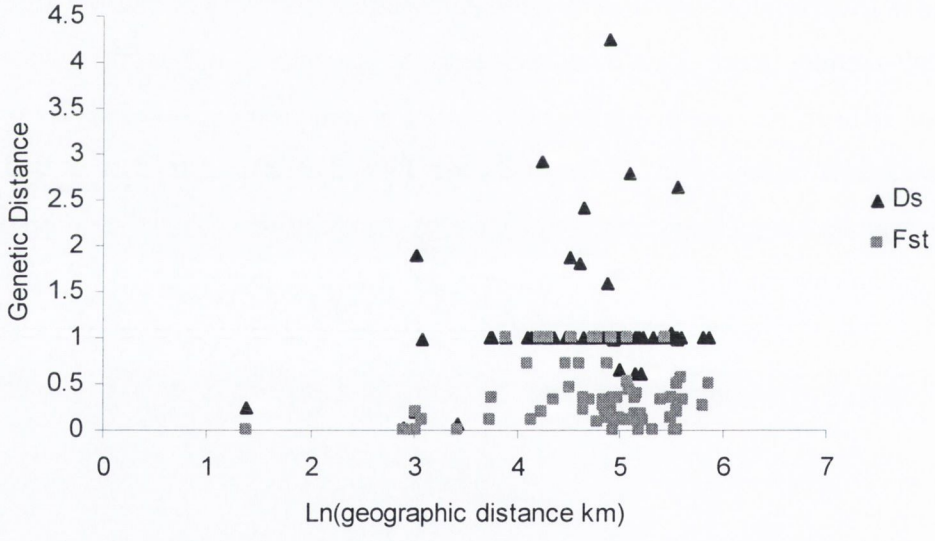


Figure 4.10. Isolation by distance. Scatterplots of genetic distance (F_{ST} and D_S) v ln geographic distance for microsatellite (a) and mtDNA (b) data. Significant IBD was detected between ln geographic distance and D_S from the microsatellite data (a) and F_{ST} from the mtDNA data (b).

As a result of this lack of IBD within regions, and the effect of the removal of the Cam from the north/eastern region on the analysis, populations were grouped together based on their geographic distance from each other and reanalysed in the program STRUCTURE to determine at which distance IBD occurs. There were 8 separate groupings; populations which were 0-24, 25-49, 50-99, 100-149, 150-199, 200-249, 250-299 and 300+ km apart. All pairs of populations within each distance class were analyzed with a K = 2 and the resulting q_2 values plotted to determine at which distance the bimodal distribution of q_2, whereby q_2 was either 0 - 0.1 or 0.9 - 1, indicative of divergence between populations, occurred.

When all populations were used in the analysis it was apparent that the distribution of q_2 was neither uni nor bimodal in the majority of the distance classes, but, instead showed trimodality – with some populations being assigned to one of the two proposed populations with either high or low q_2 and some being assigned with a q_2 of around 0.5 (not shown). When the analysis was run excluding the populations with n = 1, and, as a result of this, a much clearer picture emerged (Figure 4.11). What is first apparent is the influence that the populations with the small sample size had on the frequency of $q_2 \sim 0.5$. Once those populations were removed the extreme peak seen in the previous analysis around 0.5, indicating no clear differentiation between populations, disappeared. No distance ranges were unimodel in the rerun analysis however the frequencies of $q_2 \sim 0.5$ decrease as the distance ranges increase, indicating that IBD is occurring. Populations up to 149 km apart showed no clear genetic differentiation. Although peaks in q_2 occurred at both 0 - 0.1 and 0.9 - 1 in the distribution up to 149km, values of q_2 between 0.1 and 0.9 still occurred, and only disappeared in populations which were > 150 km apart. It is apparent that IBD is influencing the genetic differentiation between populations, however, the lack of unimodality at small geographic distances, indicates that geographic distance is not the sole influence governing genetic divergence at distances < 150km.

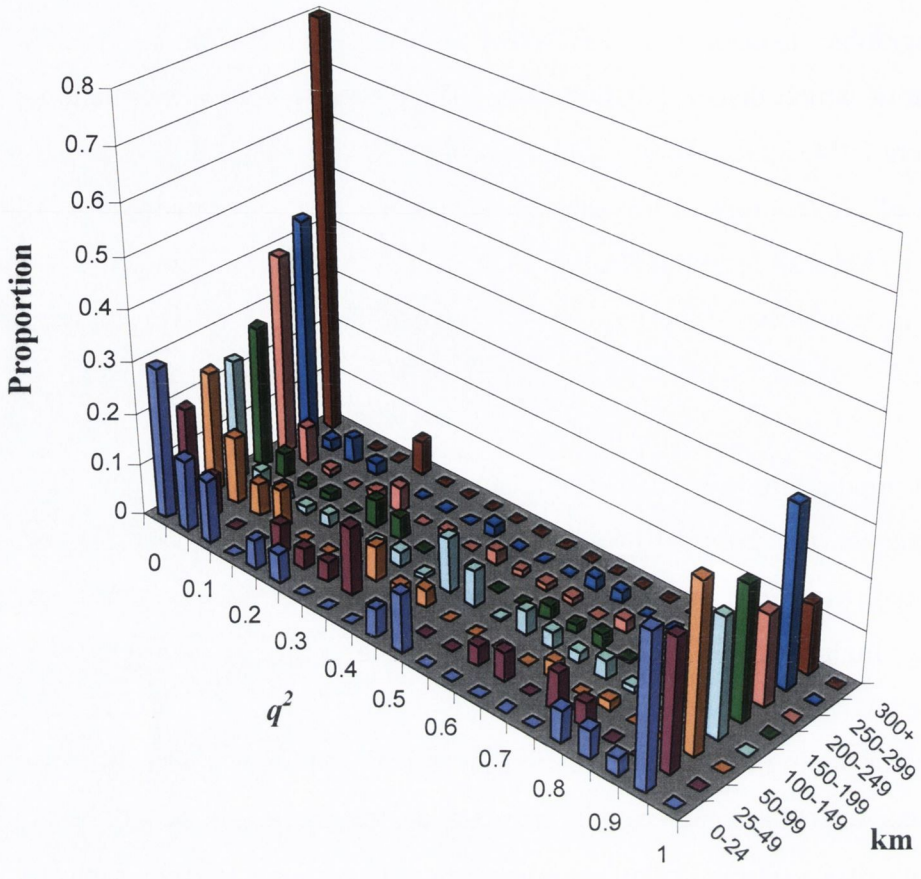


Figure 4.11. Distributions of q_2 for all pairwise combinations of populations which had sample size > 1, within 8 distance ranges. The ranges contain the following numbers of pairs of populations: 0-24: 4, 25-49: 3, 50-99: 4, 100-149: 15, 150-199: 6, 200-249: 4, 250-299: 5, 300+: 2.

Spatial and genetic variation

The marginal tests detected no relationship between any of the landscape and genetic diversity measurements with genetic structure in either the microsatellite or mtDNA data set (Table 4.17a). However, when the geographical distance between populations was taken into account in the conditional tests, significant relationships were detected in all four sets of data. In the microsatellite data set F_{ST} was related to the age of the forest and type of the forest where the population was resident, while AR within the population also influenced the genetic structure. The age of the forest was also significantly related to D_S, as was nucleotide diversity within each population. Further conditional tests on both the genetic, using landscape variables as covariates, and landscape, using genetic variables as covariates, data, revealed that much of the genetic structure seen between populations is a result of differences in genetic diversity between the populations, although the amount of variation explained by genetic diversity in both data sets was low (F_{ST} = 19%, D_S = 0.8%).

However, when the conditional tests were run on the landscape variables, taking into account the variation explained by both differences in genetic diversity and geographic distance between populations, the age of the site still remained significantly related to genetic structure in the D_S data set, accounting for 36% of the variation within the data set.

In the mtDNA data set F_{ST} was significantly related to the landscape variables once geographic variation was taken into account, as was AR. The relationship between genetic structure and AR remained significant after variation in landscape variables was taken into account while the association between landscape structure and genetic structure was explained by the geographical and genetic variation between populations (Table 4.17b).

There were significant relationships between D_S and both genetic and landscape variables when geographic distance was a covariate in the analysis (Table 4.17a), with all of the landscape variables ($p < 0.007$ in all cases) and AR, nucleotide diversity and the mean number of pairwise differences between haplotypes in a population, related to the genetic distance between populations. However, further conditional tests revealed that none of the genetic variables were related to genetic structure when the landscape variables were covariates, while a single landscape variable, the age of the forest, remained significantly related to genetic structure once geographical and genetic variation was taken into account (Table 4.17b).

When the data were reanalysed with only the 6 populations whose $n > 2$ (not shown) there were some significant differences in the marginal tests. In the microsatellite data set F_{ST} was related to the age of the forest ($F = 5.45$, $p = 0.001$), and the number of polymorphic sites in the haplotypes found within the population ($F = 20.52$, $p = 0.031$), with the latter explaining 57% of the variation within the data, and the former 83%. However, these differences were no longer apparent in the conditional tests, and no variable had a significant relationship with genetic structure, measured with either F_{ST} or D_S, over and above that explained by the covariates in the model in the microsatellite data set.

In the mtDNA data set F_{ST} was related to AR ($F = 3.82$, $p = 0.032$) and the type of habitat in which the population was resident ($F = 765$, $p = 0.001$). After geographic distance between populations was incorporated into the model, this difference was no longer apparent, however, there was a relationship with gene diversity ($F = 5.18$, $p = 0.001$), and the age of the forest was significantly related over and above the variation explained by

genetics and geographic distance ($F = 19.7$, $p = 0.001$). When D_S was used as a distance measure the landscape variables and the type of site where the population was resident were significantly related to genetic structure (Land: $F = 707$, $p = 0.001$; Hab: $F = 765$, $p = 0.001$) with both sets of variables explaining over 99% of the variation within the data set each. With geographic distance as a covariate, only the region into which the population was assigned was significant ($F = 25.9$, $p = 0.007$) of the spatial variables, while of the genetic variables, nucleotide diversity was also significantly related ($F = 2.36$, $p = 0.001$). However, after genetic and geographic variation were taken into account, no landscape variables were significantly related to genetic structure, although mitochondrial gene diversity, and the number of polymorphic sites, did have a significant relationship with genetic structure (Poly: $F = 15.14$, $p = 0.24$; MgeneD: $F = 7.41$, $p = 0.001$).

Table 4.17. a and b(over): Relationship between genetic structure and spatial and genetic predictor variables calculated from the dbRDA *F* – statistic. *P* values < 0.05 are indicated in bold. Prop is the proportion of variation within the data set explained by that set of predictor variables.

a)	Marginal Tests			Conditional Tests: Geo		
	F	P	Prop	F	P	Prop
Microsat						
Fst				**Fst**		
Land	*0.979*	*0.502*	*0.246*	*Land*	*1.234*	*0.188*	*0.240*
Reg	1.139	0.415	0.094	Reg	2.892	0.945	-0.063
Age	-0.531	0.925	-0.051	Age	5.601	**0.001**	-0.030
Hab	0.012	0.826	0.001	Hab	3.128	**0.001**	-0.054
Genetics	*0.478*	*0.831*	*0.255*	*Genetics*	*5.718*	*0.060*	*-0.025*
AR	0.701	0.508	0.060	AR	4.756	**0.001**	-0.033
Nuc	-0.632	0.958	-0.061	Nuc	1.005	0.836	0.020
He	1.633	0.307	0.129	He	3.730	0.999	-0.042
Poly	1.265	0.395	0.103	Poly	3.218	0.882	-0.051
Pair	-0.566	0.915	-0.054	Pair	3.303	0.693	-0.049
Ds				**Ds**		
Land	*0.815*	*0.600*	*0.196*	*Land*	*1.472*	*0.139*	*-0.155*
Reg	2.422	0.068	0.168	Reg	1.596	0.787	0.058
Age	0.156	0.922	0.013	Age	2.496	**0.002**	-0.074
Hab	0.114	0.928	0.009	Hab	1.478	0.757	0.042
Genetics	*1.574*	*0.167*	*0.529*	*Genetics*	*1.207*	*0.586*	*-0.323*
AR	0.365	0.859	0.030	AR	2.113	0.321	-0.275
Nuc	1.523	0.222	0.122	Nuc	2.045	**0.001**	-0.086
GeneD	1.310	0.310	0.106	GeneD	1.534	0.596	0.006
Poly	0.840	0.555	0.071	Poly	2.094	0.826	-0.042
Pair	1.373	0.278	0.111	Pair	1.974	0.978	0.146
MtDNA							
Fst				**Fst**		
Land	-0.208	0.998	-0.098	*Land*	9.240	**0.001**	-0.264
Reg	1.345	0.458	0.130	Reg	0.911	0.241	0.189
Age	-1.003	0.954	-0.125	Age	1.222	0.490	0.355
Hab	-1.790	1.000	-0.248	Hab	3.643	0.270	-0.501
Genetics				*Genetics*			
AR	0.010	0.837	0.001	AR	-0.021	**0.030**	-0.002
Nuc	-2.816	1.000	-0.455	Nuc	-1.421	1.000	-0.094
He	3.625	0.111	0.287	He	-9.060	1.000	-0.185
Poly	-3.240	1.000	-0.562	Poly	1.931	0.348	6.319
Pair	-2.970	1.000	-0.492	Pair	0.978	0.698	0.216
Ds				**Ds**		
Land	*3.553*	*0.099*	*0.604*	*Land*	*3.075*	***0.001***	*0.164*
Reg	1.675	0.341	0.157	Reg	30.665	**0.002**	0.099
Age	3.078	0.222	0.255	Age	1.405	**0.006**	-0.218
Hab	-0.972	0.928	-0.121	Hab	3.065	**0.003**	0.267
Genetics	*1.250*	*0.423*	*0.556*	Genetics	0.011	1.000	-0.001
AR	-1.097	0.967	-0.139	AR	-0.540	**0.009**	0.020
Nuc	0.198	0.654	0.022	Nuc	1.322	**0.004**	-0.180
He	-0.090	0.767	-0.010	He	3.916	0.663	0.189
Poly	-1.182	0.983	-0.151	Poly	1.964	0.481	-5.112
Pair	0.027	0.720	0.003	Pair	2.572	**0.001**	0.416

b)	Conditional Tests: Gen (Gen*Geo)							Conditional Tests: Land (Land*Geo)					
	F	(F)	P	(P)	Prop	(Prop)		F	(F)	P	(P)	Prop	(Prop)
Microsat							**Microsat**						
Fst							**Fst**						
Land	0.209	2.518	0.605	0.642	0.101	-0.127	*Genetics*	*0.136*	*2.181*	*0.999*	***0.002***	*0.110*	-0.195
Reg	0.618	14.694	0.388	0.197	-0.086	0.046	AR	0.146	-3.588	0.739	0.642	0.014	-0.014
Age	0.261	5.742	1.000	0.690	0.023	-0.111	Nuc	-0.576	3.065	0.977	**0.001**	-0.059	0.054
Hab	-0.816	0.142	1.000	0.870	0.015	-0.013	He	0.676	5.983	0.570	0.229	0.059	-0.207
							Poly	-0.141	-122.579	0.872	0.983	-0.014	-0.033
							Pair	-0.570	14.279	0.967	**0.037**	-0.058	-0.052
Ds							**Ds**						
Land	0.762	5.823	0.633	0.204	0.171	0.661	*Genetics*	*1.167*	*-0.295*	*0.426*	*0.086*	*0.437*	-0.008
Reg	2.180	3.097	0.165	0.611	0.126	-0.254	AR	1.492	4.245	0.243	0.618	0.116	0.328
Age	0.453	60.539	0.729	**0.024**	0.033	0.363	Nuc	0.920	1.740	0.482	0.555	0.076	0.031
Hab	0.227	7.360	0.910	0.099	0.017	6.551	He	1.279	13.912	0.297	**0.001**	0.102	-0.091
							Poly	0.896	6.193	0.499	1.000	0.074	-0.303
							Pair	0.837	-7.239	0.540	0.981	0.070	-0.189
MtDNA							**MtDNA**						
Fst							**Fst**						
Land	0.431	2.270	0.758	0.346	0.286	0.127	*Genetics*	0.595	55.587	0.756	**0.001**	0.656	-0.961
Reg	2.534	6.816	0.141	0.476	0.282	1.514	AR	0.904	41.205	0.483	**0.020**	0.144	-1.058
Age	0.588	0.973	0.592	1.000	0.093	0.007	Nuc	-0.522	-4.845	0.997	1.000	-0.105	-0.458
Hab	0.364	4.093	0.789	0.412	0.061	0.057	He	4.793	-10.361	0.059	0.865	0.488	-0.627
							Poly	-0.885	-11.130	1.000	1.000	-0.190	-0.642
							Pair	-0.605	-0.351	1.000	0.681	-0.123	-0.061
Ds							**Ds**						
Land	0.901	2.420	0.558	0.344	0.255	-0.390	*Genetics*	0.439	1.311	0.872	0.534	0.207	-0.160
Reg	4.456	6.812	0.124	0.513	0.234	-3.392	AR	-0.177	-2.885	0.818	0.968	-0.012	0.022
Age	-0.422	29.342	0.856	**0.001**	-0.052	0.123	Nuc	0.802	-6.617	0.469	0.945	0.047	0.034
Hab	-0.564	9.516	0.855	0.065	-0.073	0.354	He	0.094	6.871	0.745	0.319	0.006	0.219
							Poly	-0.725	4.319	0.968	0.695	-0.055	-0.378
							Pair	0.724	3.933	0.488	0.296	0.043	-0.220

Barriers to dispersal

In the microsatellite data set (Table 4.18) there was only one correlation between F_{ST} and a barrier to dispersal, and overall both the coefficients of determination (r^2) in the simple tests, and the coefficients of multiple determination (R^2) in the partial tests, were low. The number of mountains between populations was significantly negatively correlated with genetic distance between populations (Figure 4.12a). The highest r^2 or R^2 values were achieved when all the variables were input into the model, although the coefficient for the partial test of F_{ST} v discontinuous habitat v number of rivers was also high (Figure 4.12b), as was the correlation of the F_{ST} v mountain v discontinuous habitat v number of rivers, although the fact that no difference occurred when mountain was fitted to the model implies that it was contributing nothing to the model, either positive or negative, which is unexpected given the reasonably high correlation coefficient between mountain and F_{ST} in the simple tests. There were no significant correlations between any of the variables and genetic distance, once geographic distance was taken into account, in the partial tests.

When genetic distance was measured using D_S there was a significant positive correlation between genetic distance and the maximum distance of non forested area between populations (Figure 4.12c) and the number of roads between populations (Figure 4.12d), and these remained significant even after taking into account differentiation due to geographic distance. The maximum variation in the data was explained by all of the variables, although similar values were achieved when discontinuous habitat, road and geographic distance were included in the model (Figure 4.12e), or indeed when any of the partial tests with more than three habitat variables including discontinuous habitat and roads were used. The addition of the number of rivers between populations did not increase the R^2 values in the model. In the partial tests which included discontinuous habitat and roads, those variables were the only variables which had a significant correlation with genetic distance in the model ($p < 0.5$ in all cases), and the correlation was positive in all cases. The exception was the partial test between discontinuous habitat and number of roads, where neither correlation was significant ($p > 0.1$), indicating an interaction and possibly cumulative effect, of the two variables on dispersal of the red squirrel.

Table 4.18. Results of simple and partial Mantel tests investigating the relationship between genetic structure, F_{ST} and D_S, calculated from microsatellite data and possible barriers to dispersal between populations. The partial geographic tests are the correlations between genetic distance, variables in the first column, and geographic distance. Significant relationships are indicated in bold, where partial tests were carried out the variable which had a significant relationship with genetic distance is also in bold. In no instance was there more than one variable in the partial test significantly related to genetic structure.

				Partial: Geographic distance		
	p	r	r^2/R^2	p	r	r^2/R^2
Fst						
River (R)	0.150	0.157	2.47			5.87
Road (RD)	0.570	-0.058	0.33			2.69
Mount(M)	**0.039**	-0.217	4.70			4.78
Discon(D)	0.390	0.093	0.87			4.65
M v D			4.65			4.85
M v R			6.23			6.47
M v RD	**0.029**	-0.217	5.27			5.41
D v R			2.59	**0.04**	-0.211	7.06
D v RD			2.51			4.74
R v RD			4.02			6.00
M v D v R			6.38			7.06
D v R v RD			5.11			5.76
M v D v RD			5.46			6.66
R v M v RD			6.24			7.15
All			6.42			
Ds						
River (R)	0.220	0.126	1.59			9.87
Road (RD)	**0.002**	0.321	10.28	**0.02**	0.243	10.74
Mount(M)	0.680	-0.116	52.16			5.2
Discon(D)	**0.007**	0.297	8.79	**0.03**	0.297	9.87
M v **D**	**0.005**	0.302	10.46	**0.025**	0.229	10.47
M v R			3.49			5.41
M v **RD**	**0.003**	0.308	10.85	**0.02**	0.308	10.95
D v R	**0.010**	0.269	8.81	**0.04**	0.297	9.87
D v RD			12.43			12.84
R v **RD**	**0.005**	0.295	10.29	**0.03**	0.233	10.74
D v R v RD			12.51			12.94
M v D v RD			12.43			13.05
R v M v **RD**	**0.010**	0.273	10.95	**0.024**	0.237	11.01
All			12.54			13.06

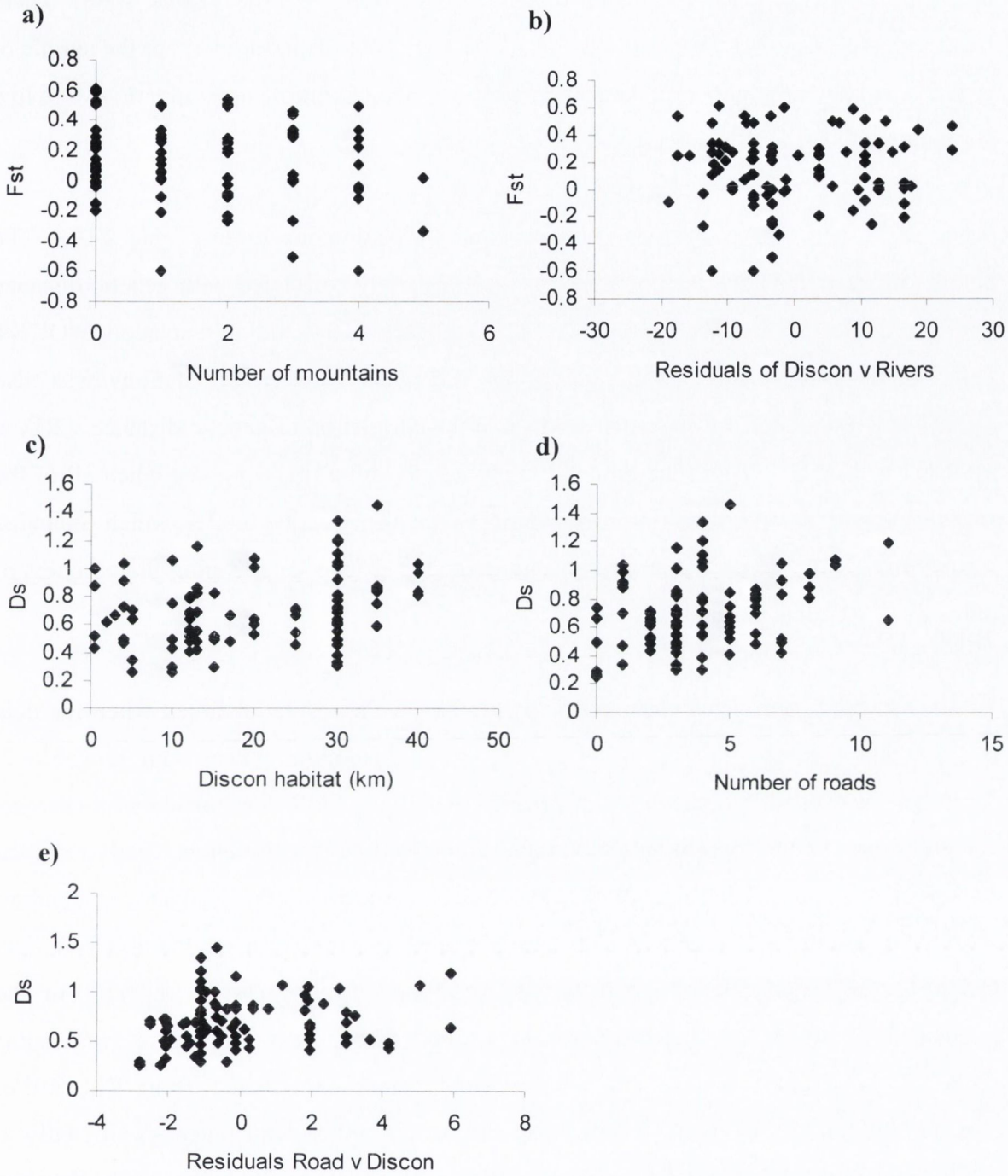


Figure 4.12. Scatterplots showing the relationship between microsatellite genetic distance and various barriers to dispersal. Where residuals are plotted they are the residuals of a linear regression between the variables. Fst was negatively related to the number of mountains between populations, $p = 0.039$ (a), however the amount of discontinuous habitat, and the number of rivers, between populations explained a high amount of variation in the model, $R^2 = 7.06$, (b). Both the amount of discontinuous habitat (c) and the number of roads (d) between populations were correlated with D_s in both the simple and partial tests. Those 2 variables explained a high proportion of variation within the model ($R^2 = 12.84$, e).

There were few significant relationships between genetic distance measured from the mtDNA data and possible barriers to dispersal between populations (Table 4.19). There were no correlations between F_{ST} and any of the barriers to dispersal in either the simple or partial tests and both the coefficients of determination and multiple determination were low throughout all comparisons ($r^2/R^2 \leq 10\%$ in all cases).

When D_S was used as a distance measure some correlations occurred (Table 4.19). The number of mountains between populations was positively correlated with genetic distance in the partial test of distance v MOUNT v GEO (Figure 4.13a), and in distance v MOUNT v ROAD (Figure 4.13b). The number of mountains between populations was also significantly correlated with genetic distance in the correlation of genetic distance v RIV v MOUNT v ROAD. Within the model, the highest R^2 value was achieved when all of the variables were incorporated into the model, and also in the model which included mountains, discontinuous habitat, rivers and geographic distance, meaning the numbers of roads were not contributing to the model.

There were few differences between these results and the results obtained when the data was reanalysed using information only from the 6 populations with a sample size > 2. Although the r^2 and R^2 values were much higher (upto 61%) the correlations between genetic distance and different habitat variables remained largely the same. In all cases the R^2 values were highest when all the variables were used in the analysis, and values obtained from the microsatellite data were higher than those from the mtDNA data (Microsat: F_{ST} = 41.2%, D_S = 61%; MtDNA: F_{ST} = 29%, D_S = 30.4%). In the microsatellite data set, as with the entire data set, both discontinuous habitat and mountains produced the highest correlations with genetic distance calculated from F_{ST} (40%), although the number of rivers between populations no longer contributed significantly to the model (with rivers: 40.8%, without: 40%). Again the number of roads between populations was related to D_S (p = 0.013) however, contrary to that found in the entire data set, between the 6 populations it was the number of mountains (p = 0.018), rather than the amount of discontinuous habitat between populations (p = 0.9), which was also related significantly to genetic distance, and these geographic distance, mountains and roads explained almost all the genetic structure seen (D_S v MOUNT v ROAD: 56.7%, D_S v all variables: 61%).

Table 4.19. Results of simple and partial Mantel tests investigating the relationship between genetic structure, F_{ST} and D_S, calculated from mtDNA data, and possible barriers to dispersal between populations. The partial geographic tests are the correlations between genetic distance, variables in the first column, and geographic distance. Significant relationships are indicated in bold, where partial tests were carried out the variable which had a significant relationship with genetic distance is also in bold. *G indicates a correlation with geographic distance.

				Partial: Geographic distance		
	p	r	r^2/R^2	p	r	r^2/R^2
Fst						
River (R)	0.38	0.107	1.16			2.53
Road (RD)	0.53	-0.078	0.60			0.69
Mount(M)	0.49	-0.087	0.76			0.81
Discon(D)	0.97	0.004	0.00			0.8
M v D			0.76			0.86
M v R			1.70			2.71
M v RD			0.85			0.86
D v R			1.35			2.53
D v RD			0.89			0.96
R v RD			2.49			2.68
M v D v R			1.85			2.72
D v R v RD			2.51			2.69
M v D v RD			0.97			0.99
R v M v RD			2.50			2.89
All			2.52			3.00
Ds						
River (R)	0.11	-0.189	3.59			3.67
Road (RD)	0.92	0.0122	0.01			0.43
Mount(M)	0.22	0.152	2.32	0.032	0.152	6.93
Discon(D)	0.97	-0.004	0.00			0.09
M v D			2.33	M 0.01 G 0.03*	0.152 -0.263	9.27
M v R			5.25			7.22
M v RD			3.34	0.03	0.152	7.22
D v R			4.25			4.25
D v RD			0.03			0.45
R v RD			4.12			4.33
M v D v R			5.78			**9.74**
D v R v RD			4.41			4.58
M v D v RD			4.01			9.30
R v **M** v RD			5.30	0.017	0.182	7.61
All			6.36			9.74

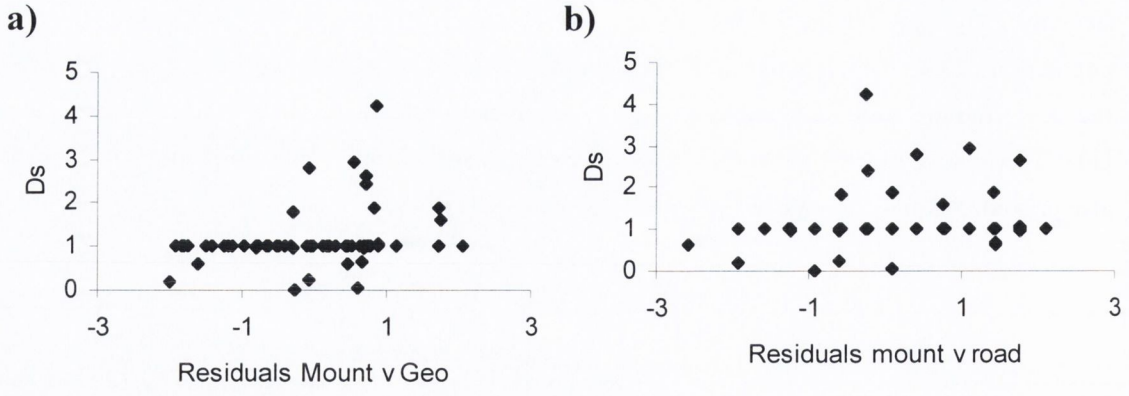


Figure 4.13. Scatterplots showing the relationship between mtDNA genetic distance and various barriers to dispersal. Where residuals are plotted they are the residuals of a linear regression between the variables. D_S was significantly related to the number of mountain and geographic distance between populations (p = 0.032, a), and the number of mountains and roads between populations (p = 0.03, b). Further investigation revealed that the number of roads was not contributing to the model (all variables R^2 = 9.74, all variables except roads R^2 = 9.74).

In the reduced data set there were even fewer correlations between the genetic distance between populations calculated from the mtDNA data set and habitat variables. However, although no relationships were significant the partial Mantel test including mountains, geographic distance and discontinuous habitat explained the highest amount of variation within the model when distance was calculated from F_{ST} (28.26%), with the model improving little after the number of roads and rivers was taken into account (28.96%).

When distance was calculated using D_S only one significant correlation occurred, that between genetic distance and the number of roads between populations (r = 0.52, p = 0.045, R^2 = 27.86%). Similar to that found in the entire data set, a high amount of variation was explained in the model when all variables except the number of roads were included (28.93%, all variables: 30.38%) however, a similar relationship occurred when all variables except discontinuous habitat were included (28.55%), implying that both discontinuous habitat and the number of roads are contributing in equal amounts to the model. In fact, the Mantel tests of D_S v GEO v ROAD v DISCON had a R^2 of 24%, meaning, in the models including only the 6 populations, it was these two variables, and not the number of mountains or rivers between populations which was having the greatest influence on genetic structure.

4.3.3. European variation

4.3.3.1. Genetic variation

Table 4.20 gives the microsatellites used in this study which were amplified in other studies in Europe, as well as mean figures for the measures of diversity used in the comparison. Despite the different primers used in the different countries, comparisons between Ireland, Britain and Italy were made with the entire data sets for each country, as raw data was not available from the other countries. However, raw data was available for Belgium and Germany; therefore, the comparisons between Ireland and those countries were carried out using the data from primers Ru1-6 only, with the Irish diversity measurements recalculated with only those 5 loci included (Table 4.21).

There was no difference between the number of alleles found at the 11 loci which were amplified in this study and in a corresponding study in northern England (Hale *et al.*, 2001a; $t_{(20)} = 1.492$, $p = 0.151$), nor between the 5 loci which were sampled in Ireland, Germany and Belgium ($F_{(2,67)} = 0.686$, $p = 0.5$). However the number of alleles is dependent on the sample size, and the measure of AR is preferable to that of the number of alleles, where comparisons are being made between groups where the sample size is unequal. Data available facilitated a statistical comparison of AR between Ireland and Italy, and between Ireland, Belgium and Germany. Italy had a significantly higher level of AR than Ireland ($t_{(12)} = 21.11$, $p < 0.001$), while there was no difference between Ireland, Germany and Belgium ($F_{(2,67)} = 1.751$, $p = 0.181$).

The proportion of heterozygous individuals in northern England, Ireland and Italy did not differ significantly ($F_{(2,22)} = 1.03$, $p = 0.373$) however there was a difference between Ireland, Belgium and Germany ($F_{(2,22)} = 11.25$, $p < 0.001$). LSD post hoc tests revealed a significantly lower level of heterozygosity in Ireland ($p < 0.001$ in both cases). There was no difference in gene diversity between Ireland and Italy ($U_{(22)} = 56$, $p = 1$), nor between Ireland, Germany and Belgium ($F_{(2,67)} = 3.04$, $p = 0.54$).

Table 4.20. Primers which were amplified in studies in both this study and in other studies in Europe, and the measures of genetic diversity available for comparison for each country. *Sample size for GB varied between primers. The number of samples amplified at each primer in GB in given in parentheses after the primer number.

	Number of individuals	Primers amplified	MNA	Ho	He	AR	Reference
Ireland	100	1,3,4,6,10,13,14,18.19.23.31 Ru1,3,4,5,6	6.875	0.39	0.63	1.604	This study
Italy	70	4,13,19 Ru4,5,6	5.088	0.53	0.570	4.569	Trizio *et al.* 2005
Wales	48	3,10 Ru4,5	1.5				Ogden *et al.* 2005
GB	*	1(27), 3(23), 4(22), 6(14), 10(32), 13(33) 14(37), 18(32), 19(33), 23(36), 31(30)	6.182	0.479			Hale *et al.* 2001a
Germany	27	Ru1,3,4,5,6	6	0.507	0.487		Todd 2000a
Belgium	136	Ru1,3,4,5,6	5	0.543	0.51		Todd 2000a

Table 4.21. Mean diversity measures ± SEM in this study and the study carried out by Todd (2000) in Belgium and Germany calculated from Ru1, 3, 4, 5 and 6.

	Ireland	Belgium	Germany
MNA	3.3±0.2	3.56±0.28	3.8±0.46
AR	1.61±0.03	1.56±0.03	1.49±0.06
He	0.65±0.04	1.56±0.03	0.49±0.06
Ho	0.32±0.04	0.56±0.04	0.6±0.05

4.3.3.2. Genetic structure

F_{ST} was compared between Ireland and the other four countries in the comparison, again, using F_{ST} calculated from only primers Ru1, 3, 4, 5 and 6 in the comparison of Ireland, Germany and Belgium. F_{ST} varied between Ireland, Italy and Britain ($\chi^2_{(2,147)} = 26.03$, $p < 0.001$) with F_{ST} values higher in Britain than Italy ($U_{(80)} = 235$, $p = 0.002$), while Ireland had significantly higher genetic differentiation than Britain ($U_{(132)} = 1443$, $p = 0.001$) and Italy ($U_{(81)} = 135$, $p < 0.001$; Figure 4.14). When F_{ST} values were corrected for the geographic distance between populations, there was no longer a difference between the two island populations; Ireland and Britain ($p = 0.732$), while the significant difference between those populations and the Italian population remained ($p < 0.02$ in both cases; Figure 4.15). No differences were apparent in the comparison of Ireland, Germany and Belgium in either the comparison of F_{ST} ($F_{(2,19)} = 1.62$, $p = 0.224$) or F_{ST}/geographic distance ($t_{(17)} = 1.74$, $p = 0.1$; Figure 4.15 and 4.16).

Contrasting results were detected in the comparison of D_S between countries, although this was only compared between Britain and Ireland. Before and after taking into account the geographic distance between populations D_S was significantly higher in Ireland than in Britain (D_S: $U_{(130)} = 312$, $p < 0.001$; D_S/Geo: $U_{(130)} = 1689$, $p = 0.036$).

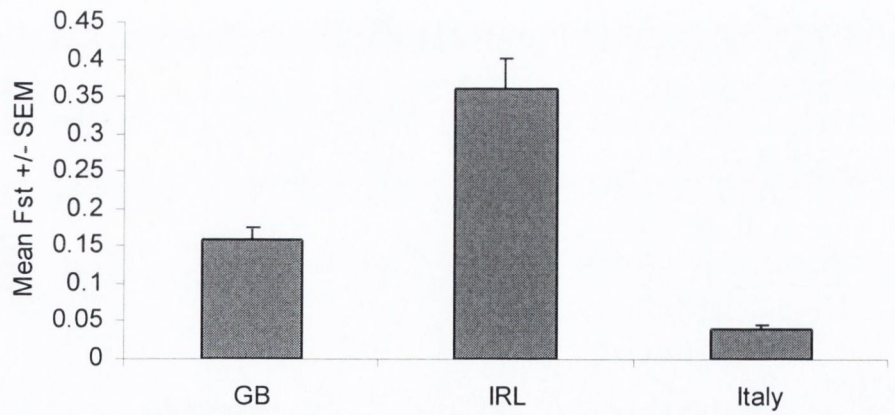


Figure 4.14. Mean F_{ST} ± SEM in Britain, Ireland and Italy. Ireland had significantly higher genetic distance between populations than Britain and Italy ($p < 0.005$).

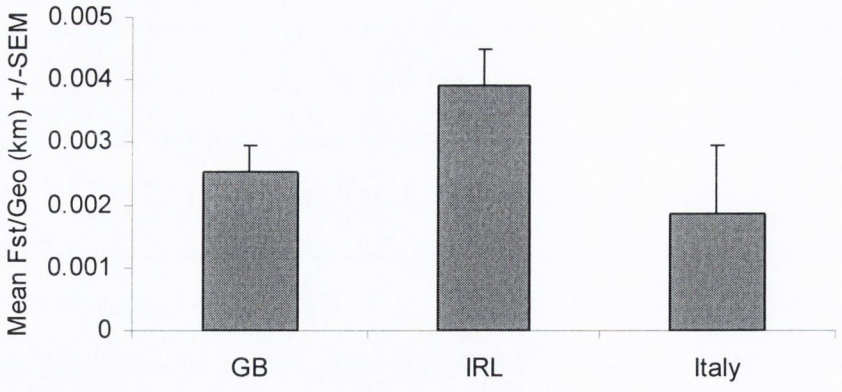


Figure 4.15. Mean F_{ST} /Geographic distance (km) ± SEM in Britain, Ireland and Italy. After correction both Ireland and Britain had significantly higher F_{ST} than Italy ($p < 0.002$).

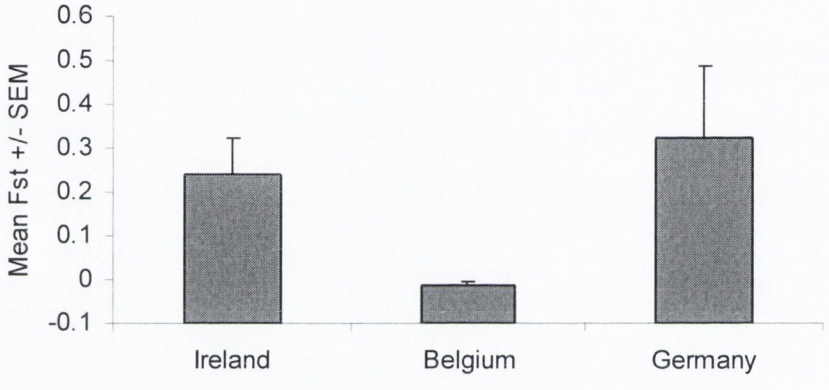


Figure 4.16. Mean F_{ST} ± SEM in Ireland, Belgium and Germany. There was no difference between the three countries ($p = 0.224$).

Figure 4.17. Mean F_{ST} /Geographic distance (km) ± SEM in Ireland and Belgium. No information on geographic distance between German populations was available. There was no difference between geographic distance corrected F_{ST} in Ireland and Belgium ($p = 0.1$).

4.4. Discussion

The presence of null alleles in this study is not unusual, as they have been reported to occur at microsatellite loci in such varied species as red deer (Pemberton *et al.*, 1995), alpine butterflies (Keyghobadi *et al.*, 1999), squid (Shaw *et al.*, 1999) and humans (Callen *et al.*, 1993). Although, when loci with null alleles are included in data analysis, estimations of diversity may be conservative (Garner *et al.*, 2004), the fact that the data set was adjusted for the presence of these alleles, and that both data sets (original and corrected) combined were used in the interpretation of genetic diversity, means that any differences in diversity which are detected, will be accurate. Also, as only the corrected data set was used in the analysis of genetic structure, and van Oosterhout *et al.* (2004) were confident that the adjusted genotypes were accurate representations of the actual allele frequencies within each population; genetic structure will reflect the actual genetic structure within the data set.

Another possible issue with the data in this study is the sample size. This varied widely from 1 – 44 individuals sampled from a population. Although this will, of course, effect the genetic diversity estimate of a population, only populations with > 2 samples collected from them are discussed with respect to genetic diversity, and these populations had similar sample sizes to those used in other studies in which genetic variation was detected (e.g. pocket gopher, Alvarez-Castañeda & Patton, 2004; Ethiopian wolf, Gottelli, *et al.*, 2004; common vole, Fink *et al.*, 2004; lynx, Hellborg *et al.*, 2002; pine voles, Machólan *et al.*, 2001), including squirrels (Barratt *et al.*, 1999; David-Gray *et al.*, 1998; Ogden *et al.*, 2005), consequentially this study should have sufficient sample sizes to accurately assess genetic variation. Also, despite the sampling and loci amplifications being lower than that suggested for measures of genetic diversity (Nei (1987) recommended 20 loci typed in 20 to 30 individuals), sampling was large enough to detect both rare and low frequency alleles in the populations. This, together with the number of polymorphic loci typed in this study, and the combination of results from both microsatellite and mtDNA analysis, will make interpretation of inter population genetic diversity results in this study, accurate.

The criteria suggested for estimates of genetic differentiation between populations are not as clear. Generally, polymorphic loci are expected to provide better estimates of genetic distance than loci with fewer alleles (Lowe *et al.*, 2004), however, Kalinowski (2002) stressed the importance of the total number of alleles across the entire study used to

estimate genetic distance, rather than the number of alleles at each loci, especially when divergence between populations is low. In this study a number of loci (16 microsatellites, one mtDNA) were amplified from a large number of individuals (100 and 87 respectively) and a total of 110 alleles were found in the microsatellite data set and 29 haplotypes in the mtDNA data. This large number of loci, reasonable number of samples and the combination of results from different markers, and also the determination of structure from two separate distance measures, increases the reliability of the results. Nevertheless, genetic distance was still assessed in two data sets; that with all the populations, and that with populations with $n > 2$, and these results were compared and contrasted with each other in order that any influences of the small sample size on genetic structure could be identified.

4.4.1. Genetic variation

On the whole, the genetic variation seen in the samples in this study was similar, both among populations and among regions, and when compared to other studies on the same species (Hale *et al.*, 2001a, b; Trizio *et al.*, 2005). Although, of the 6 populations examined in this study, the mean number of alleles (MNA) was highest in GY (MNA 5.31), this was only slightly above mean values reported by Trizio *et al.* (2005) in Italy and, on closer examination, lower than the mean number of alleles found in 3 of the 8 populations used in that study (MNA 5.4 - 5.8). However, the value was higher than that reported from Britain (northern Britain: MNA 4; Hale *et al.*, 2001b; Wales: MNA 1.5; Ogden *et al.*, 2005), and also higher than that found in all any of the 8 populations from Belgium, and the 3 from Germany, studied by Todd (2000b; MNA 2.6 - 5.2), although only very slightly above the values found in Waldhäuser in Germany (MNA 5.2) in that study. All other populations MNA were within the range calculated from those studies (Hale *et al.*, 2001b; Ogden *et al.*, 2005; Todd, 2000b; Trizio *et al.*, 2005).

AR was the only other diversity measure which varied between populations, with lower AR in some of the populations which had 2 or less samples collected from them. In fact mean AR (1.5) in all of the populations with sample size ≤ 2 was lower than that reported for any population studied on continental Europe (Todd, 2000b; Trizio *et al.*, 2005), while the other 6 populations had values similar to those reported elsewhere, supporting the fact that genetic measures from these populations with low sample sizes were skewing the results.

The lower heterozygosity recorded in GY resulted in it being the only population which was not in Hardy-Weinberg equilibrium. It is possible that GY was experiencing the Wahlund effect (Wahlund, 1928 in Lowe *et al.*, 2004), whereby an area is sampled as a single population but, in fact, is made up of 2 or more genetically differentiated populations, which have little or no gene flow between them. The likelihood of this scenario was supported by the STRUCTURE analysis, which assigned the GY population to three separate 'populations' (see section 4.2.5) however, this was not supported by trapping carried out in the site (A. Poole, pers comm.), with no evidence of distinct 'populations' within the study site. Another possible cause of a deviation from equilibrium is large amounts of gene flow into, and out of, the population, and trapping in the site seemed to support this hypothesis, with few individuals retrapped despite an exhaustive trapping program (A. Poole, pers comm.).

For the mtDNA data there was also little variation in Ireland at either level of analysis, with values relatively homogeneous across populations and regions, and similar to that described in both red squirrels (Hale *et al.*, 2004; Ogden *et al.*, 2005; Trizio *et al.*, 2005, discussed in more detail in the European comparison) and other species of rodent (e.g. pocket gopher, Alvarez-Castañeda & Patton, 2004; Australian bush rat, Hinten *et al.*, 2003). The exception was Cor, where low diversity was detected in all of the diversity measurements.

The most striking result emerging from both the comparison of mtDNA between populations, and the bottleneck analysis on the microsatellite data, is the fact that there was evidence that Cor had undergone a recent population bottleneck. Although there was evidence from both the mutation models, and examination of allele frequency distributions, that all six populations, except Glen, had recently undergone a population bottleneck, Cor was the only population where a bottleneck was detected using all three mutation models. The fact that the allele frequencies of all populations except GY detected a population bottleneck is not conclusive, as using this mode shift to indicate a population bottleneck when less than 30 individuals are typed from a population, is inaccurate (Luikart *et al.*, 1998), therefore the only reliable result was that obtained from GY (n = 44).

The bottleneck analysis works on the basis of detecting non normally distributed allele frequencies and heterozygosity, however, these parameters only remain in disequilibrium for around 3 generations (Keller *et al.*, 2001), in a squirrel population around 10 years

(Gurnell, 1987). Therefore, the detection of a bottleneck in Cor, and the occurrence of only two haplotypes within the population, the lowest frequency per squirrel in the population, together with the fact that it was the only population in which no unique haplotypes were identified, suggest that the forest was colonised within the last 10 years by possibly only 2 individuals, but certainly at least 2 different maternal lineages.

As mentioned above, bottlenecked red squirrel populations can return to equilibrium in as little as 10 years, meaning any bottlenecks occurring longer than a decade ago will probably not be detected from microsatellite analysis. However mtDNA, with its slower mutation rate, can give clues to bottlenecks which occurred in the more distant past. Avise *et al.* (1984) described low nucleotide diversity and high haplotype diversity as characteristics of populations which are expanding from a low population size. Although none of the populations sampled in this study show extreme high haplotype diversity and low nucleotide diversity, KNP, Lar and GY all had nucleotide diversities < 0.05 and gene diversities of > 0.6, suggesting that these populations may have undergone a distant population bottleneck. Whether this is a result of the recent reintroductions from Britain, or a more distant event, is investigated in more detail in Chapter 3.

In the populations in this study there were few relationships between genetic diversity and habitat variables. The increase in gene diversity from west to east can be explained by the regional difference in gene diversity between the east and the west, rather than by any gradient which exists across longitude, as no samples were collected from the centre of the country. Similarly, the lack of correlation between any of the diversity measures and forest size is understandable as red squirrel populations in very small forests are rare (Koprowski, 2005), the species needing either continuous forests, or food supplementation, to maintain a viable population (Celeda *et al.*, 1994; Margris & Gurnell, 2002). The isolation of the forest, or the interaction between isolation and forest size, would be more of an influence on genetic diversity (Verboom & van Apeldoorn, 1990; Celeda *et al.*, 1994, Roderíguez & Andrén, 1999), with the maximum distance a red squirrel will move between forests, over unfavourable habitat, estimated at around 1.5 km (Hale *et al.*, 2001a).

Only two such relationships were detected in the populations used in this study; between AR and forest isolation, and between AR, forest size and forest isolation, although, in the latter, it was only the relationship between AR and size which was contributing significantly to the model. This lack of relationship between diversity and forest

fragmentation is unexpected but not unusual. Other research has shown little correlation between recent habitat fragmentation and genetic diversity at microsatellite loci (Todd, 2000a), and through DNA fingerprinting (Wauters *et al.*, 1994). Conversely, an effect of habitat fragmentation on mtDNA variation has been described in fragment populations (Todd, 2000a), but was not detected in this study, possibly because of the high regional specificity of haplotypes in the populations in this study.

A possible reason for the lack of correlation between genetic diversity and isolation of populations is the corridors for dispersal which may occur between populations. Although, red squirrels are habitat specialists (Gurnell, 1987), and, as already mentioned above, their maximum dispersal distance over unfavourable habitat has been estimated at 1.5 km (Hale *et al.*, 2001a), observations have recorded dispersal of up to 3 times this distance (G.Verbeylen pers comm. in Todd, 2000a), and just because the forest was isolated does not mean there were not possible corridors facilitating dispersal between forests (trees, hedgerows etc.), which unfortunately, could not be determined from the maps used in this study. Therefore, the presence of undetected corridors between populations may explain the lack of significant correlation between diversity and fragmentation.

The comparison of genetic diversity in different habitat types in this study was only carried out in 6 sites so results are not conclusive. However, what did emerge from the analysis were the slightly higher levels of polymorphism seen in both the old and mixed sites. It was hypothesised in Chapter 2 that the high densities calculated from trapping in mixed sites in Ireland may be a result of red squirrels in Ireland having an adaptive advantage in that site type, as this was the forest type in which the Irish red squirrel population would have evolved. Similarly, a higher level of diversity may also be predicted in old forests, as these may contain some individuals which are descendents of squirrels which have survived in the country since after the last ice age, rather than simply descendents of squirrels translocated 200 years ago.

The results obtained from this study did not statistically support either of these hypotheses; however, the slightly higher diversity in both old and mixed sites may be reflecting significantly higher diversity in those site types in non neutral markers. Although both microsatellites and mtDNA are generally referred to as being selectively neutral (Kimura, 1983) (but see sections 1.2.2.2.and 1.2.2.3), and generally show little correlation with phenotypic traits (Pfrender *et al.*, 2000; Willis *et al.*, 1991) or fitness (Reed & Frankham,

2003), Pfrender *et al.* (2000) demonstrated that, although genetic variation measured from these variables may not accurately measure fitness or variation in quantitative characters, they can be useful as a guide to fitness and genetic differentiation, and they can actually underestimate the diversity of the population in question. The possibility that both old and mixed sites had higher diversity will be investigated further by correlating genetic and phenotypic traits in the next chapter.

4.4.2. Genetic Structure

Overall there was strong between, and within, regional differentiation in this study; evident from measures of genetic distance, population trees, STRUCTURE, and genotype assignment. Significant F_{ST} values were detected between populations in all three regions in both data sets, and between populations within all regions, except the south/southwest, in the microsatellite data. This loosely agreed with the AMOVA whereby, although the highest percentage of variation was within study sites, population differentiation was evident in both the microsatellite and mtDNA data sets, although conversely no significant between region differentiation was detected. Similar distributions of variation have been found in Britain and Italy in studies using microsatellites (Hale *et al.*, 2001a; Trizio *et al.*, 2005).

The lack of regional difference in differentiation in the mtDNA data can be explained by the fact that although there were a large number of regional, and in many cases, population, unique haplotypes amplified in this study, two (IRL and SW+W+E) of the three dominant haplotypes within the Irish population were not region specific (W11 was region specific to the west). Nevertheless, there seems to be some geographical differentiation within Ireland, unlike what has been described in red squirrel populations from other countries (Barratt *et al.*, 1999; Ogden *et al.*, 2005; Trizio *et al.*, 2005). This geographical differentiation was also evident from the neighbour joining trees constructed from both the microsatellite and mtDNA data, with most populations, except those with the low sample sizes, clustering with other populations within their region, albeit bootstrap support was generally low. The fact that IRL was only dominant in one region, but found in all, was curious. There are a number of possible explanations for this. It could be that this is a haplotype which was introduced into Ireland and has simply become fixed in some areas and not in others due to drift, gene flow, or possibly some adaptive advantage. However, the dominance of this haplotype in a region into which no reintroductions were recorded (Barrington, 1880), suggests that it may be a remnant Irish haplotype, which is less

frequent in the east and west due to dilutions through translocations in those areas (Barrington, 1880), this hypothesis, as well as possible origins of other Irish haplotypes, are discussed in Chapter 3.

As a habitat specialist, IBD would be expected between red squirrel populations, however, given the history of translocations into Ireland, and the mtDNA data discussed above, the situation may not be clear, and it was hypothesised that IBD from microsatellite DNA, (reflecting contemporary gene flow) may be more apparent than that from mtDNA (reflecting sources of translocated individuals and possible remnant Irish haplotype distribution).

Analysis found evidence for IBD in both sets of data, but the hypothesis of microsatellite IBD and no mtDNA IBD was only supported by D_S. However, although IBD was detected, it was by no means a linear relationship (Figure 4.10), and there was a lot of noise in the data set which probably resulted in the differences in significant relationship between the different markers and distance measures i.e. D_S from the microsatellite data and F_{ST} from the mtDNA data. After further analysis it was revealed that geographic distance has less of an effect on genetic differentiation of red squirrel populations at a local scale (< 150km). A similar situation was described in red squirrels in Italy, where IBD was not detected within small geographic scales, but was influencing populations > 250 km apart (Trizio *et al.*, 2005). This trend is by no means confined to habitat specialists. Jones *et al.* (2004) carried out a genetic structure analysis of the Tasmanian devil, *Sarcophilus laniarius*, within a geographic sampling distribution similar to that in this study. They found a pattern of no overall IBD, but, again, similar to this study, evidence of gene flow between populations up to 50 km apart, and between populations 150 - 250km apart there was increased genetic differentiation due to IBD. Also, a recent reanalysis of published literature on grey wolves, *Canis lupus*, in North America, concluded that IBD was not the only controlling factor in the formation of genetic differentiation, but rather a combination of geographic distance, climate differences and habitat barriers were restricting dispersal of the species (Geffen *et al.*, 2004).

The detailed investigation of the other possible influences driving red squirrel genetic differentiation and insularity of red squirrel populations revealed a number of factors influence gene flow between populations. The dbRDA results found that habitat type and habitat age did not influence the microsatellite genetic structure of the red squirrel. The

lack of correlation between habitat type and structure suggests that dispersing squirrels do not preferentially disperse to habitat of the same type as their natal habitat. This is in agreement with the generalist nature and adaptability of squirrel diet (Gurnell, 1987; Moller, 1983). It appears that squirrels which are born in a particular habitat type, but disperse to a different habitat type, are either able to adapt their diet to exploit the food resources available in the new habitat or, given the large number of common tree species in the conifer and mixed conifer/broadleaf sites, simply limit their diet to feeding on the species which were common to both sites, although the former is probably more likely. It also agrees with the results of the genetic diversity analysis, whereby adaptive mutations within the red squirrel to particular site types do not seem to be occurring, at least at the markers investigated in this study, but, again, this will be discussed in more detail in the next chapter.

Of little applicability in describing the forces behind distribution of genetic diversity in the red squirrel species as a whole, but of interest and importance in interpreting the genetic structure of the Irish red squirrel population, was the relationship between mtDNA genetic structure and the age of the forest where the population was resident. The fact that this relationship was significant, after all differences in genetic variability between populations were taken into account, indicates that the existence of red squirrel populations in both old and new forests is influencing genetic structure. The question is whether this difference arose due to more distant founder events in old forests, or due to old forest populations containing some genetic characteristics of remnant Irish red squirrels is discussed in Chapters 3 and 5.

Although first suggested by Trizio *et al.* (2005), this study has shown that barriers to dispersal have a significant effect on shaping the genetic structure of red squirrel populations on both a local and broad geographical scale, with the effect of IBD increasing at distances > 150km. Although results were not consistent across all comparisons, generally, discontinuous habitat, roads and mountains were all barriers to dispersal of the red squirrel. However, there was only one significant correlation between genetic structure and the number of rivers between populations (entire data set: Microsatellite F_{ST}), which is curious as all reports of grey squirrel spread has recorded its westward spread as restricted by the presence of the River Shannon (Hamilton, 2006; Ó Teangana *et al.*, 2000; Reilly, 1997). Consequentially, given the higher dispersal of grey squirrels over unforested areas

(Gurnell, 1987) in comparison to red squirrels, a highly significant effect on red squirrel genetic structure would be expected.

In this analysis the River Shannon was weighted as 10, while all other rivers were weighted as 1, it is possible that this weight was not sufficiently large to describe the significant barrier that the River Shannon represents, or it could be possible that, a) given the widespread distribution of red squirrels in Ireland (Reilly, 1997), the Shannon was not acting as a barrier, and squirrels were dispersing north and then east/west rather than directly east/west, or b) the sample distribution and size was not extensive or large enough to detect a difference. Unfortunately, the two nearest populations, on either side of the Shannon, were CE and LK, both represented by extremely small sample sizes ($n < 3$ in both cases), no clear effect of the river, on the genetic differentiation of red squirrel populations, which are otherwise geographically near each other, could be determined. What must also be taken into account is the fact that this correlation only occurred in the microsatellite F_{ST} analysis, which assumes populations differentiation through drift only. Although 2 populations were sampled west of the Shannon, only GY had a reasonable sample size ($n = 44$), and what is also significant is that it was also the only population where differentiation through mutation-drift was not detected. Therefore the significant relationship may be a remnant of comparing populations under mutation drift to those differentiating through drift alone.

As calculated by Hale *et al.* (2001a) and Roderíguez & Andrén (1999), and predicted by Trizio *et al.* (2005), the extent of discontinuous habitat significantly effects gene flow between populations of the red squirrel, and was significantly related to D_S calculated from the microsatellite data. This is similar to that described for another habitat specialist. An investigation of the effect of habitat connectivity on gene flow between Pacific jumping mouse populations, *Zapus trinotatus* found a high correlation between genetic distance and riparian connectivity (Vignieri, 2005). However, areas of unfavourable habitat have also been shown to shape genetic structure in wide ranging species and high gene flow species including the puma, *Puma concolor* (McRae *et al.*, 2005), butterfly, *Speyeria idalia* (Williams *et al.*, 2003) and wolverine, *Gulo gulo* (Cegelski *et al.*, 2003).

The analysis also found that the number of roads between populations significantly influenced gene flow between populations when both the microsatellite and mtDNA data were used. This agrees that which has been found elsewhere. Riley *et al.* (2006) found

that the presence of a major road, which fragmented bobcat and coyote habitat in Los Angeles, not only decreased migration between habitat fragments but, when individuals did disperse across the road, they did not reproduce, possibly due to a large number of occupied territories at the road margin. Keller & Largiadèr (2003) and Keller *et al.* (2004) also detected an effect of roads on gene flow between flightless ground bettle populations while a study on bank voles revealed similar results (Gerlach & Musolf, 2000).

Although the relationship between gene flow and the number of mountains between populations was significant in some cases, the relationship was not consistent across the different analyses. It is possible that the way in which elevated land was incorporated into the analysis influenced this result. Vignieri (2005) was investigating similar relationships to those in this study and incorporated elevated areas by calculating the total overland distance the animal had to travel and it is possible that, if the analysis in this study had been carried out using a similar method, the relationship would have been more consistent.

Overall from this analysis it seems that, depending on the number and combination of barriers between populations, red squirrel populations which are geographically near each other can by highly differentiated. This may explain the large number (~ 17) of subspecies which have been described for the species (Sidorowicz, 1971), some of which share a relatively restricted geographical range (e.g. 7 subspecies described for the Iberian Peninsula alone (Mathias & Gurnell, 1998)), and also explains the differentiation recorded between some populations on a local scale (Todd, 2000a; Trizio *et al.*, 2005).

The fact that all barriers to dispersal were affecting genetic structure in some way was expected, but there was a lack of clarity over which specific barrier may be having the largest effect on gene flow. The results demonstrated that the different barriers have an additive effect on dispersal of the red squirrel, as, in all cases the highest amount of variation was explained when all of the variables were input into the model. What could also be possible, but which was not investigated in this study was the spatial position of these barriers in relation to each other within the landscape. Squirrels would be more likely to disperse over roads which had forest on either side, likewise with rivers and possibly mountains, although the size/extent of the mountain/mountain range would also be a factor. Although the number of populations, and geographical range of these populations, made incorporation of these variables into the model unrealistic in this study,

it does suggest combining these results with GIS analysis, possibly using a more localised scale, may offer further insights into red squirrel dispersal and distribution.

4.4.3. The Irish red squirrel as a peripheral population

Diversity measurements in Ireland were not lower than those found in other red squirrel populations in Britain and mainland Europe (see Table 4.22). The only significantly lower measurement was heterozygosity, which was lower in Ireland than in either Belgium or Germany. However, the sampling range in both of those mainland European countries was quite restricted (Todd, 2000a) and was probably not a reflection of red squirrel diversity within the country as a whole. Also, AR in Italy was higher than that in Ireland, but this data was calculated from different microsatellite loci in each study so this result may not be significant.

Although a large number of haplotypes (n = 29) were found in a relatively small number of squirrels (n = 87) in Ireland, this was not unusual for red squirrels (Table 4.21) and was lower than that found in 5 populations sampled from a more restricted geographical range in Italy (Trizio *et al.*, 2005), but higher than that found in most other countries. As discussed by Trizio *et al.* (2005), Barratt *et al.* (1999), and references therein, a large number of haplotypes amplified from a small number of individuals is not unusual in rodent populations.

Table 4.22. MtDNA diversity in Ireland (this study) and 6 other countries in Europe, arranged left to right, peripheral to core populations. Welsh data is from Ogden *et al.* (2005) and other European from Hale *et al.* (2004), except Italy which is from Trizio *et al.* (2005). The number of base pairs amplified in each study is given in parenthesis. All studies involved amplification of a section of the control region of mtDNA, with the exception of the study in Italy which amplified the D loop region.

	Ireland (395)	Wales (280)	UK (395)	Spain (395)	NL (395	Italy (378)	Sweden (395)
No of individuals	87	31	106	19	10	70	13
No of haplotypes	29	4	18	2	4	23	2
No of populations	14	3	25	2	7	5	10
Gene diversity	0.7	0.3	0.52	0.53	0.73	0.87	0.154
Polymorphisms	14	8	11	2	8	9	7
Pairwise differences	4.4	1.63	2.99	1.05	3.75	3.4	1.07
Nucleotide diversity	0.05	0.012	0.023	0.008	0.03	0.06	0.008

There was no pattern of reduction in any of the diversity measures from the periphery towards the core of red squirrel range, with an increase from Wales – Italy, but then lower diversity in Sweden than Italy, and higher diversity in Ireland than in Wales, and Britain also skewed the west/east cline in diversity, as did Spain in some cases. Although a core to periphery reduction in genetic diversity has been recorded in some species (e.g. Eckstein *et al.*, 2006; Yeh *et al.*, 1979; Lammi *et al.*, 1999), other studies have shown no such relationship (e.g. Garner *et al.*, 2004; Wisely *et al.*, 2004). This investigation by no means sampled a continual distribution of the red squirrel from the periphery to the core of its range (it did not even sample a continual distribution from periphery to the core of distribution in Europe), nevertheless, this preliminary investigation suggests that diversity in peripheral populations of the red squirrel may not be reduced in comparison to more central populations, contrary to that predicted for marginal and island populations with reduced gene flow (Lesica & Allendorf, 1995). In fact, the opposite seems true, with the two peripheral, island populations, Ireland and Britain, having high levels of diversity and novel haplotypes which contribute significantly to the diversity of the European red squirrel population genetic diversity. However, it must be noted that the sampling range in Italy and Spain was restricted to one region within the country so may not be indicative of diversity within the country as a whole.

Another possible consequence of the reduced gene flow to both island and peripheral populations is increased genetic differentiation between populations (Lesica & Allendorf, 1995; Hutchinson & Templeton, 1999). Although in this study comparisons of F_{ST} were carried out between a number of different countries, with the exception of the Ireland/Belgium/Germany comparison, F_{ST} was calculated from different microsatellite loci, which may have influenced the results. Contrasting results were found in the analysis. The Ireland/Belgium/Germany comparison revealed no difference in genetic differentiation between populations, while the comparison between Ireland, Britain and Italy, detected higher levels of genetic differentiation in the two island, peripheral, populations; Ireland and Britain. Although reanalysis of all the raw data including only loci which were amplified in all countries would yield more conclusive results, this suggests that genetic differentiation is higher in peripheral populations of the red squirrel, as has been found in other mammals (e.g. fisher, Wisely *et al.*, 2004). Whether this is because both of these peripheral populations are also island populations is unknown, and further sampling is needed in populations at the edge of mainland Europe red squirrel distribution.

4.4.4. Conclusions

In conclusion, genetic diversity in the Irish red squirrel population was relatively homogeneous, with the exception of Cor, in which a recent population bottleneck was detected. Significant regional genetic structure exists in the population, despite some shared haplotypes between regions, and this regional genetic structure should be preserved in the event of translocations of populations of the species within Ireland

No reduction of genetic variability with increased habitat fragmentation was detected in this study, neither was there a significant correlation between habitat type, and genetic diversity nor structure, suggesting no adaptive divergence of red squirrels to a particular habitat type, although this is investigated further in the next chapter. Although these results confirmed that discontinuous habitat is a major factor in dispersal, it also validated the explanation for genetic differentiation proposed by Trizio *et al.* (2005), which is that a number of barriers to dispersal effect gene flow within red squirrel populations. The cumulative effects of these barriers also explain the large amounts of interpopulation differentiation, and even subspeciation, which can occur in the species within a relatively small geographical area.

As a population at the edge of a widespread species range, the genetic diversity in the Irish population seems to contribute strongly to European diversity, and does not seem to experience the effects of insularity that can occur in peripheral populations, although a more thorough survey of the European population would be needed to confirm this. The genetic structure seen in the Irish population was significantly larger than that on the continent, but this may be the result of island biogeography rather than peripheral restricted gene flow.

Overall these results show that red squirrel populations can experience insularity within quite restricted geographic areas, with insularity increasing as the number of barriers between red squirrel populations increases, but red squirrels do not seem to experience insularity at a broad geographic scale (peripheral to core of range). This means that the red squirrel is particularly susceptible to habitat fragmentation, but it also suggests the Eurasian red squirrel is a species in which adaptive microevolution to local environments would accumulate relatively quickly. Adaptive morphometric microevolution with respect to environmental pressures and possible correlates with genetic data, are investigated in the next chapter.

Chapter 5: The role of local adaptations, genetics and history in shaping the morphological diversity of the red squirrel.

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5.1. Introduction

Geographic patterns in morphological diversity may reflect natural selection (Endler, 1977) and microevolution in response to local environmental pressures (Riska, 1989), and can also be correlated with genetic diversity (Reed & Frankham, 2003). Understanding of the influences behind this microevolution can help in understanding phenotypic variation and distribution of species, and also in determining at which level significant adaptive variation should be preserved, when conservation is concerned.

The factors which influence morphological diversity are similar to those which influence genetic diversity; microevolution in response to landscape and/or habitat, insularity, and historical events. The influence of landscape and insularity on morphological diversity has long been recognised, from selection for cryptic colouring in specific habitats (e.g. Hoekstra *et al.*, 2004; Stoner *et al.*, 2003; Voipio, 1969), to the broader geographic effects of competitive release in peripheral or isolated island populations (Dayan & Simberloff, 1994, 1998, 2005; Meiri *et al.*, 2005).

Original colonisation patterns, historical habitat change, and demographic history all shape the morphological diversity of populations. Demographic history can complicate patterns of diversity, producing patterns of geographic variation which may be attributed to morphological adaptations but are, in fact, shaped by isolation by distance (IBD). To eliminate these effects Prout & Barker (1993) suggested comparison of neutral genetic markers, to track demographic patterns, with the morphological diversity presumed to be influenced by selection; thereby discerning whether any differences seen are adaptive in nature, or caused by these cryptic effects of IBD. The anthropogenic effects of translocations and introduction of alien species also influence morphology. Translocations may dilute adaptive divergence, or even cause reduced fitness due to outbreeding depression. Interactions between alien and native species may be predator/prey, direct niche competition or host/parasite, and has been documented to significantly effect, not only the survival, but also the microevolution of native species (Stauss *et al.*, 2006 and references therein).

The Eurasian red squirrel, *Sciurus vulgaris,* is a morphologically variable species, with 17 subspecies described (Sidorowicz, 1971). The majority of these are based on coat colour polymorphisms, with further 'light' and 'dark' morphs recorded within each subspecies

(Voipio, 1969; Wauters *et al.*, 2004). Although some differences in skull morphology occur, there is a lot of overlap between subspecies and little correlation between coat polymorphisms and cranial morphology (Sidorowicz, 1971). The ecological forces behind variations in Scuirid morphology have been investigated, and have been attributed to adaptive selection with respect to climate, habitat type and predation risks (Ducharme *et al.*, 1989; Kiltie 1989, 1992a, b; Sidorowicz, 1971; Voipio, 1969; Wauters & Dhondt, 1989a, b; Wauters *et al.*, 2004). However no studies have taken into account the possible genetic patterns behind these differences.

As a habitat specialist the red squirrel is intrinsically linked to its habitat and research elsewhere (Berteaux *et al.*, 2004; Réale *et al.*, 2003a, b) has shown that the North American red squirrel, *Tamiasciurus hudsonicus* can rapidly accumulate adaptive variation in response to changes in habitat type, however no studies have investigated this in the Eurasian red squirrel. Also, through much of its range, the Eurasian red squirrel has evolved in the absence of congeners, with the exception of eastern Persia, where it overlaps with *S.anomalus* (Gurnell, 1987). It has, therefore, not been subjected to the selective pressures, and subsequent niche partitioning, which occurs in squirrel species in North America (Smith & Follmer, 1972; Riege, 1991) and the tropics (Emmons, 1980).

However, recent human interference may be subjecting the Eurasian red squirrel to increased microevolutionary forces. Within Ireland, over the last 50 years, the habitat of the red squirrel has changed dramatically from a landscape once dominated by broadleaf species, to one which is primarily coniferous plantations (Neeson, 1991). Although a slight increase in broadleaf species has occurred in recent years, coniferous species, predominantly sitka spruce, *Picea sitchensis*, remain dominant in the forested landscape (Coillte, 2005), and it is unclear what effects this dramatic change in red squirrel habitat may have had, or be having, on the Eurasian red squirrel.

On a broader scale, within Europe, a number of alien competitors have been either released or escaped, and the Eurasian red squirrel is experiencing increasing resource competition, particularly from the North American grey squirrel, *S.carolinensis*, which was introduced into Britain (Corbett & Harris, 1991), Ireland (Watt, 1923) and Italy (Lurz *et al.*, 2001), and, since introduction, has replaced the red squirrel across some of its range (Gurnell, 1987, Lurz *et al.*, 2001; Ó Teangana *et al.*, 2000). While the mechanism for this replacement is unclear (see review in Lurz *et al.*, 2005), it is known that resource

competition does play a role, resulting in a selective advantage of red squirrels in conifer habitats, due both to food supply being more predictable in those sites, and the smaller size of the red squirrel allowing it to access food in the coniferous canopy that is not accessible to the larger grey (Lurz & Lloyd, 2000). Research has also shown that red squirrels, in sites where both red and grey squirrels are present, are smaller than those where no grey squirrels are present (Wauters *et al.*, 2000), but whether this is a result of simply reduced body size due to increased competition for food, or a preliminary indication of adaptive niche partitioning between red and grey squirrels, is uncertain.

Within Ireland the red squirrel is considered native but was translocated into the country, from Britain, during the 1800's (Barrington, 1880). Despite the introduction of the grey squirrel, the red squirrel in Ireland is still quite widespread (Ó Teangana *et al.*, 2000), and is common in both conifer and mixed conifer and broadleaf sites (Reilly, 1997). The Irish red squirrel population therefore represents one in which a number of questions can be addressed. Its status as an island population, with the red squirrel population isolated for at least 100 years, means patterns of morphological diversity, which may be diluted by continual gene flow elsewhere in its range, might be more apparent in the Irish population. Also, the continual presence of the species in sites which contain both conifer and broadleaf species, allows an investigation of possible habitat adaptation, incorporating genetic data, which has not been previously addressed. Additionally, possible microevolution in the species in response to habitat change, and an introduced competitor, can also be assessed.

Furthermore within the British Isles, the red squirrel has been described as a separate subspecies, *S.v.leucourus,* based on bleaching of the tail and ear tufts in the summer months (Sidorowicz, 1971). However, the history of both island populations has been complicated by numerous translocations. Recent studies in Britain (Hale & Lurz, 2003) have shown that the light colour morph is relatively rare in Britain, confined to one population in the northwest of England, and this was attributed to the rapid spread of translocated squirrels throughout Britain from mainland Europe. Although, as previously mentioned, the Irish population has been subject to translocations, it is unlikely, as these individuals were from Britain, and therefore presumably the same subspecies, that these translocations greatly affected patterns of morphological diversity within the Irish population, at least with respect to the mainland European population. This may mean that

the Irish red squirrel population represents the sole representatives of the subspecies, although no thorough survey of tail colour polymorphisms has been carried out in Ireland.

In this part of the study morphological data; tail colour, shin bone lengths (representing body size) and body weights, were compared between regions and habitat types, in order to answer a number of questions about adaptive morphological diversity within the species. Data on tail colour and cranial measurements were also recorded from both contemporary and museum red squirrels, again to address adaptive divergence, but also to investigate the effects that niche competition with alien competitors, and habitat change, may have on native species' morphology. By incorporating genetic data from the same individuals it was hoped to firstly determine whether any variation seen is indeed morphological adaptation, and secondly whether there is any correlation between genetic and morphological traits. There were 3 main aims to this part of the study:

- To determine the selective pressures behind morphological variation in the red squirrel.
- To assess whether any temporal changes in morphology have occurred in a native species due to habitat change or the presence of a recently introduced competitor.
- To determine the subspecific status of the Irish red squirrel and whether morphology gives clues as to the origin of the Irish population.

5.2. Materials and Methods

5.2.1. Sample collection

Samples were collected from around Ireland (Figure 5.1) as outlined in detail in Chapter 2. Morphological data were recorded from a total of 62 individual contemporary squirrels, 56 of which were adults (Table 5.1) collected from old and new forests (see Chapter 2) and conifer and mixed forests. Data collected from the museum collections are described in detail in Chapter 2. Data on tail colour was recorded from a total of 34 individual museum specimens, 3 of which also had skulls from which measurements were taken. Samples were grouped into regions according to their geographical location (Figure 5.1). Although the western region is geographically close to both the south and southwest, it is separated from those regions by the considerable barrier of the River Shannon.

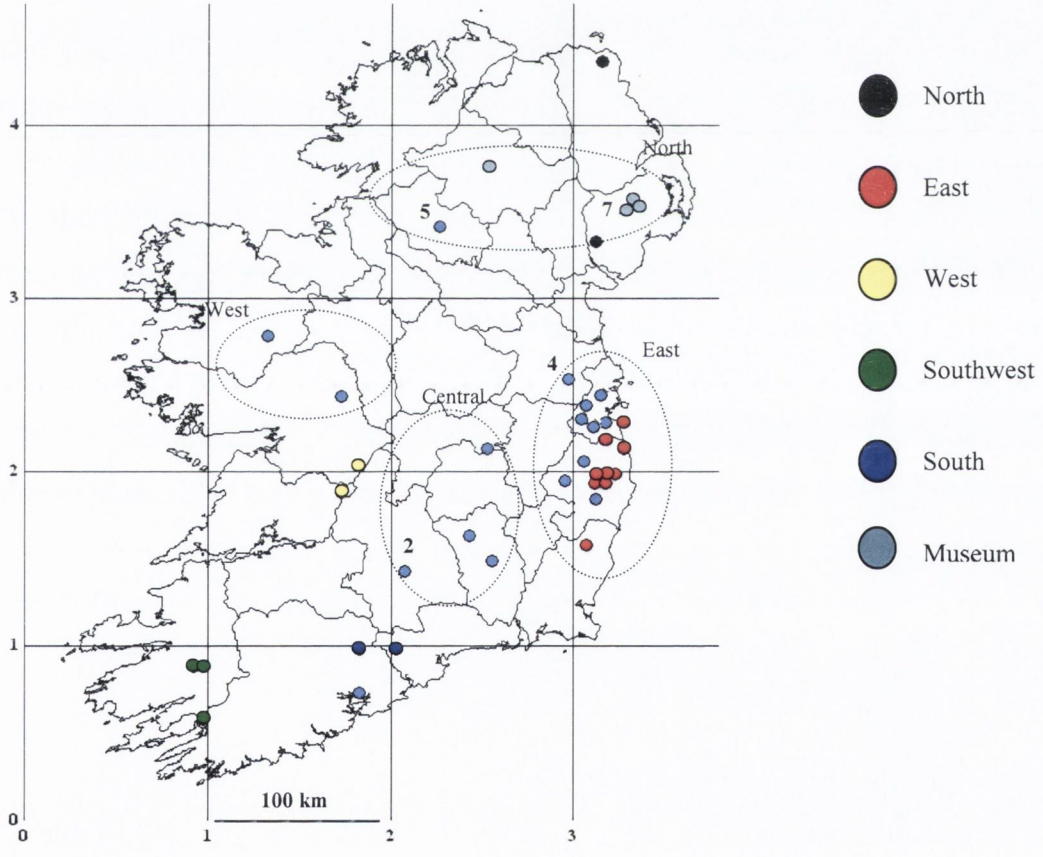

Figure 5.1. Map showing the locations of samples from which data were collected for the morphological analysis. Museum samples with numbers beside them had skull data recorded from them and are referred to by those numbers in the text, they are grouped into regions; east, west, central and north, indicated by the dashed lines. The number of contemporary samples collected from each region and mean morphological data for those regions are given in Table 5.1.

Table 5.1. Total number of contemporary samples collected, mean measures of body weight and shin length (± SEM; adults only), and proportion of light tailed individuals, in each region. The number of skulls collected from each region is also shown. Only tails were recorded from the north of Ireland.

Regions	East	West	South	Southwest	North
Number of samples	11	25	7	10	2
Number of skulls	4	1	0	0	0
Mean body weight (g) ± SEM	295 +/-10.7	243 +/-7.5	260.7 +/-11.5	260.7+/-11.5	n/a
Mean shin length (mm) ± SEM	62 +/-1.16	66.6 +/-0.75	65.6 +/-0.91	65.66+/-0.9	n/a
Proportion light tails	0.5	0.74	0.86	0.85	0.5

5.2.2. Tail colour

Although it would have been preferable to analyse coat colour polymorphisms with respect to both body and tail colour, the nature of the sample collection limited the investigation to variation in tail colour alone.

Tail colour was classified as one of 6 separate classifications, outlined by Hale & Lurz (2003): bleached, yellow/white tip, red, darker than the body, black edges and totally black. Bleached tails were the traditional colour described for *S.v.leucourus*, with the entire tail bleached white or cream coloured. Yellow/white tipped had bleaching only at the distal part of the tail, with the rest of the tail red or the same colour as the body. Red tails were the same colour as the body. Darker tails were still reddish in colour but the hue was slightly darker than that found on the body. Black edge was again generally red in colour, but the distal part of each hair was black. Black tails were entirely black in colour.

A 7th tail colour was also observed; bleached tail with the root of the hairs black in colour (see Plate 1.1). However, this was only observed in 2 Irish museum samples and one individual from the contemporary population in Galway and was classified under 'bleached'.

5.2.3. Cranial and body measurements

Fourteen skull measurements were taken from each individual (Figure 5.2, Table 5.2), chosen so as to allow comparisons with published data. Body measurements were taken as outlined in Chapter 2.

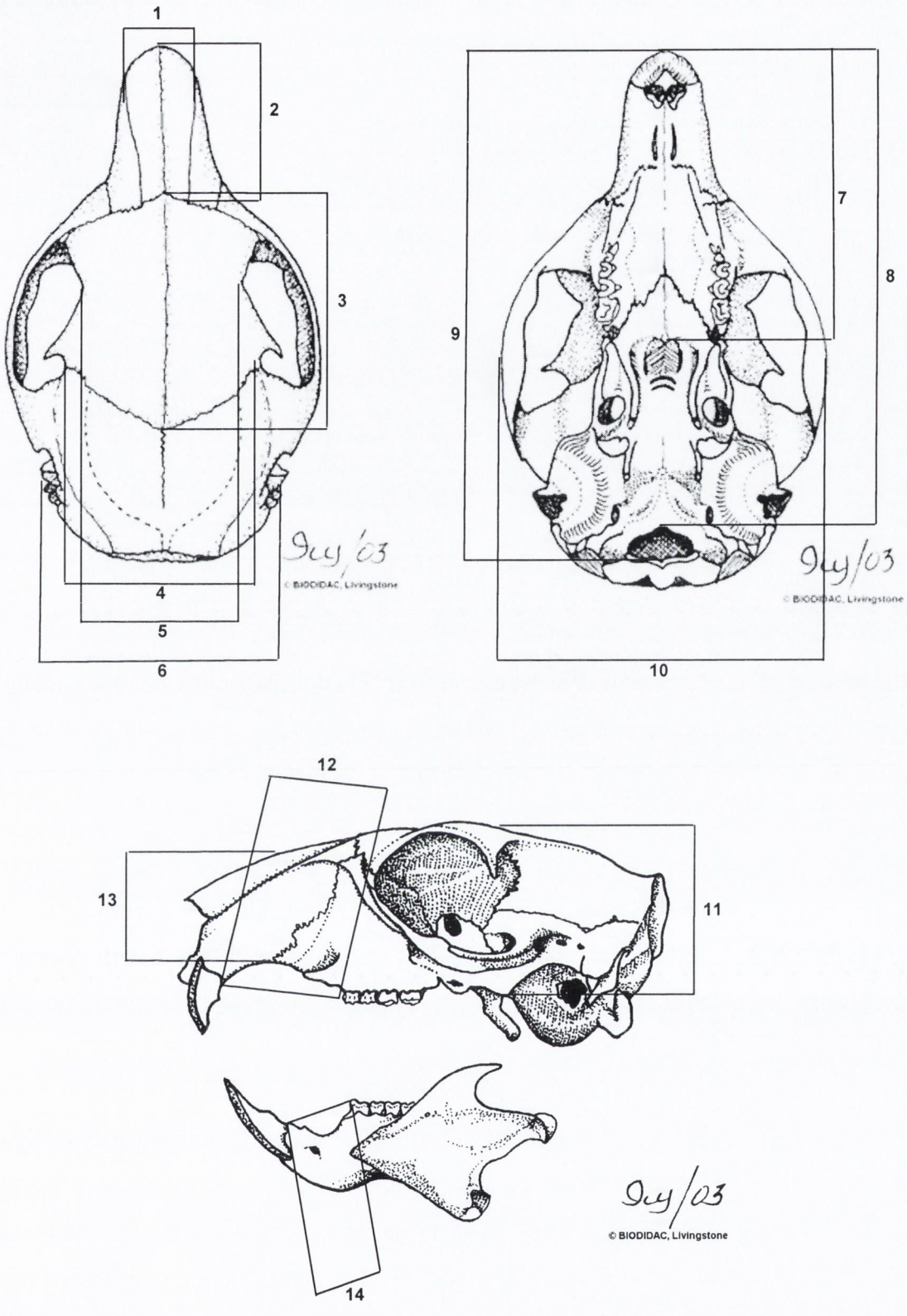

Figure 5.2. Dorsal, ventral and lateral views of red squirrel skull showing the 14 cranial measurements taken in this study. Numbers allocated to each measurement are explained in Table 5.2. Skull drawings were adapted from those copyrighted by Biodidac Livingstone and available online at http://biodidac.bio.uottawa.ca

Table 5.2. The 14 cranial measurements recorded in this study. Codes refer to those shown on Figure 5.2.

Code	Measurement
1	Nasal width: Maximum breadth of the nasals
2	Nasal length: Maximum length of the nasals
3	Frontal length: Maximum length of the frontals
4	Posterior intraorbital: Width of the posterior intraorbital constriction
5	Anterior intraorbital: Width of the anterior intraorbital constriction
6	Cranial width
7	Palate length
8	Basal length
9	Condylobasal length
10	Zygomatic width: width of the skull across the zygomatics
11	Cranial depth
12	Diastema upper: Length of the diastema of the upper jaw
13	Nasal depth: depth of the nose
14	Diastema lower: Length of the diastema of the lower jaw

5.2.4. Data analysis

Principal component, canonical and discriminant function canonical analysis, along with tests of normality, correlations, ANOVA and MANOVA were carried out in SPSS 12.0.1. Where another program was used in the analysis it is indicated in the text.

Data were analysed in three parts 1) quantitative morphological data, 2) comparisons between morphological and genetic data and 3) morphological and genetic data. Due to the small sample size tail colours were pooled into 'light' and 'dark', with bleached and white tipped tails classified as light, and red, dark edge, darker and black tails classified as dark.

In the first part of the analysis there was no difference between the frequency of light and dark tail colours in either male or female, juvenile or adult squirrels ($\chi^2_{(69)} = 0.868$, $p = 0.466$), therefore all samples were used when investigating landscape and regional differences in tail colour. However, there was age dimorphism in the sample data ($F_{(3,67)} = 15.852$, $p < 0.001$), with both adult female and male squirrels heavier than juveniles of both sexes (LSD post hoc: $p < 0.002$ in all cases), but no differences between males and females within each age class (LSD post hoc: Adults $p = 0.078$; Juveniles $p = 0.449$). Similar patterns were seen in the data with respect to shin bone length ($F_{(3,62)} = 12.406$, $p < 0.001$). Therefore, morphometric analysis was carried out on the adult data only. The only analysis for which the juveniles were used was the comparison between tail colour

and genetic diversity. Also, the nature of the cadaver collection (i.e. roadkill) resulted in very few (n =5) entire skills being available for measurement. Therefore cranial data comparisons were only carried out between contemporary and museum samples and when comparing data from Ireland and other countries.

Seasonal and regional variation in tail colour and body size (shin length and body weight) were assessed using χ^2 tests (tail colour), ANOVA and MANOVA. Data were tested for normality prior to analysis with the Levene's test of homogeneity, and, where necessary, LSD post hoc tests were carried out. Isolation by distance (IBD), the increase of differentiation with increasing geographic distance between regions was also investigated using the data on mean shin length, mean body weight and the proportion of light tail individuals in each region, and the mean geographic distance between regions. Prior to analysis morphological data were standardised to *z*-scores (mean 0, standard deviation 1) following Legendre & Legendre (1998). A Eucladian similarity distance matrix (*D*) between regions for each morphological measurement, and all three measurements, was constructed. Matrix values were converted to dissimilarity scores (*S*; $S = 1 - D$) and compared through correlation with the geographic distance matrix using the Mantel test (Mantel, 1967), with 5,000 permutations of the distance matrices in FSTAT 2.93 (Goudet, 2001), retaining $\alpha < 0.05$ using sequential Bonferroni corrections (Rice, 1989).

Adaptive variation of morphological data and habitat type (conifer or mixed), and morphological measurements and tail colour, were assessed with ANOVA and MANOVA, again, testing for normality with the Levene's test. Temporal changes in morphology, comparing contemporary data and data collected from museum specimens, and comparing data from squirrels from old and new forests, was investigated with ANOVA, t tests and χ^2 tests where appropriate. Temporal changes in skull morphology were further assessed with principal component analysis, whereby individual variation was minimised.

In the second part of the analysis, associations between morphological data and genetic diversity calculated from mtDNA and microsatellite data, details of which are in Chapter 4, were investigated with χ^2 tests and ANOVA. As described in Chapter 4, a large number of null alleles were detected in the data set and this most significantly affected the levels of heterozygosity within the data set. The correction method used in Chapter 4 corrected the population data set to account for these null alleles, but did not correct it at the individual

level. Therefore, the original microsatellite data set was used in the comparison, excluding the levels of heterozygosity as a variable in the analysis.

Relationships between shin length and body weight and microsatellite data were also assessed with partial Pearson correlations, controlling for any regional variation. PCA scores of shin length, body weight and tail colour were compared to gene diversity (He) with Pearson correlations. A canonical correlation between all morphological variables (n = 3) and all genetic variables (n = 3) was also performed.

In the third part of the analysis combined morphological and genetic data were used to assess regional and habitat variation. Discriminant function canonical analysis was used to assess divergence between regions based on these variables. Mantel tests between the variables and mean geographic distance were carried out in FSTAT 2.93 (Goudet, 2001), again producing dissimilarity matrices, and comparing through 5000 permutations as outlined above. Mantel tests between morphological data and Nei's genetic distance (D_s) between regions, the latter variable calculated in PHYLIP 3.5c (Felsenstein, 2004), were also carried out. Finally, diversity differences between conifer and mixed sites were investigated with PCA and discriminant function canonical analysis.

5.3. Results

5.3.1. Morphological variation

5.3.1.1. Seasonal and regional variation

Although the data collected suggested that bleaching of the tail was seasonal – with light tailed individuals recorded in all months except October, November and December, this may be a reflection of the poor sample size collected from those months (n = 4; Figure 5.3). Over all the samples, the light colour morph was dominant, found in 76% of the squirrels sampled. The only seasonal bias in tail colour occurred in March and June, whereby 70% and 61% respectively of individuals sampled from those months were light tailed (Figure 5.3), and no trend of increasing proportion of light tailed individuals as the summer progressed was observed. Unfortunately, the low number of samples did not allow a statistical month by month, or seasonal, comparison, and data had to be pooled into 'Winter' (October-March) and 'Summer' (April-September). Statistically there was no difference in tail colour between squirrels sampled in those two 'seasons' ($\chi^2_{(75)} = 0.532$, $p = 0.597$) and, in the sites where both colour morphs were recorded, the mean ratio of light:dark individuals was 1.5:1.

There was no overall regional pattern in tail bleaching ($\chi^2_{(4)} = 8.249$, $p = 0.083$) with almost equal number of squirrels in the east and west with each tail colour (light:dark; east 1.1:1; west 0.7:1), however there was a higher proportion of individuals in the southern region with light tails (80%), and a higher proportion of dark tailed individuals in the southwest (66%) and north (82%).

A MANOVA comparing body size (body weight and shin bone length) to the season and region in which the measurements were taken (Figure 5.4) revealed a significant difference between regions ($F_{(6,90)} = 3.364$, $p = 0.005$), but not between seasons ($F_{(6,90)} = 1.196$, $p = 0.316$), and there was no interaction between regions and seasons ($F_{(12,90)} = 0.971$, $p = 0.482$). Post hoc tests found that adults caught in the east were heavier than those caught in other regions ($p < 0.03$ in all cases).

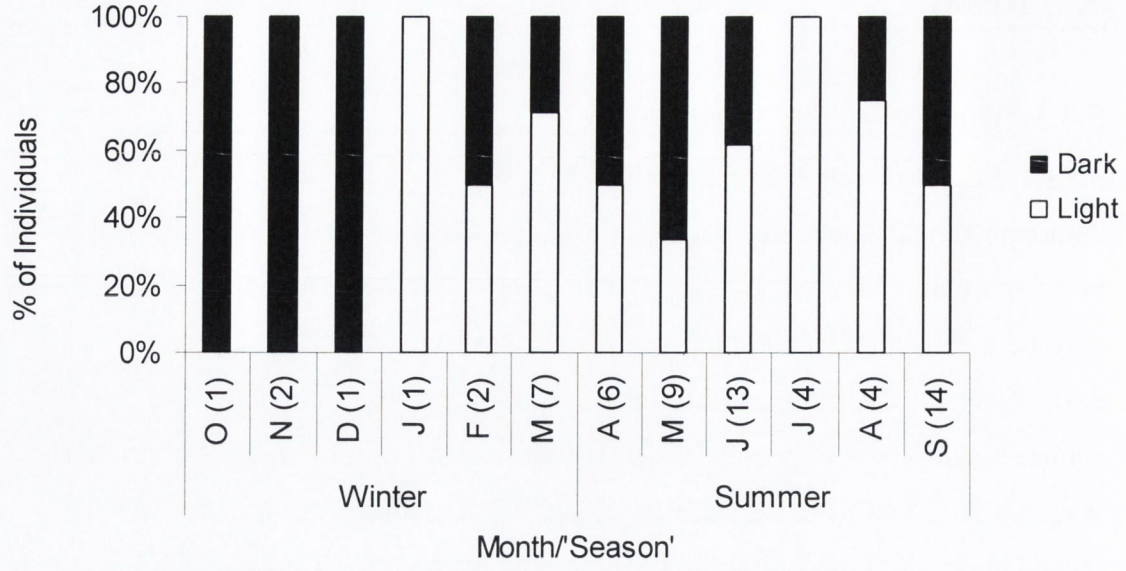

Figure 5.3. % of individuals sampled each month which had light and dark tails. There was no difference between seasons, and the proportion of light to dark individuals was relatively equal (mean 1.5:1) across the months when both colour morphs were recorded. Sample size for each month is given in parentheses.

The Mantel tests, investigating the association between morphological diversity and geographic distance between regions, found no evidence of IBD occurring with respect to body size (Weight: r = -0.57, R^2 = 32.94, p = 0.25; Length: r = -0.71, R^2 = 50.54, p = 0.07), although the association between length and geographic distance was just above the 95% level of significance. However, there was an association between tail colour and geographic distance (r = -0.84, R^2 = 71.85, p = 0.02), with the difference in proportion of light to dark individuals between regions inversely related to the geographic distance between regions. When all three morphological variables were used there was no evidence of IBD (r = -0.73, R^2 = 53.59, p = 0.057), although, again, this was just outside the 95% level of significance.

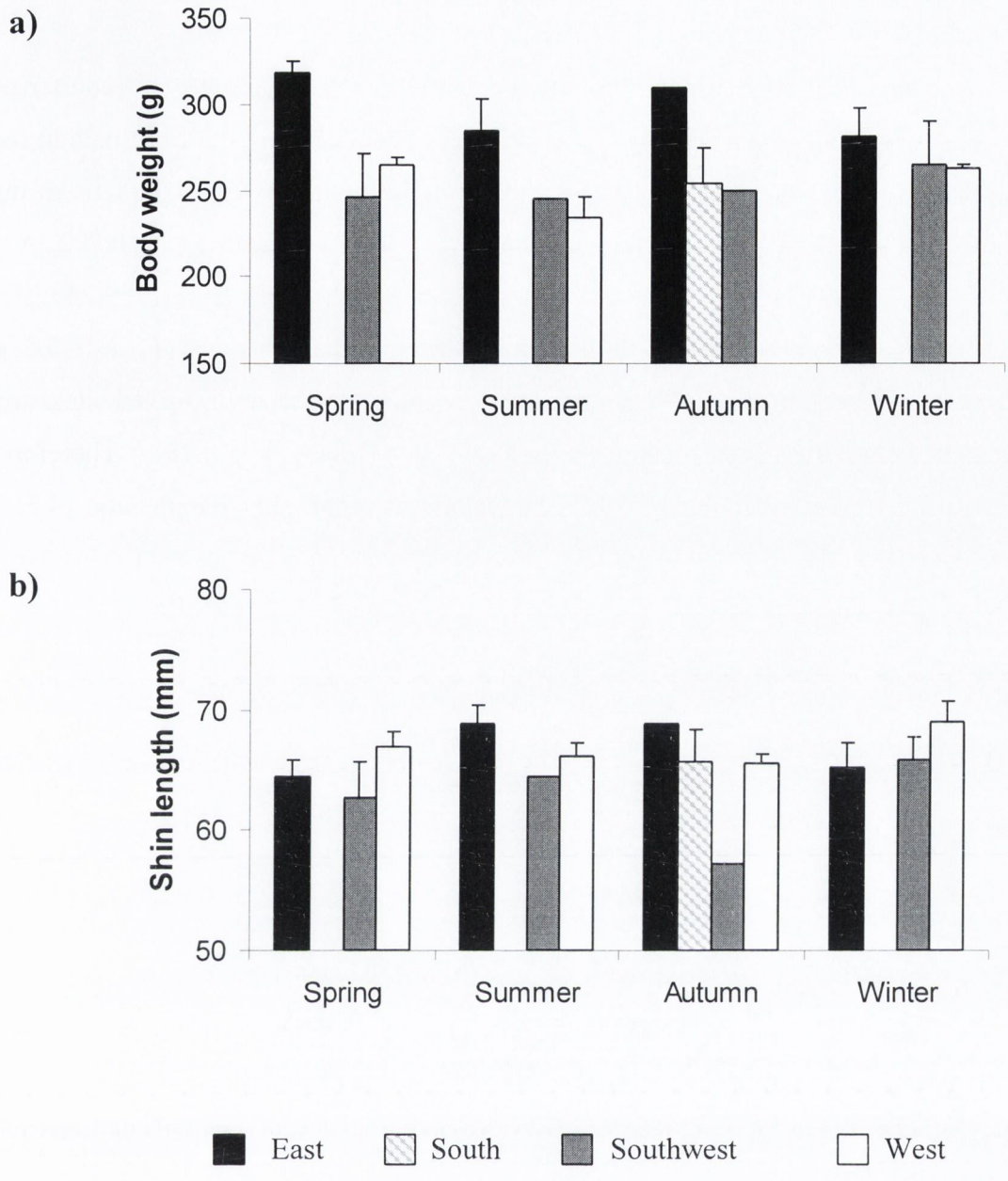

Figure 5.4 Mean ± SEM of a) body weight and b) shin lengths in the four regions and seasons from and in which of adult squirrels were sampled in this study. Squirrels from the east were significantly heavier than those in other regions ($p < 0.03$ in all cases).

5.3.1.2. Adaptive variation

Habitat variation

There seemed to be no clear association between tail colour and habitat type (conifer or mixed). Although there was a slightly higher number of individuals with light tails in the conifer forests (Light:Dark; 1:0.087) and a slightly higher number with dark tails in the mixed forests (Light:Dark; 1:1.42) this difference was not significant ($\chi^2 = 0.792$, $p = 0.419$). However, body measurements did vary between habitat types ($F_{(2,56)} = 9.243$, $p < 0.001$). This difference was between shin lengths in different habitat types ($F_{(1,57)} = 5.5$, $p = 0.023$), with squirrels in conifer sites having longer shins than those in mixed sites, and not correlated with differences in body weight ($F_{(1,57)} = 1.845$, $p = 0.18$). Therefore, squirrels in mixed sites were 'fatter' with a larger body weight: shin length ratio (4.5:1) than those in conifer sites (3.7:1; Figure 5.5).

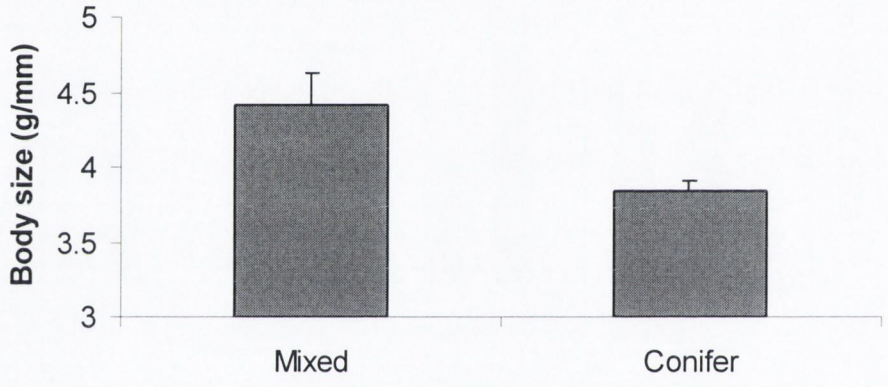

Figure 5.5. Mean measures ± SEM of 'Body size' (body weight (g)/shin length (mm)) of adult squirrels in mixed and conifer forests.

Correlates between tail colour and morphometrics

There was no relationship between tail colour and any of the morphometric measurements recorded in this study. Tail colour was not related to either body weight or shin length ($F_{(2,44)} = 1.24$, $p = 0.3$). Unfortunately, again, the low number of skulls collected prevented any detailed statistical investigation of possible associations between cranial features and tail colour, and, unluckily, 4 out of the five entire skulls had dark tails, and only one of the museum specimens, from which both information on tail colour and cranial measurements were collected, also had a light tail.

5.3.1.3. Temporal variation

Skull morphology did not differ significantly between the contemporary data (n = 5) and the data collected from the museum samples (n = 8; $F_{(1,11)} = 0.757$, $p = 0.725$; Table 5.3). However both frontal and palate length differed between the two data sets (Frontal: $p = 0.032$; Palate: $p = 0.038$l), with contemporary specimens having longer frontal lengths and shorter palates. Although these differences were no longer significant (Frontal: $p = 0.094$; Palate: $p = 0.072$) when the juvenile was excluded from the contemporary data set, mean frontal and palate lengths were still different between the two data sets. There was no difference in the length of the lower diastema between the two data sets ($t_{(10)} = 0.275$, $p = 0.789$).

Table 5.3. Cranial measurements (mm ±SEM) of red squirrel skulls from around Ireland and Britain. The number of samples from which data were recorded in each data set is shown in parenthesis.

	Ireland (n = 5)	Museum Ireland (n = 4)	Museum GB (n = 4)
Condylobasal	49.2±0.6	50.8±1.9	50.9±0.8
Palate length	22.3±1.1	19.7±0.2	14.2±4.8
Frontal length	22±0.2	22.6±0.1	22.5±0.2
Upper diastema	11±0.2	11.8±0.4	12.7±0.7
Lower diastema	7.1±0.2	6.5±0.2	7.7
Nasal length	16.4±1.4	15.7±0.8	15.7±0.4
Zygomatic width	29±0.9	30.1±0.5	29.9±0.5
Cranium width	22.5±0.3	23.2±1.9	24.7±1.2
Interorbital posterior	17.6±0.1	17.6±0.3	18.6±0.3
Interorbital anterior	17.1±0.4	17.4±0.6	19.4±1.2
Nasal Width	7.8±0.3	8.7±0.5	8.2±0.5
Cranium depth	21.1±0.4	21.8±0.6	23.9±1.9
Nasal depth	11.7±1.2	10.8±0.6	10.3±0.4

In the principal component analysis of the two data sets, the first three principal components explained 73% of the variation in the data set. Both principal component loadings on axis 2 and 3 were different in the two data sets, both when the juvenile was included (PCA 2: $F_{(1,11)} = 8.235$, $p = 0.015$; PCA 3: $F_{(1,11)} = 5.503$, $p = 0.039$), and when the juvenile was removed from the analysis (PCA 2: $F_{(1,10)} = 6.028$, $p = 0.004$; PCA 3: $F_{(1,10)} = 4.993$, $p = 0.049$). There was no difference between the data sets on axis 1 (Juvenile included: $F_{(1,11)} = 0.084$, $p = 0.778$; Juvenile excluded: $F_{(1,10)} = 0.133$, $p = 0.724$). Figure 5.8 shows the scatterplot of loadings of each individual in each data set on PCA 2 and PCA 3. PCA 2 was negatively correlated with nasal length and depth, and positively

related to frontal length and zygomatic width. PCA 3 was positively related to the length of the upper diastema and palate length.

Although generally the specimens collected from Ireland fell within the measurements taken from the British museum specimens (Table 5.3), in the contemporary data, samples 1, 4 and 5 were separated from the other contemporary samples, and from the museum samples, on the second principal component, while samples 2 and 3 were separated from the museum samples on the third principal component (Figure 5.6). Therefore, samples 1, 4 and 5 had larger (longer and deeper) snouts relative to the total length of the skull and also had wider skulls than the other samples, while 2 and 3 had shorter upper diastemas and palates than the other samples. Within the museum specimens there was little temporal association. The two most recent museum samples, #8 collected in 1981 and #2 collected in 1964 were similar to those collected at the beginning of the 1900's.

Also, the four samples from England (1, 3, 6 and 8) were similar to specimens collected from Ireland, with sample 8 almost identical to the skull data collected from Northern Ireland. What is striking is the linear distribution of the majority of the museum data on the second principal component. The length of the palate and upper diastema remains relatively constant while the length and the depth of the snout in relation to the overall skull length, along with skull width, varies between the samples.

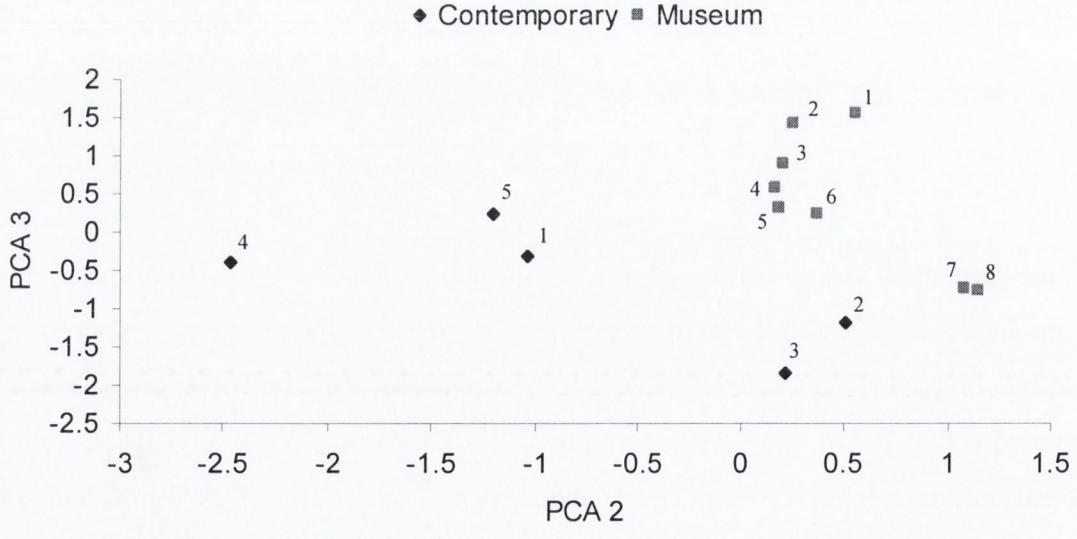

Figure 5.6. Principal component analysis of the contemporary and museum squirrel skulls. Museum samples: codes refer to those in Figure 5.1. Samples 1, 3, 6 and 8 are English skulls.

There was no difference in the frequency of light and dark colour morphs in the two data sets ($\chi^2_{(1)} = 1.423$, $p = 0.325$), neither was there a difference when the data were analysed with respect to the decade in which the specimen was collected ($\chi^2_{(8)} = 12.345$, $p = 0.136$). When only the Irish samples were used there was still no difference between museum and contemporary data ($\chi^2_{(1)} = 0.287$, $p = 0.612$). Likewise, there was no difference when the data were split into decade of collection ($\chi^2_{(7)} = 0.28$, $p = 0.725$; Table 5.4), whether data were collected before or after 1950 ($\chi^2_{(1)} = 1.11$, $p = 0.338$) or before or after 1910 ($\chi^2_{(1)} = 0.28$, $p = 0.725$). However, although there were no significant differences it is worth noting from Table 5.4 that before 1960, 75% of the specimens had light tail colours while, in the samples collected in the 1980s, the tail colour had shifted to a higher frequency of dark morphs (83%), and in the most recent samples, light morphs were once again dominant (62%). At a regional level (eastern region only) there was no temporal change in the frequency of light or dark colour morphs ($\chi^2_{(1)} = 0.038$, $p = 1$). Overall, 60% of contemporary, and 46% of museum, specimens sampled had light tails.

There was no difference in tail colour frequency ($\chi^2_{(75)} = 3.5$, $p = 0.075$), shin bone length ($t_{(60)} = -1.2$, $p = 0.22$) or body weight ($t_{(57)} = -0.84$, $p = 0.4$) between squirrels caught in old forests and those sampled from new forests.

Table 5.4. Frequency distribution of light and dark tail colours in samples collected from eight decades in Ireland. Expected values are in parenthesis. There was no temporal difference in tail colour (p = 0.725).

Decade of Collection		1890	1900	1940	1950	1960	1970	1980	2000
Tail colour	Light	5(4.09)	2(1.75)	1(0.58)	1(0.58)	2(1.75)	1(1.75)	1(3.51)	25(23.97)
	Dark	2(2.91)	1(1.25)	0(0.42)	0(0.42)	1(1.25)	2(1.25)	5(2.49)	16(17.03)
Total		**7**	**3**	**1**	**1**	**3**	**3**	**6**	**41**

5.3.2. Correlates between genetic and morphological traits

5.3.2.1. MtDNA and morphological traits

Where a mtDNA haplotype was amplified in more than one individual, body size was significantly related to the mtDNA haplotype which the squirrel had ($F_{(14,58)} = 1.935$, $p = 0.041$) with individuals with haplotype E6 heavier than those with all other haplotypes (LSD post hoc: $p < 0.04$ in all cases), except those with haplotype E+N ($p = 0.878$), and E3 ($p = 0.224$). Individuals with haplotype E+N were also heavier than those with haplotype S1 ($p = 0.048$). However, a further ANOVA on only the eastern samples revealed this

relationship occurred due to variations of body weight between the east and other regions, rather than any association between body weight and haplotype (ANOVA eastern size v haplotype: $F_{(4,8)} = 2.803, p = 0.1$).

There was no association between tail colour and haplotype, either when all haplotypes were included ($\chi^2_{(20)} = 20.826, p = 0.407$) or when only haplotypes which were found in more than one individual were included ($\chi^2_{(6)} = 6.029, p = 0.42$). The haplotype network (Figure 5.7) showed no clear association between tail colour and the number of mutations between haplotypes. A more in depth investigation between phenotypic variation and mutational differences between haplotypes was carried out using the nesting design originally produced in section 3.3.3.2.

Body size varied between the 1-step clades ($F_{(8,50)} = 4.716, p < 0.001$) and 2-step clades ($F_{(6,64)} = 2.251, p = 0.03$) with post hoc tests revealing identical results to those found in the ANOVA described above, with the only differences occurring among clades which consisted solely of eastern individuals and clades which did not have haplotypes found in the east nested within them.

There was no association between tail colour and haplotypes at the 1-step ($\chi^2_{(15)} = 16.486, p = 0.351$), 2-step ($\chi^2_{(6)} = 9, p = 0.174$) or 3-step ($\chi^2_{(4)} = 4.095, p = 0.393$) levels. The low number of samples collected from the cranial measurements, and the fact that haplotype data were only available from one skull, made any interpretations of the relationship between skull size and haplotype impossible.

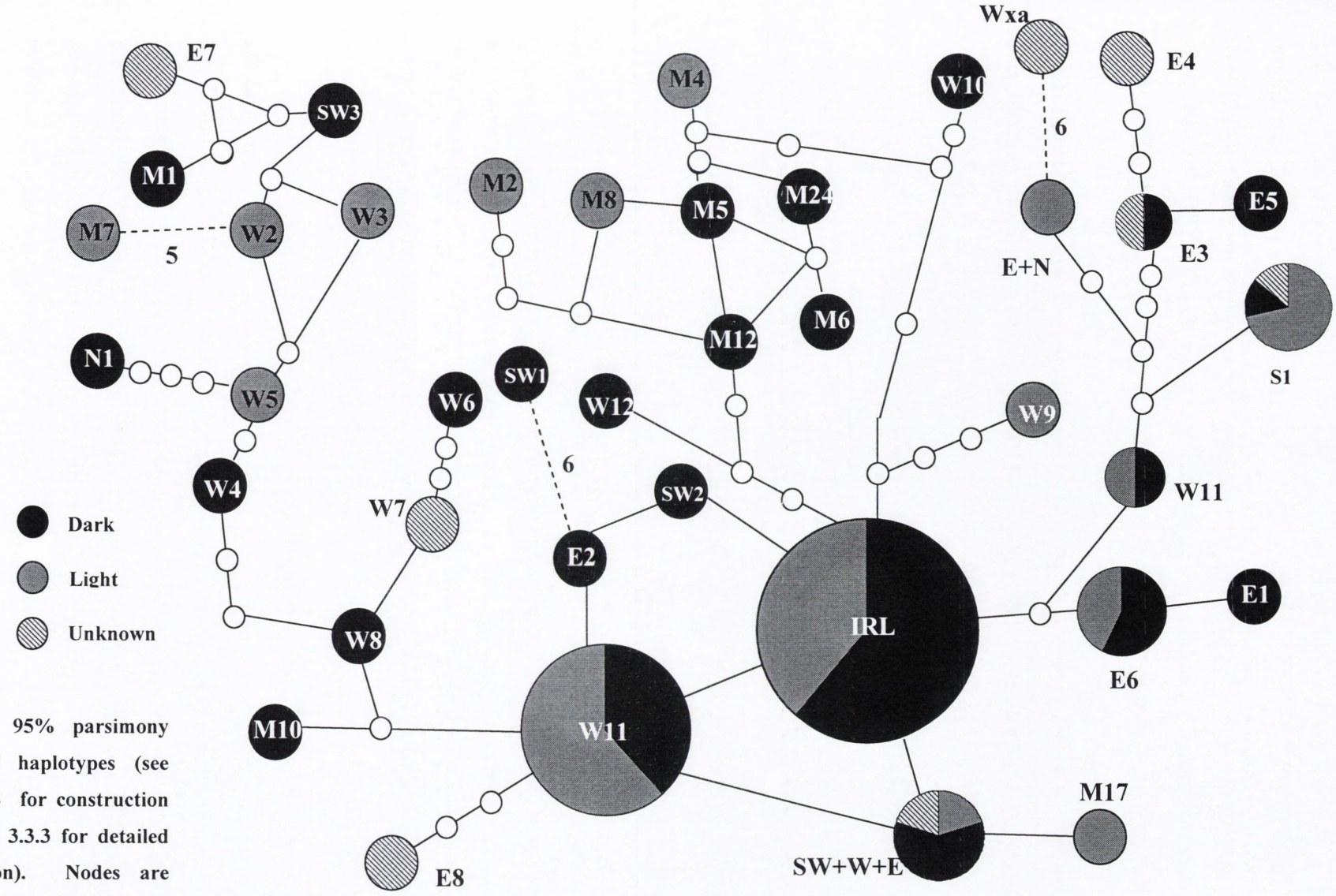

Figure 5.7. 95% parsimony network of haplotypes (see section 3.2.4 for construction method and 3.3.3 for detailed interpretation). Nodes are labelled with respect to the tail colour(s) of the individual(s) with that haplotype.

5.3.2.2. Microsatellites and morphological traits

There were no correlations between shin length and diversity measured from the microsatellite data even after controlling for any regional variation (Partial correlation Number of alleles: $r_{(35)} = 0.106$, $p = 0.531$; He: $r_{(35)} = 0.531$), however, body weight was significantly correlated to both the number of alleles an individual had ($r_{(35)} = 0.406$, $p = 0.013$), and He ($r_{(35)} = 395$, $p = 0.016$) over and above any regional variation (Figure 5.8).

There was no association between tail colour and genetic diversity estimated from microsatellite data (Number of alleles: $F_{(1,50)} = 1.293$, $p = 0.261$; He: $F_{(1,50)} = 2.294$, $p = 0.136$).

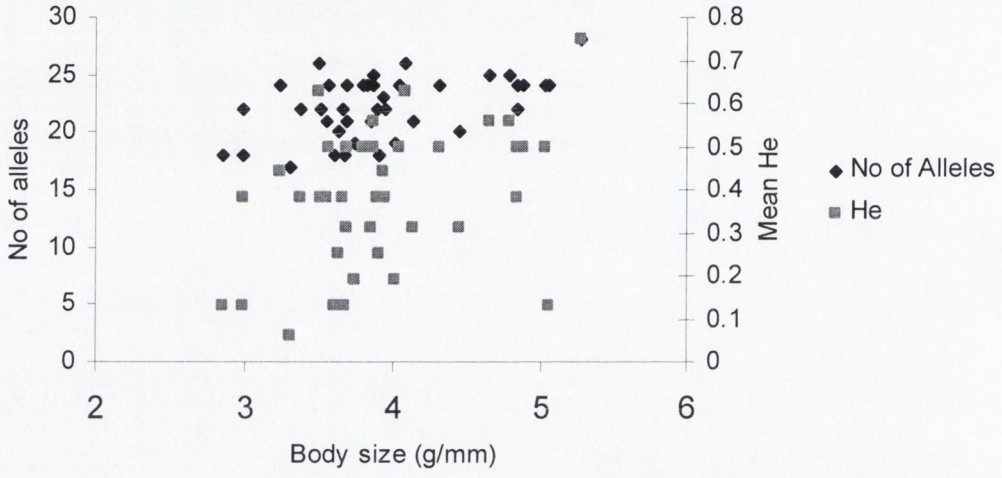

Figure 5.8. Relationship between body size (body weight (g)/shin length (mm)) and the number of alleles and He found in each squirrel. Body weight was positively related to each measure of genetic diversity ($p < 0.05$)

5.3.2.3. All genetic and morphological traits

The first two canonical variables explained 99% of the variation within the data and the two sets of variables were different on the first canonical variable (Wilks' $\lambda_{(9)} = 0.457$, $p < 0.001$), and there was little correlation between the two sets of variables. The genetic traits scored low on both the first and second canonical axes (Figure 5.12) while the morphological traits scored high on the first axis and was quite variable on the second axis, representing shin length and tail colour. It appears that, over all, the genetic variables sampled in this study had little correlation with squirrel morphology.

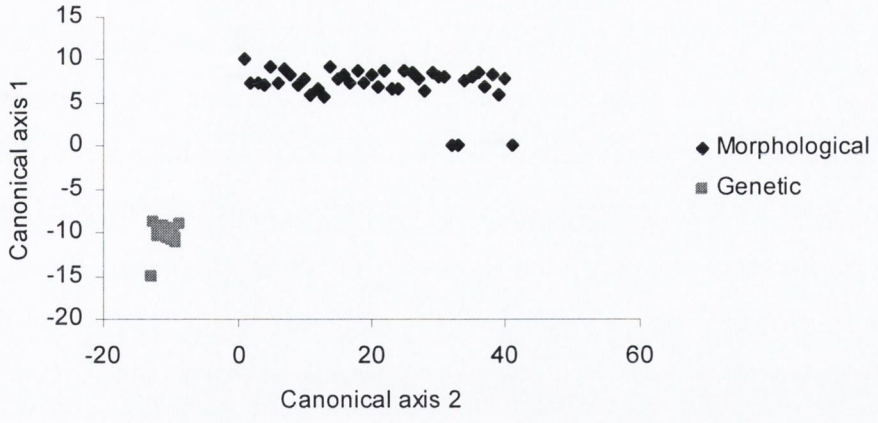

Figure 5.9. Scatterplot depicting the relationship between genetic and morphological diversity. The two sets of variables were not highly correlated.

5.3.3. Genetic and morphological variation

5.3.3.1. Regional variation

Diversity, based on both genetic and morphological characteristics, varied between regions (Wilks' $\lambda_{(18)} = 0.098$, $p < 0.001$). The first two discriminant functions explained 97.9% of genetic and morphological variation, and varied significantly between regions (Discrim 1: $F_{(3,34)} = 38.66$, $p < 0.001$; Discrim 2: $F_{(3,34)} = 12.44$, $p < 0.001$). Although the addition of the third discriminate function would have explained 100% of the data variation, this variable, representing tail colour and He, did not vary between regions ($F_{(3,34)} = 1.122$, $p = 0.354$) and was therefore not used in the analysis. Figure 5.10 shows the mean and SEM of diversity in each region based on the first two discriminant functions. The first discriminant function described haplotype diversity, body weight and the number of microsatellite alleles while the second was correlated with shin length.

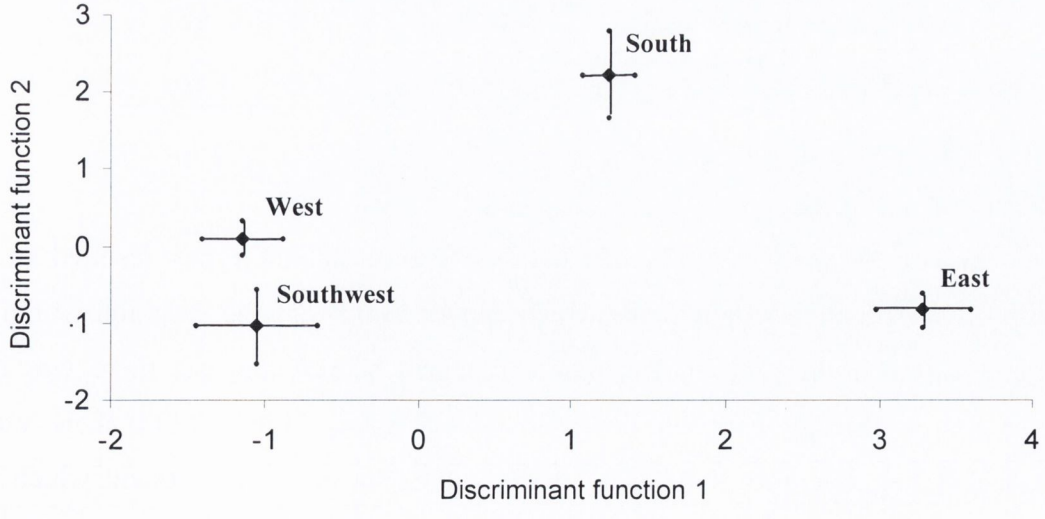

Figure 5.10. Discriminant function analysis of morphometric and genetic diversity in four regions in Ireland. Mean loadings on each axis ± SEM are shown.

Of the four regions the south was the most divergent, separated from the other regions with respect to both of the discriminant functions, while the east was similar to the west and southwest on the second discriminant function, and the west and southwest were similar on both functions. Therefore, individuals from the south had longer shin bones than those in other regions, but their genetic diversity and body weight fell between those found in other regions. Eastern individuals had similar shin bone lengths to those in the west and southwest but had higher genetic diversity and body weight than those found in the west and south west.

These differences were further explored by assessing the percentage of correct regional classification of individuals based on these discriminant functions (Table 5.5). This supported the differentiation between the east and the south and other regions, whereby 100% of individuals within those regions were correctly assigned, and also supported the similarities between the west and southwest with almost half of the southwestern individuals assigned to the western region. However, despite the seeming geographical partitioning occurring between regions, no association between geographical distance between regions and morphological and genetic distance was detected (Mantel test: $r = -0.6$, $R^2 = 37.12$, $p = 0.2$), neither was there a relationship between any of the morphological characters and genetic distance between regions calculated with either the microsatellite ($p > 0.7$ in all cases) or mtDNA ($p > 0.6$ in all cases) data sets.

Table 5.5. Re-classification of individuals into regions based on the morphological and genetic data.

From \ To	South	West	Southwest	East	% Correct
South	5	0	0	0	100
West	1	17	1	0	89.5
Southwest	0	3	4	0	57.1
East	0	0	0	7	100

5.3.3.2. Habitat variation

Principal component analysis of diversity in difference habitat types resulted in the extraction of two principal components which explained 64% of the variation within the data sets. Although the plot of principal component scores suggests there was little difference in morphology between different habitat types (Figure 5.11), there was a significant difference between habitat types on the first principal component which was positively correlated with genetic diversity and body weight ($F_{(1,36)} = 4.292$, $p = 0.046$), with individuals from the mixed sites scoring higher on this component.

When the variation within the different groups was minimised through discriminant function analysis even more division occurred. A single discriminant factor was extracted which accounted for 100% of the variation in different site types and this was significantly different between the different habitats ($t_{(36)} = -5.269$, $p < 0.001$). Squirrels from mixed sites had more alleles, higher He, and were heavier with shorter shin bones, than those in conifer sites.

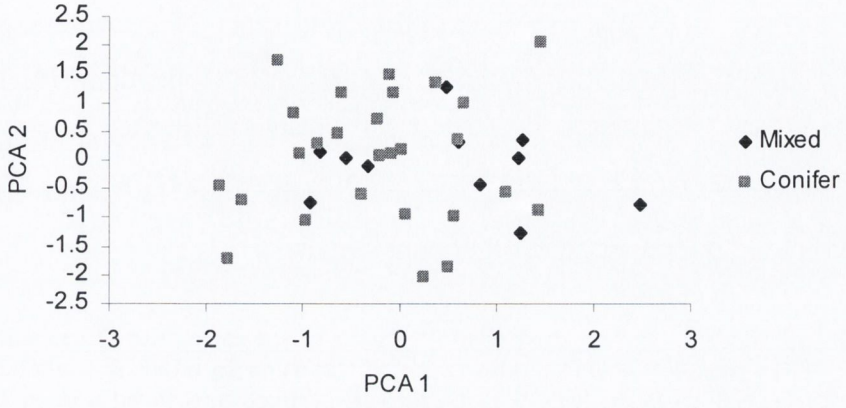

Figure 5.11. Scatterplot showing distribution of individuals in mixed and conifer sites with respect to their scores on the first two principal component axes summarizing genetic and morphological diversity.

5.3.4. Subspecific variation

Table 5.6. shows the mean measurements taken from Irish specimens (this study) as well as those recorded by Hale & Lurz (2003) and Sidorowicz (1971) for *S.v.leucourus* and other subspecies of the Eurasian red squirrel, for the three cranial features common to all the three studies. Generally, Irish red squirrel measurements fell within the range of those from other regions, and there was no clear division of the three groups of data collected from *S.v.leucourus* populations from the other data sets. Nevertheless, when the three variables are considered together a pattern emerges in the Irish data set. The Irish skulls had relatively large condylobasal: diastema length and condylobasal:zygomatic width ratios than those recorded elsewhere, suggesting the Irish red squirrel skull may be narrower, with a shorter snout, than other populations.

Table 5.6. Mean cranial measurements (in mm) of some of the subspecies described for the Eurasian red squirrel. Irish samples are from this study. * recorded by Hale & Lurz (2003). All other measurements from Sidorowicz (1971). The number of specimens measured is in parenthesis.

Subspecies	Country/Region	Condylobasal	Upper diastema	Zygomatic
S.v.leucourus	Ireland (9)	49.9	11.4	29.5
	Britain* (30)	47.4	12.3	26.2
	Britain (28)	45.8	12.4	30.4
S.v.vulgairs	Scandinavia (158)	46.5	12.3	31.0
S.v.infuscatus	Spain (30)	50.4	13.1	32.9
S.v.fuscoater	France (65)	47.9	12.6	31.3
S.v.italicus	Italy (40)	46.9	12.5	31.1
S.v.balcanius	Former Yugoslavia (32)	47.9	13.3	31.6
S.v.lis	Japan (5)	45.1	12.8	29.3
S.v.bashkiricus	Ural mountains (37)	46.9	12.6	31.1
S.v.argenteus	Western Siberia (52)	48.7	12.7	30.9

5.4. Discussion

5.4.1. Morphometric variation

Red squirrel body size varied between regions sampled in this study. Squirrels were significantly heavier in the eastern region (mean 295g), but similar to measurements of red squirrel adult body weights described elsewhere (approx 300g, e.g. Holm, 1990; Lurz, 1995; Reilly, 1997; Wauters *et al.*, 2000), while weights in other regions in this study were considerably lower than those described elsewhere (243-260g). A possible cause of this could be seasonal effects on body mass, with Magris & Gurnell (2002) describing heavier females in summer (April and July) than winter (January and October). However, the region*season interaction showed no seasonal difference, neither did the within east comparison. Also, at a glance, the time of sampling in each region would predict *lighter* squirrels in the east, as the majority of the samples from that region were collected in the winter months, while samples in the other regions were collected from April-September. Also worth noting is the lack of difference between regions in shin bone length. Therefore, not only were eastern squirrels heavier, they had better body condition, with larger body weight to shin bone ratios.

An analogous pattern in differences in body weights was found when squirrels from the mixed and conifer sites were compared. Although, in this case, there was no difference between body weight in the two habitat types, shin bone length was shorter in the mixed sites, meaning that, overall, red squirrels in mixed sites were in better body condition than those in conifer sites, suggesting some ecological advantage to inhabiting mixed sites. Although traditionally described as a species which occurs in boreal conifer forests, and this is the habitat which it inhabits over much of its range (Gurnell & Anderson, 1996 in Lurz *et al.*, 2005), the advantage of mixed conifer/broadleaf forests as a habitat for the species as been documented, with higher densities recorded in those site types when compared to conifer sites (Gurnell, 1983; Lurz *et al.*, 1995), and, although generally body weights in the different habitat types are the same, larger skull measurements have been recorded in subspecies associated with mixed coniferous and broadleaf and pine, *Pinus* sp., forests (Sidorowicz, 1971). The differences in body weight to shin bone relationships found in this study suggest that mixed conifer and broadleaf habitats are better red squirrel habitat, probably due to a number of variables; more reliable and varied food supplies,

mothers being able to raise larger litters, higher densities interacting with or producing increased genetic diversity.

Although the overall canonical correlation showed little relationship between morphological and genetic diversity, there was a correlation between genetic diversity measured from the microsatellite data and body weight, and the discriminant function analysis of morphological and genetic diversity showed clear division between regions (Figure 5.13) and site type (Figure 5.14). Genetic diversity is often used as a measure of the 'health' of a population, with more diverse populations having a higher evolutionary potential to adapt to changes in environmental conditions (Frankham *et al.*, 2002). Although generally there is little correlation between neutral genetic markers, such as mtDNA and microsatellites, and phenotypic traits (Willis *et al.*, 1991; Kjær *et al.*, 2004; Pfrender *et al.*, 2000), they have been found to underestimate the fitness of a population (Reed & Frankham, 2003). Therefore the correlation between genetic diversity, measured from microsatellite data, and body weight found in this study, firstly, may indicate that levels of genetic diversity at neutral genetic markers in red squirrel populations may reflect diversity which is correlated with phenotypic variation, and therefore, fitness, and secondly, that levels of genetic variation have an effect on the general body condition of the squirrels.

However, the opposite could also be true, and it could be body weight that is influencing genetic diversity and not vice versa. Within red squirrel populations body weight directly affects recruitment within the population. Female red squirrels do not come into oestrus until a minimum threshold in body weight of approximately 300g has been reached (Lurz, 1995; Wauters & Dhondt, 1989a). Therefore, the higher body weights in the east may result in more females coming into oestrus than in other regions, therefore reducing the effect of diversity loss due to genetic drift in the eastern region, relative to other regions. However, the analysis detected no significant difference in female body weights between regions so it is likely that genetic diversity is influencing body weight and not vice versa.

In an experimental study carried on the North American red squirrel, McAdam *et al.* (2002) found inherited genetic diversity was positively correlated with growth in body size and body weight, but also that the maternal effects of rearing ability etc contributed strongly to this phenotypic variation. Although the maternal effects on body weight and body size were not accounted for in this study, the correlates between genetic diversity,

calculated from the microsatellites, which are inherited in a Mendelian fashion, and body weight found here, suggest that a similar pattern exists in Eurasian red squirrel populations.

Overall the genetic and morphological results suggest that mixed sites are better red squirrel habitat, maintaining squirrels with higher body weights and harbouring larger amounts of genetic diversity. This suggests that habitat composition affects both morphology and genetics of red squirrel populations, and therefore, changes in habitat composition have the potential to stimulate microevolution in the red squirrel as a species, although this will depend on the scale at which insularity is experienced by red squirrel populations and this is discussed in Chapter 4. The reason for the higher genetic diversity and body weights in the east is unclear but could be due to recent genetic bottlenecks in other regions, or a product of more historical processes, and is discussed in more detail in Chapters 3 and 4.

Throughout all the comparisons there was no association between tail colour and any of the geographical, morphological or genetic measurements recorded in this study. Traditionally, the light colour morph is associated with the summer months (Sidorowicz, 1971), and Hale & Lurz (2003) found a higher frequency of bleached tails in June, July and August. However, Lowe & Gardiner (1983) predicted little association between bleaching and season, due to patterns and timings of tail and body moults over the year and that was seen is this study. The occurrence of tail colour does not seem to be related to fading of the hair in the summer months and instead may be related to camouflage or some other selective pressure.

The various selective pressures on pelage polymorphism have been well researched. Wauters *et al.* (2004) reported a correlation between darker fur and dense spruce forests in the Italian Alps; attributing this association to lower costs of thermoregulation in winter and lower visibility to predators. Although the former is well supported by research, evidence for the latter is not as evident. Voipio (1969, 1970) concluded that ecological adaptations explained the difference in coat colour between red squirrel populations in Finland, whereby differences in coat colour between regions remained relatively stable, returning to near original ratios over time, even after considerable immigration into the area. Darker coat colours in squirrels have been described as denser, increasing thermoregulation in the winter months, therefore producing a selective pressure on this polymorphism at high altitudes and/or latitudes (Ducharme *et al.*, 1989; Voipio & Hissa,

1970). As only tail colour, and not overall coat colour, was recorded in this study, interpretation of the tail colour as a means of thermoregulation is not possible. However, within the relatively small area of the island of Ireland, it is highly unlikely that altitude or climate would be a driving force behind coat colour.

The interaction between predation and cryptic colouration has been investigated in detail in the fox squirrel, *Sciurus niger*, by Kiltie (1989, 1992a, b). He assessed the levels of predation of fox squirrels with different coat colours which affected their level of camouflage with respect to their habitat, and found little correlation between the visibility of the squirrel and predation. Within this study a selective pressure for lighter tails in the less dense mixed forests, and vice versa for conifer forests, increasing crypsis and decreasing the chances of predation, might be expected. However, no correlation between coat colour and habitat type was found, in fact there was a slightly higher proportion of light tailed individuals in the conifer forests and more dark tailed individuals in the mixed forests. In Ireland, red squirrel mortality due to predation is not a major factor, with little evidence of red squirrel remains in dietary studies of fox, pine marten or domestic cat (e.g. Constable, 2003; McCann, 2005; Warner & O'Sullivan, 1982). Therefore, the lack of correlation between habitat type and tail colour is understandable, as within Ireland, predation risk as a microevolutionary force behind tail colour is unlikely.

The lack of correlation between tail colour and any of the genetic measurements is interesting but lack of association between pelage and molecular markers has been detected in other species (e.g. pocket mice, *Chaeotodipus intermedius*; Hoekstra *et al.*, 2004). It also explains the contradictory results described through genetic work carried out by Barratt *et al.* (1999) and morphological work by Kitchener *et al.* (2003) on the same red squirrel populations in Britain. Also, Hale & Lurz (2003) and Hale *et al.* (2001a) described the genetics and morphology respectively in the same populations in northern England and found that at the microsatellite level, the single population which had the light colour morph was not significantly genetically divergent from other populations.

Due to the lack of correlation between any of the other variables and tail colour it is unclear what, if any, evolutionary pressures could have a) initially led to the existence of the light colour morph in the Irish and British red squirrel populations, resulting in its description as a subspecies and b) retained this colour morph in the Irish population in almost equal ratios to darker tails. Regarding the first, as already mentioned, darker colour

morphs have better thermoregulation (Ducharme *et al.*, 1989; Voipio & Hissa, 1970) but, within Ireland, selective pressures against a light tail with respect to climate, which may be apparent elsewhere, is probably not a factor. Therefore the occurrence of the light colour morph only in the British Isles may simply be a product of a small founder effect combined with no selective pressure against that colour morph, allowing the morph to persist.

In the temporal analysis of data, although the shape of the skull was slightly different between museum and contemporary samples, with contemporary samples having shorter snouts than those in museums, this difference was not significant and the small samples sizes make any conclusions tenuous. Nevertheless, the validity of the results is supported by the fact that it was the size of the snout which was varying between data sets. The study in Britain found the length of the snout, relative to the overall length of the skull, to be the main discriminating factor between populations (Hale & Lurz, 2003). The Irish red squirrel population may be undergoing a change from a low palate length:frontal length ratio in the museum samples to a high ratio in the contemporary samples, which may be preliminary evidence for microevolution in the red squirrel population in Ireland.

As previously outlined, research has suggested a competitive advantage of grey squirrels over reds in sites where broadleaf species are present, and vice versa in conifer sites, where red squirrels smaller body sizes allow it to avail of food on slender upper branches which the grey squirrel cannot access (Gurnell *et al.*, 2004; Wauters *et al.*, 2004). Therefore, the spread of the grey squirrel in Britain and Ireland, may be resulting in a selective pressure of smaller body size of red squirrels in order to decrease niche overlap between the species. This is evident from Tables 5.3 and 5.6 where a clear decrease in overall skull length (condylobasal length), with Irish museum specimens > Contemporary Irish samples > British samples, is apparent, which inversely relates to the length of time over which both the red and grey squirrel were present in both countries. However, it is more likely, as no red squirrels sampled in this study were living in sympatry with grey squirrels, that this decrease in size has been driven by the increase in conifer planting throughout Ireland in the last 50 years, selecting for smaller squirrels which can better exploit food resources in coniferous sites. In addition, as already discussed, genetic diversity and correlated body size are greater in mixed sites. Therefore, the loss of broadleaf species across Ireland may have dramatically reduced both genetic diversity and morphological diversity in the Irish red squirrel population. It will be interesting to see whether genetic diversity increases with the recent increase in broadleaf species in state forest plantations (Coillte, 2005).

These results suggest that the grey squirrel may not be just threatening the distribution of the red squirrel, but may more subtly, through forcing range retraction into coniferous forests, be having significant effects on the morphological and correlated genetic diversity of the Eurasian red squirrel.

Conversely, the lack of difference between the occurrence of light colour morphs in the contemporary and museum samples support the apparent lack of selective pressure influencing this morphological characteristic, as discussed above. Why the light tail colour has virtually disappeared from the British population (Hale & Lurz, 2003), is unclear.

Finally, worth noting, is the entire lack of correlation between mtDNA diversity and any of the morphological traits. Hale *et al.* (2004) suggested the rapid spread of an introduced Swedish haplotype may be due to this haplotype being associated with an adaptive advantage in the conifer plantations. From the results from this study it seems that this is not the case, and, with respect to the morphological characteristics measured in this study, no evidence of a correlation between mtDNA and adaptive variation to a particular habitat type was detected. Whether there is absolutely no correlation between haplotypes and survival in a specific habitat type could only be ascertained through detailed phenotypic investigation. However, with respect to the environmental variables in this study, mtDNA appears to be a neutral marker, and the mechanisms and reasons behind the increasing dominance of haplotype H1 in the British population is unknown, but are likely related to energetics of the red squirrel, given the role of the mitochondria in the cell. Further work, looking at correlations between this haplotype and morphological and/or biochemical responses related to energetics, may yield more answers.

5.4.2. Subspecific status of the Irish red squirrel

S.v.leucourus is characterised primarily on the basis of tail colour, the 'whitening' of the tail, as it is morphologically similar to *S.v.vulgaris*, described for much of mainland Europe, except the British Isles, Iberia and Scandinavia (Sidorowicz, 1971), and *S.v.fuscoater*, the Scandinavian subspecies (Lowe & Gardiner, 1983). The cranial measurements taken in this study show largely that the Irish red squirrel skull is similar to that of both British specimens, and those described for other subspecies of red squirrel (Table 5.6), although there was a slight difference between Irish and museum specimens which has already been discussed.

Sidorowicz (1971) detected a pattern of larger skull size associated with subspecies found in broadleaf or mixed conifer broadleaf habitats (e.g. *S.v.infuscatus* and *S.v.fuscoater*) in comparison to those common in coniferous habitats (*S.v.vulgaris*), with transitional subspecies occurring between those major habitat types (*S.v.bashkiricus* and *S.v.argenteus*). Therefore, when the woodland history of both Ireland and Britain are taken into account, a larger skull size might be expected for *S.v.leucourus*. The data from Britain collected by Hale & Lurz (2003) and Sidorowicz (1971) did not support this, with the subspecies having a smaller skull size than all others except the other island population, *S.v.lis*. Sidorowicz (1971) attributed this smaller skull size in the two island populations, to the milder climate on the two islands. However, the inclusion of the Irish data collected in this study disagreed with that recorded from Britain, with condylobasal length in the Irish samples towards the higher end of the range of data collected from the species, and may indeed further support the larger skull size in mixed or broadleaf dominated habitats as previously discussed.

The defining characteristic of the *S.v.leucourus* subspecies is tail colour rather than cranial divergence (Lowe & Gardiner, 1983; Sidorowicz, 1971). Although that statement was largely supported by the data collected in this study a slight difference was detected in the skulls measured from Ireland. The Irish skulls had a relatively narrow skull when compared to their length and also had relatively short snouts in comparison to overall skull length (Table 5.6). This suggests that the squirrels found in Ireland may be morphologically distinct from those found elsewhere although a larger number of samples would be needed to confirm this.

On the basis of coat colour alone, the Irish red squirrel population could be considered a separate subspecies. Certainly the light colour morph is far more dominant in the Irish population than in the British (Hale & Lurz, 2003), and the stability of the colour morph frequencies over time indicate that there is no danger of it disappearing in Ireland as a result of selective pressures or competition. The lack of agreement between the colour polymorphism and cranial measurements was also described in Britain (Hale & Lurz, 2003) and in the *S.vulgaris* species as a whole (Sidorowicz, 1971), and merely supports the fact that cranial variation is not a distinguishing feature of the subspecies and possibly reflects introgression or differentiation between populations as detected elsewhere (Hale and Lurz, 2003) or simply different selection pressures, as previously discussed.

Lastly, the cranial measurements gave few clues to the origin of the Irish population. Skull width in Ireland was similar to that recorded from *S.v.lis* in Japan. Skull length was most similar to those of the specimens collected from Spain, while snout length was similar to British and Scandinavian individuals, but overall, no other specimens from which data was available had a similar overall skull shape to those from Ireland. Without further sampling throughout Europe, the use of cranial morphometrics to determine the origin of the Irish red squirrel population and divergence between red squirrel populations and/or subspecies is not possible. However, the difficulties of obtaining samples from this species, especially given its protected nature throughout the British Isles and its status as an increasing conservation priority in mainland Europe, suggest that it is non-invasive sampling, leading to molecular analysis, which will be of importance in defining evolutionary significant units for conservation of the species.

5.4.3. Conclusions

This part of the study has shown that habitat influences red squirrel body size and genetic diversity, and therefore as a habitat specialist species, the potential for microevolution of the red squirrel in response to habitat variation is high. Changes in forestry practices and increasing restriction of red squirrel in coniferous sites may have, and be, selecting for smaller body size in red squirrel, and a correlated reduction in genetic diversity.

The high proportion of light tailed individuals distinguished the Irish red squirrel population as *S.v.leucourus*. The selective pressures, if any, behind tail colour polymorphism are unclear, correlating neither with body size nor genetic data. Further work could focus on a more widespread investigation of correlates between morphological and genetic traits to determine whether this is an Irish or an island phenomenon. Also, whether the tail colour follows an annual pattern, or varies from year to year in response to habitat, environmental etc conditions could reveal more on the selective influences which govern the existence and persistence of this morph in the Irish population.

Chapter 6: General discussion and further work

6.1. General discussion

The primary aim of this investigation was to increase understanding of the factors which have shaped, and are shaping, the current range, and distribution of genetic and morphological diversity, of the Eurasian red squirrel. It was found that both past and current biogeography have been intrinsic in determining the extent of both species distribution, and distribution of diversity within the species, however, anthropogenic effects of habitat fragmentation and alteration, and possibly introduced competitors, had a marked effect, and it will be these factors, rather than natural topographical features, which will be fundamental in determining both distribution, and microevolution to local environments, in the species in the future.

Although the limited sampling within Europe made any conclusions relating to the postglacial spread of the species tenuous, the reanalysis of the published literature carried out in this study, suggests that, in mainland Europe at least, the red squirrel could be a useful species through which to track the postglacial spread of forests. Also, what may also be important is that fact that red squirrels need relatively large tracts of forests (in comparison to bank voles etc.) to maintain a viable population. Therefore, phylogeographical work on the red squirrel may reveal evidence of refugia, at higher latitudes than the Mediterranean, which not only had forests, as has been found in other phylogenetic studies (Bilton *et al.*, 1998; Deffontaine *et al.*, 2005; Kotlík *et al.*, 2006; Schönswetter *et al.*, 2005), but in fact had substantial forests, large enough to maintain a red squirrel population.

Unfortunately, in the British Isles, although the red squirrel could be an important species in determining the origin of fauna, and also the spread of forests throughout the islands, the numerous translocations both into, and between, the two islands, has resulted in a phylogeographical pattern of the species on the two islands which reflects recent human interference, rather than natural postglacial spread, or prehistoric human introductions. However, the haplotype network revealed a few tantalising hints as to the origin of the red squirrel in both islands. In both Britain and Ireland there appeared to be a 'Lusitanian' association, with some haplotypes from both countries being mutationally similar to haplotypes from the Iberian Peninsula, supporting a colonisation pattern which has been found through work on both mammals (Corbet, 1961; Piertney *et al.*, 2005; Yalden, 1982) and trees (Mitchell, 2006; Petit *et al.*, 2002). Also, there were similarities between British,

Irish, and western European haplotypes, which also suggest a colonisation event, possibly via a landbridge, from mainland Europe. On the whole, regrettably, it seems that, due to the spread of translocated haplotypes in both Britain and Ireland, the original source(s) and/or colonisation routes of the red squirrel into the British Isles, will never be known.

The population genetic analysis confirmed that, although geographic distance is a factor in genetic differentiation between populations, at a more local scale habitat fragmentation significantly affects gene flow between red squirrel populations with all barriers used in the analysis having a cumulative positive effect on the amount of differentiation between populations, thereby explaining the high levels of genetic structure (Trizio *et al.*, 2005), and possibly the large variations in coat colour (Sidorowicz, 1971), which have been described within relatively small geographic areas in other studies.

This first landscape genetic analysis of gene flow between red squirrel populations confirmed results which have been found elsewhere. Research on both wide ranging, and more habitat generalist, species, has revealed the significant effect that landscape features, like areas of unfavourable habitat, have on shaping the gene flow and dispersal patterns between natural populations (e.g. Bockelman *et al.*, 2003; Coulon *et al.*, 2006; Geffen *et al.*, 2004). Increasingly, the significant effect of recent habitat fragmentation by roads is being revealed by both radiotracking and molecular investigations; a relationship which was also found as significant in this study. Research on ground beetles (Keller & Largiadèr, 2003; Keller *et al.*, 2004), bank voles (Gerlach & Musolf, 2000) and large carnivores (Riley *et al.*, 2003, 2006), have found through examination of rapidly mutating markers the decreased gene flow caused by roads over even a short length of time. While traditional radiotracking and behavioural observations (Clarke *et al.*, 1998; Develey & Stouffer, 2001), and combinations of both molecular and ecological methods (Riley *et al.*, 2006), are also being used to fully understand the influence of this anthropogenic factor on fragmenting wildlife populations.

Overall, these results mean that the red squirrel is a species which would be susceptible to the deleterious effects of habitat fragmentation. Although no evidence for decreased diversity in fragmented habitat was found in either this or other studies (e.g. Todd, 2000a; Wauters *et al.*, 1994), it is logical that this reduced gene flow between populations will eventually correlate with increased inbreeding, and possibly a lack of adaptive genetic diversity (David, 1998; Ledig *et al.*, 1993; Mitton & Grant, 1984). Numerous studies have

reported correlations between reduced genetic diversity and bottleneck events in wildlife population, as can occur in fragmented landscapes. Randi *et al.* (2000) found that in a study sample of 101 wolves, *Canis lupus*, in Europe, a population which was reduced to 100 individuals isolated in 10 separate areas across Europe in the 1970's, a single mtDNA haplotype was fixed within the study sample. Extreme loss of genetic diversity in the species has also been reported as a consequence of culling (Leonard *et al.*, 2005), and habitat fragmentation and hybridisation with other canids (Wayne *et al.*, 1992). Similar effects of demographic bottlenecks on genetic variability have been reported in lynx, *Lynx lynx* (Hellborg *et al.*, 2002) and elephant seal, *Mirounga angustirostrus* (Hoelzel, 1999) while Keller & Largiadèr (2003) detected lower levels of genetic variation in populations of ground beetles, *Carabus violaceus*, which occurred in smaller forests fragments, isolated for approximately 30 years.

However, a question which has been of issue is whether genetic variation at so called 'neutral' markers, which are not under strong selective pressure, is a reflection of molecular diversity which may be correlated with fitness. An investigation of brown trout, *Salmo trutta*, found a strong correlation between the MHC gene, one which is strongly under the influence of selection, and microsatellite variation (Campos *et al.*, 2006). Research investigating associations between genetic variation and phenotypic traits associated with fitness have also found correlations. Reed & Frankham (2003) found a strong correlation between population growth rate and fecundity, and genetic diversity. Hoelzel (1999) found increased asymmetry in elephant seal cranial measurements correlated with decreased genetic diversity. Wisely *et al.* (2002) found reduced skull and overall body size in a captive bred population (n = 3000) of black footed ferret, *Mustela nigripes*, which derived from only 7 individual animals. The results from this study on red squirrels also revealed a correlation between genetic diversity and a trait which reflects fitness, with a body weight, in relation to body size, higher in individuals which had higher genetic diversity. All of these results show therefore, as proposed by Reed & Frankham (2003), 'neutral' genetic markers can seem to reflect variability at genes which are under selection and are of use in investigations of the effects of demographic bottlenecks on fitness of populations.

This investigation also revealed that mixed sites may harbour important pools of diversity within the red squirrel species. It could be possible that this indicates adaptive microevolution of red squirrels in Ireland to forests which have some element of broadleaf

species in them, as, presumably, this was the forest type in which the red squirrel in the British Isles would have existed in since its colonisation of the islands. Local scale adaptation to environments has been found elsewhere. Research has found habitat composition to be a significant factor in genetic differentiation between populations (e.g. *Elymus athericus*, Bockelmann *et al.*, 2003; *Canis lupus*, Geffen *et al.*, 2004, Pilot *et al.*, 2006) However, likewise, the results could be interpreted as a combination of the ecological factors which red squirrels in mixed sites experience in comparison to those in conifer sites (i.e. more food resources), and further research into these factors, will allow more definite conclusions to be made. Nevertheless, this study has shown that habitat composition can have an effect on the genetic diversity of a red squirrel population and this will have to be taken into account in both further population genetic, and phylogenetic, investigations, of the species.

At a wider geographic perspective, the insularity of a red squirrel population seems to influence diversity and structure within the species, with peripheral populations indeed showing the increased genetic structure but, conversely, not the decreased diversity described for marginal populations (Gapare & Aitken, 2005; Eckstein *et al.*, 2006; Faugeron *et al.*, 2004; Lammi *et al.*, 1999; Li & Adams, 1989). In fact, levels of diversity within the two marginal populations in this study were higher than those found elsewhere. The reason for this pattern of diversity is unclear. It may simply be a remnant of the widespread sampling carried out in the two marginal populations in comparison to the sampling range elsewhere. However, it could also be a result of the relative isolation of the two marginal populations; both also island populations, which has resulted in little gene flow in comparison to mainland European populations. Antunes *et al.* (2006) found that peripheral populations of brown trout had higher levels of genetic diversity than populations sampled elsewhere in their study, attributing this to the longer demographic stability of populations in the marginal region. A similar situation may be occurring in Ireland and Britain, with the relative isolation over a longer period of time allowing the populations to accumulate adaptive genetic diversity.

A final explanation may be a combination of the postglacial colonisation routes of red squirrels in Europe and the history of the species on both islands. Although generally increased diversity in populations sampled in glacial refugia is expected (Hewitt, 1996, 2000, 2004), Petit *et al.* (2003) found higher levels of diversity in areas of where introgression between genetic lineages from separate refugia occurred, rather than in the

refugia themselves. The haplotype network constructed in this analysis found close associations between many of the mainland European populations and those in Britain and Ireland which may suggest a number of different postglacial colonisation routes of the islands, which may mean the high genetic diversity is caused by introgression between these different lineages. When this is combined with the numerous artificial movements of red squirrels from Europe into Britain (Hale *et al.*, 2004) and between Ireland and Britain, high amounts of genetic variation in these two peripheral island populations would be expected. Clearly more studies incorporating populations from elsewhere within red squirrel range is needed to fully understand the observed patterns of diversity.

Nevertheless, the analyses of red squirrel populations carried out so far, combined with the morphological analysis of tail colour also carried out in this study, and the results described by Hale & Lurz (2003) suggest that the peripheral red squirrel populations contain levels of diversity which are important to species conservation as a whole. Furthermore, this increased diversity, combined with the isolated nature of the Irish population, means this diversity may be correlated with adaptive traits in specific habitat types, which could be important in the future in the face of global climate change (Alleume-Benharira *et al.*, 2005; Guo *et al.*, 2005; Hampe & Petit, 2005; Lesica & Allendorf, 1995).

A secondary, and more localised, aim of this study was to increase the understanding of the origin of the Irish red squirrel population, and investigate any patterns of diversity within the population which can be taken into account in conservation of the species in Ireland. This investigation found evidence that the red squirrel can be considered a native Irish species (i.e. is not solely derived from the British translocations) and therefore has answered the question which caused much debate in the early 1900's (Barrington, 1880; Moffart, 1923b; Scharff 1922, 1923).

Although the analysis found evidence to support all of the suggested faunal colonisation events of Ireland, support for the through glacial persistence of the species in Ireland was tenuous, and no evidence for a refugium in the southwest was found. To date genetic analysis of species in Ireland (see Table 1.1.) has found that only two, the hare, *Lepus timidus* (Hamill *et al.*, 2006) and stoat, *Mustela erminea* (N. Martínková pers comm.), were significantly divergent to both support their persistence in a glacial refugium in Ireland, and to describe them as separate subspecies. Both of these species are tundral

adapted and therefore their survival in a refugium in Ireland is not implausible. However, after the phylogenetic analysis of the red squirrel carried out in this study, survival of more temperate species in that area remains uncertain. However, it is probably unlikely, as considerable evidence exists to refute the persistence of temperate conditions in Ireland through the last glacial maximum (Coxon, 2005)

Nevertheless, although the Irish population is largely a product of the translocations from Britain, and there were a number of haplotypes shared between regions, significant regional genetic and morphological structure did exist in the Irish red squirrel population and the eastern region was more diverse, both genetically and morphologically, than other regions in Ireland. This high diversity in the east could be explained by that region containing individuals from a number of different founder events. As already discussed, the eastern region had haplotypes which were strongly associated with some found on mainland Europe, while both the west and southwest had possible remnant haplotypes, although these were present at extremely low frequencies within the populations. However, in comparison to other regions, the eastern region was subject to a larger number of translocations than other regions. Likewise, the geographical proximity of the central translocation points to the eastern region mean that the higher diversity in the east, in comparison to other regions, may simply reflect that region consisting of individuals which are a result of introgression among descendents from a number of different translocation points.

It must also be remembered that, unlike in other regions, the haplotypes which were predominant in the eastern region were those which were most strongly associated with mainland Europe. If these haplotypes represent a more distant colonisation event of that region, it is possible that the higher diversity in that region is a product of a longer time period during which to accumulate adaptive traits to local conditions, similar to that found in the results on brown trout previously described (Antunes *et al.*, 2006), and further sampling in Ireland may confirm this.

Finally, from a conservation and management point of view is the Irish red squirrel population an 'Evolutionarily Significant Unit' (ESU) (Moritz, 1994a, b; Ryder, 1986) or a 'Management Unit' (MU) (Mortiz, 1994a)? Moritz (1994a) defined ESUs as 'reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies. Although others support the criteria that all geographically divergent populations should be

preserved (Lesica & Allendorf, 1994), in most cases this approach is impractical (Moritz, 1994a). Conversely, MU do not need to be phylogenetically distinct, but are populations which are significantly divergent from others at both nuclear and mitochondrial loci (Moritz, 1994a).

From the genetic results from this study, there was no pattern of the mtDNA haplotypes found in Ireland being reciprocally monophyletic; therefore, following Moritz's (1994a) criteria, it seems that the Irish red squirrel population as a whole is not an ESU. However, the Irish population, and indeed each region within Ireland, do meet the criteria for MU and are therefore each valuable for conservation, and should be preserved separately to maintain the genetic structure within Ireland, and the patterns of geographic divergence, and adaptive habitat diversity detected in this study.

Although Moritz (1994a) did not consider that phenotypic diversity should be considered as part of the ESU definition, earlier definitions (Dizon *et al.*, 1992; Ryder, 1986; Vogler *et al.*, 1993) considered both molecular and non molecular divergence important. Therefore, given the widespread distribution of the light tail colour in Ireland, a morphological trait which has not been described elsewhere in the Eurasian red squirrel species (Sidorowicz, 1971), and which has relatively disappeared from Britain (Hale & Lurz, 2003), the Irish red squirrel might be considered an ESU, or even a separate subspecies. Whether the Irish red squirrel population is an ESU or an MU, it does represent significant morphological and genetic diversity which is not found elsewhere within Eurasian red squirrel range, and has been lost from the British population (Barratt *et al.*, 1999; Hale & Lurz, 2003; Hale *et al.*, 2004) and is therefore valuable to conserve.

In summary, this investigation has provided insights into the factors which have, and are, shaping distribution, diversity and insularity of the Eurasian red squirrel as a species, and also, at a more localised scale, the Irish red squirrel population. It has revealed that the Eurasian red squirrel is a species which is particularly susceptible to habitat fragmentation, but also can respond rapidly to habitat change. This can be used both to assess large scale effects of climate change on species, and also the effectiveness of conservation strategies and habitat management, on the species, within short time scales. It has also highlighted the effectiveness of, and insights gained through, addressing questions of both ecology and genetics through simultaneous analysis of landscape, genetic and phenotypic variation, and

subsequent investigations of any correlations between the data sets, an approach, which, before now, had not been used in the study of the Eurasian red squirrel species.

6.2. Implications for conservation

This investigation has produced a number of results which may be of use in both conserving the red squirrel as a species, particularly in Europe, where the introduced grey squirrel is threatening distribution, and in conservation of the species in Ireland.

Most notably was the statistical support for the effect that barriers to dispersal have on gene flow between red squirrel populations. Although, as a habitat specialist, these barriers are logical factors in the distribution of genetic diversity in the species, and other research has shown the effect that discontinuous habitat can have on a red squirrel population (Hale *et al.*, 2001a), this study has revealed the important effect that habitat fragmentation by roads has on gene flow. The existence of these barriers should be taken into account where habitat patches are being used to either join distinct red squirrel populations, or manage a number of small discrete metapopulations in habitat fragments, as a single population. Public relation actions, and to a lesser extent conservation action, on the island of Jersey and in northern England has resulted in the connection of red squirrel habitat, fragmented by roads, by rope bridges. Although these were primarily erected to decrease the extent of red squirrel roadkill in these areas, this research has found that they may be extremely important corridors to gene flow between red squirrel populations.

The results from the mixed sites imply that mixed conifer/broadleaf sites are important sinks of diversity of the red squirrel. Biodiversity conservation in Ireland has increased the levels of broadleaf planting in conifer plantations and this has been argued as aiding the replacement of the red squirrel by the grey (Hamilton, 2006; Reilly, 1997). However, although large seed species provide grey squirrels with a foothold in a forest, this advantage is not as apparent when small seeded species are planted. Therefore planting of small seeded broadleaf species in red squirrel habitat may aid in the maintenance of genetic and morphological diversity in the species.

The mitochondrial analysis did not find marked geographical structuring within Europe; however most haplotypes were region specific, meaning, if translocations were to occur these should be within, rather than between, these regions. The exception was Ireland and Britain, where, although no haplotypes were shared between the two islands, there was a strong association between Ireland and Britain, and, in fact, Ireland has some haplotypes which have since become extinct in Britain. Therefore, in the future, if augmentation of the British red squirrel population is needed, the Irish population could act as a source population. Also, from a British point of view, the conservation of the Irish red squirrel population is important, particularly in the face of the rapid spread of introduced European red squirrels across Britain (Hale *et al.*, 2004), as it contains genetic diversity which appears to have disappeared from the British population.

Within Ireland, although a large number of haplotypes were shared between populations there was significant regional structure, and this should be preserved if translocations are taking place. At a national scale the Irish red squirrel population includes high levels of both genetic and morphological diversity which warrants conservation. As all regions had at least one haplotype which was not considered a translocated British haplotype, all equally, should be conserved, but, the eastern population, with its high levels of diversity, and possible Lusitanian and western European haplotypes, and more imminent threat of replacement due to grey squirrel spread, may warrant more urgent conservation measures than others. Lastly, as it appears that Irish red squirrel population is the sole representative of *S.v.leucourus* in Europe, although this is not correlated with any genetic distinctiveness, it does represent considerable morphological diversity of the species which should also be conserved.

6.3. Further work

The analysis of red squirrels in mixed sites in Ireland was preliminary and further investigations of populations within these site types, will give further insights as to the value of these sites to the species. Also, the initial sampling was difficult due to the lack of information on current red squirrel status within Ireland and a more updated distribution survey of the species in Ireland is needed.

The phylogeographical work in this study was difficult to interpret due to the large unsampled areas of Europe. A detailed phylogeographical study of the red squirrel in mainland Europe, and possibly into Asia, will reveal more about the postglacial expansion of both the species, and forests, and may reveal further evidence for central European glacial refugia. Regarding colonisation of the British Isles, other species, which have not been subject to such high levels of recent human interference will have be studied to determine the patterns and/or methods of faunal colonisation of the islands.

Although the landscape genetics did find associations between genetic structure and barriers to dispersal, this was based on a relatively small number of populations and more thorough analysis, on a more localised scale, possibly incorporating GIS analysis, will provide data on not just the factors which govern red squirrel population structure, but also the suitability of specific habitats and/or habitat mosaics for establishment of red squirrel populations, and the subsequent maintenance of genetic diversity.

Though the various factors which drive red squirrel population differentiation and gene flow are of interest, perhaps, more significant from a red squirrel conservation point of view, would be a similar study carried out on grey squirrel populations. Conservation action in Britain and Northern Ireland are now focusing on habitat management to conserve the red squirrel, creating buffer zones between red and grey squirrel populations and planting red squirrel favourable tree species. Landscape genetic analysis of the grey squirrel, similar to that which was carried out here, will provide data on the usefulness of specific forests as red squirrel refugia, and the measures and/or habitat designs which will most effectively conserve red squirrel populations in the face of continual grey squirrel spread.

A final interesting phenomenon which could be investigated in more detail is the selective pressures behind small body size in red squirrels. Whether this is associated with habitat type could perhaps be solved by collection of more data from mixed forests, combined with continual monitoring of red squirrel populations in response to habitat alteration, like that currently occurring in Ireland. Whether red squirrel microevolution is being shaped by competition with the grey squirrel due to niche partitioning may only be able to be answered in future, if the two species manage to coexist.

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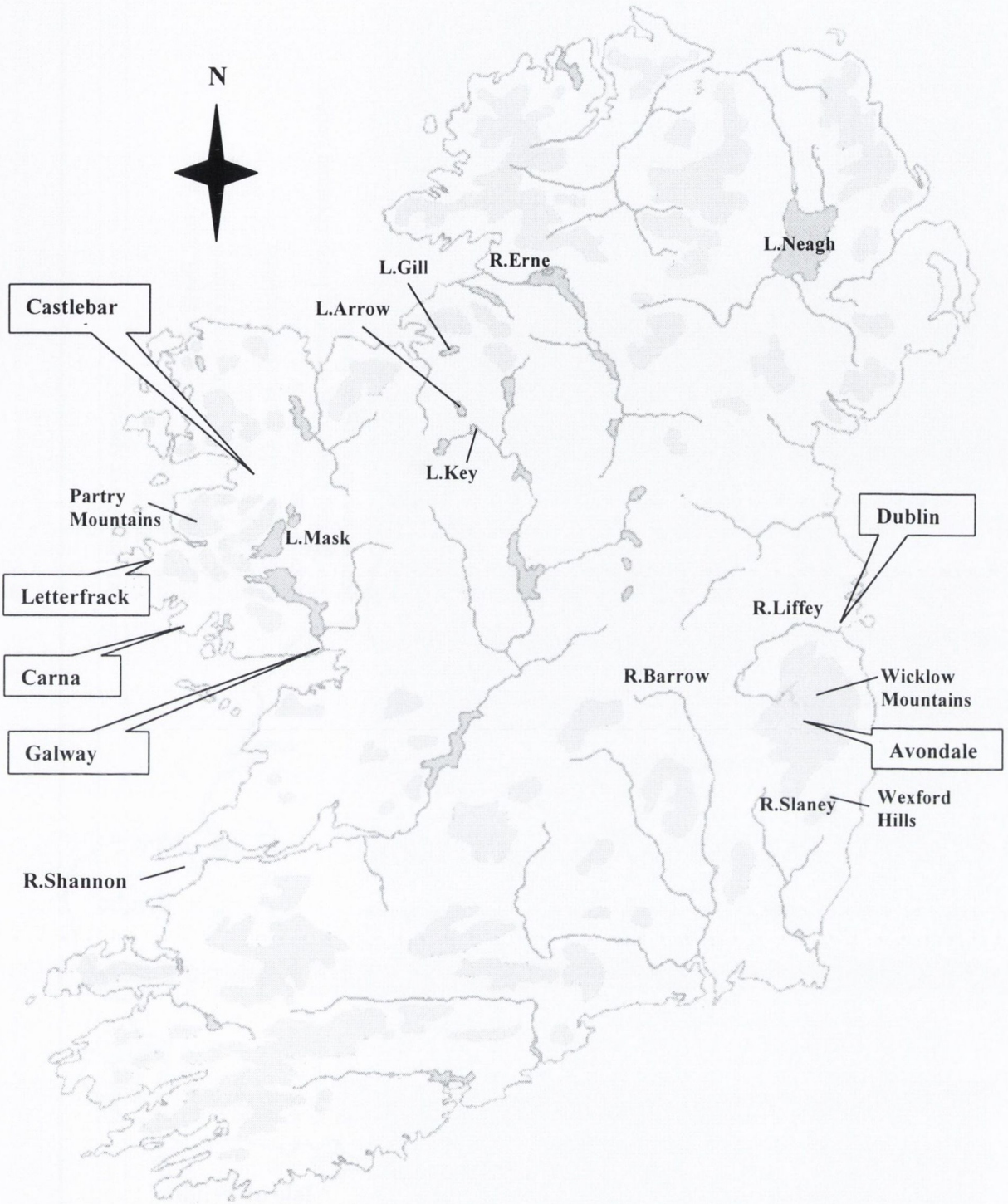
8.1.1. Map of Ireland showing the 32 counties of Ireland, the provinces into which they fall (Leinster, Munster, Connaught and Ulster) and the Northern Irish border.



8.1.2. Map showing the location of major rivers, lakes and mountain ranges in Ireland. Features and locations referred to in the text are named.

8.1.3. Map of Britain showing the areas referred to in the text.

8.2. Site descriptions

County	Site	Grid Ref	Age	Dominant tree species
Clare	Cratloe	R490600	Old: 1st Ed OS 1839	Sitka spruce
	Lakeside (CE)	R720863	Old: 1st Ed OS 1839	Sitka spruce, oak, beech
	Raheen	R650830	New	Sitka spruce, Scot's pine, larch, beech, holly, oak
	Violet Hill	R570750	Old: 1st Ed OS 1839	Sitka spruce, beech, birch, Scot's pine
	Woodpark	R690870	New	Sitka spruce, beech, oak
Cork	Castlemartyr	W960720	Old: 1st Ed OS 1839	Scot's pine, beech, birch, rhododendron
	Corrin (Cor)	W825965	New	Sitka spruce, Douglas fir
	Curraghbinny	W760620	New	Scot's pine, beech, oak, sycamore, horse chestnut, juniper
	Glenbower	W990780	New	Scot's pine, sitka spruce, beech
	Rostellan	W870660	Old: 1st Ed OS 1839	Scot's pine, oak, beech
Galway	Garryland	M420030	Old: 1st Ed OS 1839, old estate	Scot's pine, sitka spruce, oak, ash
	Kilcoran	M420195	New	Sitka spruce
	Mountbellew	M660460	New	Scot's pine, sitka spruce
	Portumna	M830030	Old: 1st Ed OS 1839	Scot's pine
Kerry	Kilderry	V810005	New	Sitka spruce, beech, oak
	KNP	V940860	Old: 1st Ed OS 1839, old estate	Oak, holly, ash, Scot's pine
Sligo	Glenwood	G618273	New	Sitka spruce
	Hazelwood	G723345	Old: 1st Ed OS 1839, 1600 maps	Oak, ash, holly, Scot's pine
Waterford	Colligan	X205970	New	Sitka spruce, larch
	Lismore	X020998	Old: 1st Ed OS 1839, old estate	Scot's pine, beech, birch
	Portlaw	S460140	Old: 1st Ed OS 1839	Sitka spruce
	Stradbally	X365982	Old: 1st Ed OS 1839	Beech, Scot's pine
Wexford	Camolin (Cam)	T050550	Old: 1st Ed OS 1839	Beech, Scot's pine
Wicklow	Glendalough (Glen)	T115975	Old: 1st Ed OS 1839	Sitka spruce, Scot's pine, beech
	Kindelstown	O270125	New	Beech, sitka spruce, holly
	Laragh (Lar)	T150975	New	Sitka spruce
	Trooperstown	T162962	New	Sitka spruce, Scot's pine

8.3. Individual level microsatellite, for each of the 16 primers, and haplotype data.

	3	4	6	18	23	31	1	10	13	14	19	r1	r3	r4	r5	r6	MtDNA
KNP 1	180	215	195	242	161	187	186	74	171	200	202	184	163	276	137	125	SW+W+E
	184	215	195	254	161	193	186	78	171	202	202	192	163	276	137	128	
KNP 2	178	213	195	254	157	191	190	76	171	196	202	184	163	272	137	125	SW+W+E
	182	213	195	254	159	191	192	78	171	196	204	192	165	272	139	125	
KNP 3	180	215	193	254	157	187	186	74	171	198	198	184	165	272	137	128	
	206	215	193	254	159	187	186	78	171	202	198	192	165	276	137	128	
KNP 5	178	213	195	254	157	191	190	76	171	196	198	192	163	276	137	125	IRL
	182	213	195	254	157	191	192	80	173	196	198	192	165	276	139	125	
KNP 6	176	213	197	254	157	193	186	80	171	198	198	192	165	276	137	122	IRL
	176	215	199	254	157	193	188	80	173	200	202	192	167	276	137	122	
KNP 7	178	215	193	258	157	193	186	80	173	198	198	184	165	276	137	125	IRL
	180	215	195	258	157	193	188	80	173	200	202	184	167	276	137	125	
KNP A	178	215	193	258	153	191	188	78	167	192	198	184	163	276	137	128	SW2
	180	215	195	258	155	191	188	78	167	192	202	184	163	276	137	128	
KNP B	180	215	193	242	155	191	186	76	173	192	198	184	163	276	135	128	IRL
	180	215	195	258	155	191	188	78	181	194	202	184	165	276	135	128	
KNP C	176	215	197	258	155	191	186	76	173	194	202	192	165	276	135	128	SW3
	180	215	197	258	155	191	188	78	181	194	202	192	165	276	135	128	
KNP D	178	215	197	258	153	189	186	78	173	198	202	184	165	268	135	128	IRL
	198	215	197	258	155	191	188	80	181	198	202	184	165	276	139	128	
KNP E	176	215	195	258	157	191	186	78	173	198	198	184	163	276	135	128	SW1
	180	215	197	258	157	191	188	78	173	198	202	184	163	276	139	128	
KNP F	176	215	195	254	155	191	188	78	171	200	198	192	165	276	139	125	IRL
	176	215	197	258	155	191	188	78	171	200	202	192	165	276	139	128	
KNP G	176	215	195	258	155	195	188	78	173	194	202	184	163	276	139	128	IRL
	176	215	197	258	155	197	188	78	173	194	202	192	163	276	139	128	
LK 1	180	215	195	254	155	193	182	70	181	198	200	184	165	272	135	128	W1
	180	215	195	254	157	197	182	78	181	200	202	184	167	276	141	128	
Cor 1	176	215	195	242	157	187	182	78	171	198	200	184	165	272	139	125	
	176	215	199	258	157	187	184	78	181	200	202	192	165	272	139	125	
Cor 2	176	213	195	256	155	187	182	74	171	196	202	180	163	276	129	125	IRL
	176	215	199	258	157	187	184	78	181	200	202	180	165	276	129	125	
Cor 3	176	213	197	242	155	187	182	78	181	196	198	180	163	272	137	125	S1
	180	215	199	242	157	187	184	80	181	200	200	180	163	272	139	125	
Cor 4	176	215	197	242	153	187	180	80	171	196	198	184	165	276	137	125	S1
	176	215	197	258	153	189	182	80	171	198	200	192	165	276	139	125	
Cor 5	176	213	195	242	153	191	182	80	171	196	200	184	165	276	135	125	S1
	176	215	197	258	161	191	184	80	181	196	200	192	165	276	141	125	
Cor 6	176	213	197	242	155	191	184	78	171	196	200	184	165	268	135	125	S1
	176	213	199	258	155	191	184	78	181	196	200	192	165	268	141	125	
Cor 7	176	215	197	258	155	191	180	76	181	196	202	180	163	256	135	125	S1
	180	215	199	258	155	191	180	78	181	196	202	180	165	260	135	125	
Cor 8	176	215	197	242	153	191	178	76	171	196	200	184	165	268	135	125	
	180	215	197	258	161	191	180	80	181	198	200	192	165	268	135	125	
GG 9	200	213	193	240	157	191	186	76	181	202	202	184	165	276	135	125	IRL
	200	213	195	242	159	191	186	76	181	202	200	184	165	276	135	125	
GG 10	180	215	193	254	157	191	188	76	171	200	202	184	165	276	135	128	IRL
	180	215	195	254	159	193	188	76	173	200	202	184	165	276	141	128	
D 1	200	213	197	238	159	191	192	76	171	196	202	184	167	276	135	125	
	200	213	197	254	161	191	192	76	171	198	202	184	167	276	141	128	
NI 1	200	207	193	242	157	193	182	74	181	200	204	180	165	268	135	128	NI1
	200	213	193	242	157	193	182	76	181	200	204	180	169	268	135	128	
NI 2	192	207	193	242	157	181	184	76	181	200	202	176	165	268	135	125	E+N
	200	207	193	242	159	181	184	78	181	202	204	188	165	268	135	128	

	3	4	6	18	23	31	1	10	13	14	19	r1	r3	r4	r5	r6	MtDNA
WD 1	180	215	193	242	161	193	182	74	171	204	200	176	165	272	135	125	S1
	184	215	195	254	161	197	186	78	181	204	202	176	167	276	135	128	
Lar 4	184	215	195	242	161	193	182	74	171	202	202	188	163	268	135	122	IRL
	184	215	195	254	163	193	182	74	181	204	202	188	165	268	135	125	
Lar 5	202	209	193	238	153	187	180	73	181	192	200	180	163	276	137	128	E6
	206	213	195	254	159	187	184	76	181	192	200	180	165	276	139	128	
Lar 6	204	213	193	242	157	181	180	76	181	192	200	184	163	272	137	128	E6
	206	215	195	254	157	181	184	76	181	192	200	184	163	276	137	128	
Lar 1	200	213	195	258	153	183	182	78	171	196	200	180	165	264	133	125	E1
	202	213	195	258	161	183	184	78	181	196	200	188	165	268	135	128	
Lar 2	200	213	195	242	153	181	182	78	171	196	200	188	165	272	133	125	E+N
	200	215	201	258	161	183	182	80	181	196	200	188	165	272	135	128	
Lar 3	206	206	197	242	153	185	184	78	171	194	198	188	163	268	133	125	
	206	206	197	254	161	185	186	80	181	196	200	188	163	268	133	125	
Curt 4	200	213	199	258	161	175	186	80	171	202	202	184	163	272	135	122	E3
	200	213	199	258	161	181	186	80	171	202	202	184	163	272	135	128	
Curt 5	200	213	197	254	153	185	186	80	171	202	202	180	163	272	131	122	E4
	200	213	197	254	153	185	186	80	171	202	202	180	163	272	133	128	
Curt 6	184	213	193	238	153	181	178	80	171	198	198	184	163	272	131	122	E8
	200	213	193	254	157	183	180	80	171	202	200	184	163	272	133	122	
Curt 3	200	213	193	242	157	185	182	74	181	200	202	188	165	272	135	122	E3
	200	215	195	254	163	191	182	78	181	202	202	188	165	276	133	125	
Curt 7	184	213	199	238	159	183	180	78	171	198	202	184	163	272	133	128	SW+W+E
	200	213	199	238	159	183	184	80	171	202	202	184	163	276	133	128	
Curt 8	206	213	193	258	159	183	184	78	181	202	200	184	165	268	133	125	E3
	206	213	195	258	159	183	184	78	181	202	200	184	167	268	133	128	
Curt 9	206	213	197	258	153	185	184	78	171	192	200	188	165	268	133	125	
	206	213	197	258	153	185	184	78	171	192	200	188	165	268	133	128	
Curt 2	206	213	201	254	157	185	182	78	169	196	198	192	163	272	133	125	E2
	206	215	197	254	157	185	182	78	171	198	202	192	163	272	135	128	
Curt 1	202	213	193	254	161	183	186	76	181	196	202	188	165	272	135	128	
	206	213	193	258	161	183	186	76	181	196	202	188	165	272	137	128	
Curt 10	202	207	195	242	151	185	184	78	171	192	202	188	165	272	135	125	IRL
	202	213	197	254	159	185	190	78	181	192	202	188	165	276	135	128	
Glen A	206	213	193	242	153	181	184	74	171	198	202	188	165	264	139	128	E6
	206	215	195	254	155	185	186	78	173	198	202	188	165	268	139	128	
Glen B	202	213	193	242	155	181	184	78	167	198	200	184	163	264	135	125	E+N
	206	215	195	254	157	185	186	78	167	202	202	180	165	264	135	128	
Glen C	206	213	197	240	153	181	182	78	167	198	200	188	163	272	131	125	E6
	206	215	197	242	153	185	184	78	181	202	202	188	163	272	131	128	
Glen D	206	215	195	254	159	185	182	78	167	196	200	180	163	272	131	125	E6
	206	215	197	254	159	187	182	78	167	202	202	180	163	276	131	128	
Glen F	202	215	197	254	153	181	182	74	171	196	198	192	163	276	135	125	E6
	206	215	197	254	161	193	184	78	173	196	200	192	163	276	135	128	
Glen 6	206	207	197	242	159	181	180	70	181	196	202	176	163	272	129	122	E6
	206	213	197	254	151	181	184	76	181	198	202	176	163	276	129	125	
Glen 8	186	213	193	254	153	185	188	78	171	192	198	188	165	276	135	122	E3
	186	215	199	258	159	185	188	78	181	192	202	188	165	276	135	128	
Glen 7	206	213	197	258	153	185	186	78	171	202	202	184	165	268	133	125	
	206	213	197	258	153	185	186	80	181	202	202	184	165	268	133	128	
Cam 1	198	215	197	254	157	185	180	76	171	192	200	180	165	272	133	125	Wxa
	200	215	199	258	157	185	180	76	181	196	200	184	165	272	133	125	
Cam 2	180	213	195	242	157	191	180	76	181	192	198	184	165	272	131	128	
	180	213	201	254	159	191	180	76	183	196	200	184	165	272	131	128	
CE1	182	215	193	242	157	185	184	76	181	200	200	184	163	272	139	125	W11
	182	215	195	254	161	185	184	76	181	200	200	184	163	272	139	125	

	3	4	6	18	23	31	1	10	13	14	19	r1	r3	r4	r5	r6	MtDNA
CE2	182	213	195	254	157	181	180	78	171	200	200	184	163	272	131	125	
	182	213	195	254	157	181	180	80	181	200	200	184	165	272	133	125	
GY1	176	213	195	256	153	191	184	76	171	196	184	184	161	272	135	128	IRL
	176	213	195	258	157	191	192	76	181	202	202	188	165	272	135	128	
GY2	176	213	195	256	153	187	184	76	171	196	184	184	165	272	135	128	IRL
	176	215	195	258	153	187	192	76	171	202	202	184	165	272	135	128	
GY3	182	215	197	258	159	175	186	78	171	198	198	180	165	272	135	128	W9
	182	215	197	258	159	175	186	78	171	198	200	180	167	272	135	128	
GY4	182	215	197	254	153	175	186	78	171	196	198	180	165	268	135	128	W11
	182	215	199	258	159	181	186	78	171	198	200	180	165	268	135	128	
GY5	178	215	199	254	159	187	184	76	173	196	198	180	165	268	135	128	W11
	202	215	199	254	159	187	186	78	173	198	200	180	165	268	135	128	
GY6	206	215	199	254	159	185	186	76	173	196	200	184	167	268	131	122	IRL
	180	215	199	254	161	185	188	78	181	196	200	184	167	268	135	128	
GY7	180	213	195	254	153	181	186	78	173	198	198	180	167	268	133	128	W1
	180	215	195	254	159	183	188	78	181	198	200	184	167	268	135	128	
GY8	184	215	199	254	161	179	184	76	173	198	198	180	165	272	135	128	IRL
	184	215	199	258	161	179	184	78	173	198	200	180	165	272	135	128	
GY9	180	215	193	254	157	179	184	78	173	198	200	180	167	272	135	128	W10
	184	215	193	254	157	179	184	78	173	198	200	180	167	272	135	128	
GY10	178	213	199	254	157	179	180	78	173	198	200	180	167	272	135	128	W11
	178	213	199	254	157	181	180	78	173	198	200	184	167	272	135	128	
GY11	178	215	197	258	161	185	184	78	171	198	200	180	165	256	133	128	IRL
	178	215	199	258	161	185	186	78	173	198	202	180	165	272	139	128	
GY12	178	213	197	258	161	175	186	78	171	196	202	180	165	272	141	128	W11
	192	213	197	258	161	175	186	78	171	198	202	176	165	276	141	128	
GY13	176	215	195	258	159	175	186	74	171	196	194	180	165	272	135	128	W11
	180	215	195	258	161	175	188	78	171	198	202	180	167	272	137	128	
GY14	174	213	199	242	157	185	188	78	171	196	202	184	165	272	135	128	
	174	213	199	258	157	185	188	78	171	198	202	184	167	272	137	128	
GY15	174	213	195	258	153	175	186	76	171	198	194	184	165	272	133	128	W11
	176	215	197	258	159	179	186	78	181	200	198	184	165	276	133	128	
GY16	180	211	193	258	157	179	186	78	171	198	202	180	165	272	133	122	IRL
	184	215	193	258	159	179	188	78	171	198	202	184	167	272	137	128	
GY17	176	215	195	242	159	179	180	76	181	198	198	184	165	268	141	128	W8
	176	215	197	258	161	179	184	78	179	198	202	184	165	276	141	128	
GY18	178	211	195	254	161	181	184	78	171	200	198	180	165	276	133	128	W11
	178	215	199	258	161	181	180	78	181	200	202	184	165	276	141	128	
GY19	178	213	199	254	161	193	186	78	171	198	198	184	165	272	139	128	W11
	178	213	199	254	161	195	186	78	171	198	200	184	165	272	139	128	
GY20	200	213	193	254	153	175	184	76	171	198	200	180	163	268	135	128	
	200	213	193	254	159	175	184	78	171	198	200	184	165	272	141	128	
GY21	180	213	199	254	159	175	184	76	171	198	198	184	163	268	139	128	
	180	213	199	254	159	183	184	78	171	198	202	184	165	272	139	128	
GY22	178	213	197	254	159	179	184	78	171	198	200	180	165	272	139	128	W11
	180	215	199	258	159	179	186	78	171	198	200	180	167	272	139	128	
GY23	180	213	199	254	153	183	186	76	167	198	202	184	165	276	141	128	W11
	180	213	199	254	159	183	188	78	167	198	202	184	165	276	141	128	
GY24	176	215	195	254	161	175	184	76	167	196	202	184	165	272	135	128	
	176	215	199	258	161	185	184	78	173	198	202	184	167	272	139	128	
GY25	176	213	195	254	161	175	186	78	173	196	202	180	165	272	135	125	W6
	176	215	195	258	161	175	186	78	173	200	202	184	167	272	139	125	
GY26	176	213	199	254	157	175	182	78	173	196	198	180	165	268	139	128	IRL
	180	213	199	254	157	175	184	78	173	196	198	184	167	272	139	128	
GY27	180	215	199	254	157	175	186	76	173	198	202	180	169	264	137	128	W11
	180	215	199	254	161	181	186	78	173	198	202	180	169	272	137	128	

	3	4	6	18	23	31	1	10	13	14	19	r1	r3	r4	r5	r6	MtDNA
GY28	180	213	197	254	157	173	182	78	173	202	202	184	165	268	137	128	W12
	180	215	199	254	159	173	182	78	173	202	202	184	167	272	137	128	
GY29	180	215	195	258	157	175	182	76	167	198	198	184	169	272	137	128	
	180	215	195	258	157	175	182	76	167	198	202	184	169	272	137	128	
GY30	178	215	197	254	155	181	184	74	167	196	200	188	165	268	137	128	IRL
	178	215	197	254	155	181	184	78	169	198	200	188	165	272	139	128	
GY31	180	213	193	254	155	181	184	76	169	198	184	184	163	272	137	128	W7
	180	215	193	254	155	181	184	78	169	198	200	184	163	272	139	128	
GY32	178	215	195	254	155	179	184	78	169	196	200	184	165	272	141	128	W4
	184	215	197	254	157	181	184	78	169	198	202	184	165	272	141	128	
GY33	180	215	199	258	157	177	186	78	171	202	200	180	165	268	139	125	W2
	180	215	199	258	157	177	186	78	181	202	202	184	165	268	139	128	
GY34	178	213	195	254	157	181	186	78	171	202	202	184	165	272	135	125	
	178	213	197	254	157	181	186	78	171	202	202	184	165	272	135	128	
GY35	178	215	199	258	157	177	186	78	169	194	198	184	165	264	139	122	W12
	178	215	199	258	157	181	186	78	171	198	198	184	167	272	139	128	
GY36	178	215	199	254	157	175	188	78	169	196	200	184	165	264	139	125	W3
	180	215	199	258	157	175	188	78	171	198	200	184	165	272	139	128	
GY37	180	211	199	254	155	177	188	78	169	194	200	184	165	264	137	128	W11
	180	215	199	254	155	177	188	78	171	196	200	184	165	272	139	128	
GY38	178	215	199	258	157	175	186	78	169	194	200	180	165	272	139	125	
	178	215	199	258	157	175	186	78	169	198	200	184	167	276	139	128	
GY39	176	213	199	258	153	181	186	78	169	198	198	184	165	272	137	128	
	176	213	199	258	157	181	186	78	169	198	200	184	165	272	139	128	
GY40	176	215	199	254	157	179	186	78	169	196	202	184	163	272	133	128	SW+W+E
	176	215	199	258	157	181	188	78	181	198	202	184	165	272	133	128	
GY41	180	215	199	258	157	179	186	78	169	196	202	180	165	272	137	128	
	180	215	199	258	157	179	188	78	169	198	202	180	165	272	139	128	
GY42	176	213	197	254	155	179	186	76	169	198	198	184	163	272	137	128	
	176	213	199	254	155	181	186	78	169	198	202	184	163	272	137	128	
GY43	180	215	199	254	155	175	184	78	173	198	202	184	163	268	131	125	
	184	215	199	254	155	175	186	78	181	198	202	184	163	268	133	128	
GY44	180	215	197	254	157	175	188	78	173	196	202	180	163	268	133	125	
	184	215	197	254	157	175	188	78	181	198	202	180	165	268	133	128	

8.4. Matrix of geographic distances (km) between populations

	GG	KNP	LK	Cor	WD	Curt	D	Glen	GO	Lar	Cam	NI	CE
KNP	30.5												
LK	67.8	78.3											
Cor	100	91.9	60.3										
WD	116.7	110	73.5	18.3									
Curt	275	259	187	177.4	164								
D	287	271	198	193	176.4	12.24							
Glen	258.7	244.4	174	160	146	20.55	32.1						
GO	278.5	263.8	192.5	179.9	166	10.2	5	20					
Lar	260.6	246	176.9	161.6	147	21.7	32.5	4	19.1				
Cam	232	222	160	132.2	116	63.3	74.1	43	60	41.7			
NI	355.1	332.3	254	268.8	260.5	118.7	110	138	125	140	181		
CE	118.9	127	49	87.7	92.3	150	159.3	140	156	143.3	136.6	205.7	
GY	172	147.6	69.6	103.5	105.3	134.7	144	127.6	142	131	130	185	20.5