Retracing the history and planning the future of the red squirrel (*Sciurus vulgaris*) in Ireland using non-invasive genetics

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Abstract

The Eurasian red squirrel's (*Sciurus vulgaris*) history in Ireland is largely unknown, but the original population is thought to have been driven to extinction by humans in the 17th Century, and multiple records exist for its subsequent reintroduction in the 19th Century. However, it is currently unknown how these reintroductions affect the red squirrel population today, or may do so in the future. In this study, we report on the development of a DNA toolkit for the non-invasive genetic study of the red squirrel. Non-invasively collected red squirrel samples were combined with other samples collected throughout Ireland and previously published mitochondrial DNA (mtDNA) data from Ireland, Great Britain and continental Europe to give an insight into population genetics and historical introductions of the red squirrel in Ireland. Our findings demonstrate that the Irish red squirrel population is on a national scale quite genetically diverse, but at a local level contains relatively low levels of genetic diversity and evidence of genetic structure. This is likely an artefact of the introduction of a small number of genetically similar animals to specific sites. A lack of continuous woodland cover in Ireland has prevented further mixing with animals of different origins that may have been introduced even to neighbouring sites. Consequently, some of these genetically isolated populations are or may in the future be at risk of extinction. The Irish red squirrel population contains mtDNA haplotypes of both a British and Continental European origin, the former of which are now extinct or simply not recorded in contemporary Great Britain. The Irish population is therefore important in terms of red squirrel conservation not only in Ireland, but also for Great Britain, and should be appropriately managed.

Keywords: Conservation genetics, re-introductions, phylogeography.

Introduction

The Eurasian red squirrel (*Sciurus vulgaris*) range extends across the Palaearctic region, making it the mostly widely distributed squirrel species (Thorington et al. 2012). Population declines occurred in Ireland and Great Britain between the 17th and 19th Centuries due to deforestation and trapping of squirrels for the fur trade. According to Le Fanu (1922) thousands of red squirrel skins were exported from Ireland in the 16th Century, but when such exports ceased in the 17th Century it is assumed that the population had become extinct or that numbers were so low that their export was no longer viable (Fairley 1983; Montgomery et al. 2014).

In the 19th Century, the trading and sale of live red squirrels was fashionable, and up to 20,000 animals were sold annually in London markets (Shorten 1954). Stock was brought in from the surrounding areas in southern England and imported from Continental Europe. This geographically diverse stock was used to restore the red squirrel population throughout Ireland with at least ten documented introductions occurring in the 19th Century (Barrington 1880). These introductions often consisted of a few individuals, and it was their offspring that were later moved to other locations, resulting in red squirrels being documented throughout Ireland by 1910 (Shorten 1954).

The North American grey squirrel (*Sciurus carolinensis*) was introduced into both Great Britain and Ireland in the late 19th and early 20th Centuries (Shorten 1954). Its spread contributed to declines of the red squirrel in many parts of its distribution due to a competitive advantage held by the grey squirrel, and its role in the spread of the squirrelpox virus (Chantreyet al. 2014). Despite the continued presence of the grey squirrel throughout many parts of Ireland today, the most recent national squirrel survey found that the grey squirrel distribution had retracted and the species was absent from parts of its former range, although still actively invading new areas (Lawton et al. 2015). There is now evidence that the expanding pine marten (*Martes martes*) population might be facilitating the retraction of the grey squirrel population and subsequent expansion of the red squirrel (Sheehy and Lawton 2014).

At 10.5%, Ireland has the lowest level of forest cover in Europe, after Malta (Teagasc, 2016). Despite an extensive hedgerow network in parts of the country, habitat fragmentation is a

threat to woodland species. When this is coupled with the history of translocations, the Irish red squirrel population is likely to exhibit high levels of genetic isolation. This in turn could result in a heightened risk of inbreeding, loss of locally adapted ecotypes, and an inability for new animals to move into the area and 'rescue' the remnant stock. Such risks could ultimately lead to local extinctions as has been seen and predicted in other species (Weckworth et al. 2012; Mondol et al. 2013), and may already have been a contributing factor to previous declines of the red squirrel in Ireland.

Finnegan et al. (2008) conducted a mitochondrial DNA (mtDNA) study of the Irish red squirrel population and found 26 novel haplotypes. Only one haplotype was present in contemporary Britain, H15 from Hale et al. (2004). This was likely an introduction from Continental Europe into Great Britain that was subsequently introduced into Ireland. There were two additional haplotypes found in British museum specimens that were present or formerly present in the Irish population, but have not been recorded in contemporary Great Britain to date. Finnegan et al. (2008) suggested that much of the Irish red squirrel stock was of British and Central European ancestry but due to the widespread decline of the red squirrel in parts of Great Britain, it is likely that some of the original haplotypes introduced into Ireland are now extinct in Great Britain.

Studies to date that have attempted to assess levels of genetic diversity and structure of the red squirrel have relied on DNA extracted from tissue or hair plucked from trapped animals (e.g. Trizio et al. 2005). Despite a move towards non-invasive genetic studies in other species, particularly carnivores (Mullins et al. 2010; O'Neill et al. 2013; Sheehy et al. 2014; O'Mahony et al. 2017), there have been few such studies conducted with rodents. This is most likely due to their small body size and elusive behaviour, and the difficulties of obtaining faeces from arboreal rodents (Reiners et al. 2011).

In this paper, we firstly outline the non-invasive genetic approach used to sample red squirrels in Ireland. Using these non-invasively collected hair samples, we aimed to provide information regarding local levels of genetic diversity and population structure. Secondly, other hair samples were collected from other sources throughout Ireland, and combined with previously published material from Ireland, Great Britain and Europe to gain insights into red squirrel genetic heritage. Contemporary levels of genetic diversity were then

compared to the species' recent past in Ireland to create an informed discussion regarding future conservation and management decisions.

Materials and Methods

Sample Collection

Study Area and Hair-Tube Survey Design

Non-invasive hair-tube surveys were used to collect hair samples from red squirrels in South-East Ireland between 2009 and 2013 (Fig. 1). The hair-tubes were made from 300 mm lengths of 70 mm diameter PVC piping and the hair or glue patch was as described in Mullins et al. (2010). Tubes were secured horizontally to trees with wire and were positioned at a surveyor accessible height at a minimum distance of 30 m apart, with the hair patch positioned 5-7 cm inside the tube. Between one and three transects were selected per woodland depending on the size of the woodland and accessibility within it, and ten hair-tubes were deployed per transect. Bait consisted of maize, peanuts and hazelnuts, and tubes were visited and rebaited every seven days for four weeks. Patches containing hair were removed and stored in 30 ml sterile containers at -20 °C prior to DNA extraction.

Additional hair samples were collected from different projects from the following locations Cork (n = 4 RTA (road traffic accident)), Wexford (n = 14 (plucked hairs from trapped animals)), Midlands, (Laois and Offaly) (n = 3 (hair-tube samples)), Dublin (n = 4 (hair-tube samples)), Galway (n = 11(plucked hairs from trapped animals)) and Northern Ireland, (Antrim) (n = 11 (plucked hairs from trapped animals)) (Supp. Material 1 and Fig. 1 for locations). The hair-tube samples from the Midlands were confirmed as red squirrel in Sheehy et al. (2014) and the Dublin samples were identified to species following the method described by O'Meara et al. (2012) for the Mammals in a Sustainable Environment (MISE) project www.miseproject.ie. All of these additional samples went forward for both microsatellite and mtDNA analysis. *DNA Extraction*

Genomic DNA (gDNA) was extracted from hair samples using ZR Genomic DNA™–Tissue MicroPrep (Zymo Research) according to the Solid Tissue and Hair protocol with Zymo-Spin™ II columns. Purified DNA preparations were stored at -20°C.

Species identification of non-invasive samples from South-East Ireland

Squirrel samples were genetically identified to species using real-time PCR as per O'Meara et al. (2012). Samples that failed to test positive for either red or grey squirrel DNA were subsequently tested for pine marten DNA as per Mullins et al. (2010), since this species displays sympatry with the squirrel species in the study area.

Assessing the quality and quantity of nDNA in the red squirrel DNA samples

A nuclear DNA (nDNA) real-time PCR assay was designed to target a 153-bp of sequence on the zinc-finger intron using the forward primer SQF (5'-GTCCACAGCAAGAACTTTCCTCA-3'), reverse primer SQR (5'-TCAAGTTAGAAGAGTCTGCAGACCTA-3') and TaqMan® MGB probe (5'-VIC-CACATGCGAATCCAT-MGB-3') based on the following sequence (Genbank accession no.). All novel primers and probes were designed in this study using Primer Express 2 software from Applied Biosystems (Life Technologies), primers purchased from Eurofins MWG Operon and the TaqMan probe from Applied Biosystems. Each PCR was as described in O'Meara et al. (2012). The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 ° C for 1 min. All assays were carried out using an Applied Biosystems 7300 Real-Time PCR System, and Applied Biosystems Sequence Detection Software (SDS 1.2.3.) was used for data analysis. This real-time PCR method provides a Ct value as an output which corresponds to the quantity of DNA present, and facilitates the selection of good quality DNA samples for microsatellite genotyping using non-invasive samples (O'Neill et al. 2013; Sheehy et al. 2014)

Microsatellite Panel Selection

The following microsatellite loci were chosen based on their small amplicon size and the number of alleles observed in previous studies: SCV3, SCV4, SCV6, SCV8, SCV20, SCV23, SCV31 (Hale et al. 2001b), RSU1, RSU4, RSU5 (Todd 2000), LIS3 and LIS12 (Shibata et al. 2006). The loci designed by Shibata et al. (2006) for the Japanese squirrel (*S. lis)* had not previously been tested for cross-species amplification with the red squirrel.

Each primer pair was initially tested in singleplex using the following PCR protocol: 5 µl GoTaq® Hot Start Green Master Mix (Promega), 2 µM of each primer, 4 µl DNA, and H_2O in a final volume of 10 µl and amplified using a dropdown PCR protocol involving one cycle of 95

°C for 10 min, followed by 20 cycles of 95 °C for 30 s and a touchdown from 60 °C to 50 °C for 1 min decreasing by 0.5 °C per cycle, and then 72 °C for 1.5 minutes. This was followed by 20 cycles of 95 °C for 30 s, 45 °C for 1 minute and 72 °C for 1.5 minutes, and a final extension of 72 °C for 10 minutes. Where possible, the reverse primers were redesigned to sit closer to the repeat motif to reduce the overall size of the amplicon and to increase the specificity of the primer pair to work in conjunction with non-invasive samples. Mini microsatellites were successfully designed for SCV4 (miniSCV4R: 5'-GTTTCTTGCACAGGAATCATTTGGGA-3'), SCV31 (miniSCV3R: 5'-GTTTCTTGAACAGAAGTGATATGAGGCCAG-3') and SCV8 (miniSCV8R: 5'-TGTGTTTGGGTGTCTGTGTGTT) to amplify shorter fragments of 114-148-bp, 162-166-bp and 162-166-bp, respectively. All reverse primers were also modified following Brownstein et al. (1996) to include 5'-GTTTCTT-3' sequences on the reverse primers to promote non-templated nucleotide addition.

The final panel of nine loci were amplified in one of the following three multiplex reactions using fluorescent labels to enable multiplexing: (a) SCV3 (0.2 µM 6-FAM), LIS12 (0.3 µM ATTO550), RSU5 (0.18 µM 6-FAM); (b) miniSCV4 (0.15 µM ATTO550), miniSCV31 (0.15 µM ATTO551), miniSCV8 (0.2 µM 6-FAM); (c) SCV6 (0.25 µM 6-FAM), SCV20 (0.3 µM 6-FAM) and LIS3 (0.1 6-FAM). The optimised concentrations stated are for both the fluorescently labelled forward and reverse primers that enabled pooling of the three multiplexes for cost effective simultaneous fragment analysis. The use of loci SCV23, RSU1 and RSU4 (see above) was discontinued due to poor amplification under these conditions. The PCR products were diluted (1:20) in water prior to fragment analysis, and 1 µl was added to 15 µl of HiDi Formamide with 0.15 µl size standard (GS500 LIZ™). Fragment analysis was completed on an ABI PRISM® 310 Genetic Analyser with 4% polyacrylamide (POP-4® polymer), in a 47 cm x 50 µm capillary, under default run conditions. Alleles were scored using the GeneMapper software version 3.7 (Applied Biosystems). All samples were amplified in duplicate and failed or inconsistent scores across both replicates were independently repeated from the PCR stage (e.g. Sheehy et al. 2014).

Identification of the red squirrel mtDNA haplotype

PCR primers were designed to target a 350-bp region of D-loop mtDNA: SvScF (5'-TTCACGGAGGTAGGTAGATTAAGA-3') and Svul_R (5'-TTGATGTCTATGTAATTCGTGCAT-3') using the following sequences AF1110001–AF111027 (Barratt et al. 1999). Each PCR contained 5 µl GoTaq® Hot Start Green Master Mix (Promega), 2 µM each primer, 4 µl purified DNA and H_20 in a final volume of 10 µl. The PCR conditions were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 10 min. PCR products were visualised, sequenced in both directions on ABI PRISM® 310 Genetic Analyser.

Microsatellite DNA data analysis

Genetic identification of individual squirrels from hair-tubes

GIMLET, version 1.3.4 (Valière 2002) and MICROCHECKER, version 2.2.3 (van Oosterhout et al. 2004) were used to assess the replicated data for the presence of errors, allelic drop out, false alleles, the presence of null alleles, scoring errors due to stutter peaks and the percentage of positive PCRs. Unique genotypes, observed (H_O) and expected (H_E) heterozygosities, the number of alleles (A), and probability of identity for individuals (PI) and siblings (PI_{SID}) were all assessed using GENALEX, version 6.5b (Peakall and Smouse 2006). The inbreeding coefficient (F_{IS}) was calculated using FSTAT version 2.9.3, with significance levels for F_{IS} levels calculated by randomizing the alleles among the individuals within the population and comparison to the observed data to determine deviations from Hardy-Weinberg Equilibrium using 10,000 permutations.

Descriptive statistics of all samples

A representative sample set was selected from the field study area in Co. Waterford, SE Ireland (n = 15), which included samples from all sites analysed in Co. Waterford. Populations were divided geographically and groups with at least 11 individuals were assessed for descriptive statistics. Cork, Dublin and the midlands region had too few individuals for inclusion for these analyses. H_O, H_E, A and F_{IS} were assessed as above and allelic richness (A_R), an index of genetic diversity was assessed using FSTAT version 2.9. FSTAT was also used to estimate pair-wise F_{ST} statistics, and the significance was tested using 1000 randomisations and applying a Bonferroni correction. Genotypic linkage

disequilibrium by Fisher's method (1000 dememorizations and 5000) iterations were also calculated using GENEPOP.

The number of genetic clusters present in the dataset was assessed using STRUCTURE version 2.3.1 (Pritchard et al. 2000; Falush et al. 2003). The Bayesian clustering algorithm implemented in the programme was used to analyse the data with default settings with a burn-in period of 10,000, followed by 100,000 replicates with no prior population information. The likelihood of *K* equals 1 to 10, with each *K*-value replicated three times to assess the most likely number of inferred populations. The most likely *K* was assessed by implementing the Δ*K* method (Evanno et al. (2005), using STRUCTURE Harvester (Earl and van Holdt 2012). The web server CLUMPAK was used to summarize and visualize the STRUCTURE results (Kopelman et al. 2015). To further investigate the presence of genetic structure, a principal coordinate analysis (PCoA) in GENALEX, version 6.5b (Peakall and Smouse 2006) was employed to further examine the genetic relationships among the samples. This multivariate approach was chosen to complement the STRUCTURE analysis as that might be affected by the presence of related individuals in the dataset.

To test if geographic distance was affecting genetic differentiation, tests for isolation-by-distance were conducted using Mantel's test, implemented in GENALEX using Nei's standard genetic distance (Nei 1972, 1978) with 999 permutations.

MtDNA Data Analysis

MtDNA sequences generated in this study were compared by multiple alignments using the CLUSTALW method in MEGA V.7 (Tamura et al 2011). Haplotypes were identified using ARLEQUIN (version 3.5; Excoffier and Lischer 2010). MtDNA haplotypes were compared to those previously recorded in Ireland and Great Britain (Finnegan et al. 2008) by truncating the sequences to 263-bp. A median-joining network was constructed using the median algorithm of Bandelt et al. (1999) in PopART (v1.7.1) (http://popart.otago.ac.nz/) with default settings. DNASP version 5.10.01 (Librado and Rozas 2009) was used to estimate nucleotide diversity.

Finally, the DNA sequences obtained from this study and that of Finnegan et al. (2008) were compared to those previously published across Europe to compare the genetic heritage of the Irish population today. The data consisted of samples from Great Britain (including the Channel Islands), Continental Europe (Sweden, Finland, France, Austria, Poland, Hungary, Czech Republic, Romania, Slovenia, Spain, Portugal, Italy, Albania and Greece) and eastern samples from China and Russia all previously published by Hale et al. (2004), Ogden et al. (2005), Grill et al. (2009) and Simpson et al. (2013). The dataset consisting of 208 sequences was truncated to 188-bp and visualised via a median-joining network constructed as before using PopART.

Results

Hair-tube survey

In total there were 110 mammal detections at hair-tubes across the study area in South East Ireland (min = 1, max = 21 per site).The real-time PCR species identification tests identified red squirrel (n = 86), grey squirrel (n = 4) and pine marten (n = 8). The samples that failed to amplify as red squirrel, grey squirrel or pine marten (n = 12) could not be identified to species as those samples contained a non-target species or contained DNA of poor quality that was unsuitable for genetic analyses. The positive red squirrel samples were subsequently amplified with the nDNA assay and the samples with a Ct < 35 (n = 59) were selected for amplification with the microsatellite panel.

Microsatellite Analysis

A total of 58 genotypes were obtained from the red squirrel hair-tube samples, with 31 individual red squirrels identified. The number of individual red squirrels identified per site ranged from one each in the Nire Valley and Boola Bridge to nine in Ballyscanlon (Fig. 1). Replicated genotypes were recorded at four sites which ranged from one to 16 recaptures. Low levels of allelic dropout were identified at RSU5 and SCV20 (0.01 each). The overall dropout rate across all loci and PCRs was 0.03. No occurrences of false alleles were recorded. The number of alleles per locus ranged from two to four and averaged 3.6 alleles per loci. Observed heterozygosity averaged 0.53 and expected levels of heterozygosity averaged 0.48 across all loci. Three loci RSU5, LIS12 and LIS3 showed significant deviations

from Hardy-Weinberg equilibrium (P = 0.05), and remained so after the Bonferroni correction was applied (P = 0.0056). Probability of identity averaged 3.5 x 10^{-1} across loci, with a cumulative PI of 4.7 x 10^{-5} (Supp. Material 2).

From the populations which had >11 individuals genotyped, the average number of alleles per population ranged from 2.7 in the Waterford population to 3.2 in the other populations (Galway, Wexford and Northern Ireland). Allelic richness ranged from 1.8 in Waterford to 2.2 in Galway. Average levels of expected heterozygosity ranged from 0.40 in Waterford to 0.55 in Galway. Average observed levels of heterozygosity ranged from 0.38 in Waterford to 0.60 in Northern Ireland. F_{IS} values ranged from 0.15 in Galway to -0.35 in Wexford, with both of those populations showing signification deviations from Hardy–Weinberg Equilibrium at the 5% significance level. However, no population showed significant deviation from Hardy-Weinberg equilibrium following Bonferroni correction (P = 0.00079) (Table 1).

The pairwise F_{ST} estimates showed that the geographically divided populations were highly differentiated from one another (Table 2). The overall F_{ST} = 0.237, 99% C.L = 0.158, 0.324 was found to be significantly different from zero. The result of Mantel's test revealed a significant pattern of isolation by distance (IBD) (R^2 = 0.1015; P = < 0.001) which was expected as samples were used from a broad geographical area, without sampling between the regions.

Genetic Structure

A total of 58 individuals were analysed for assignment, removing samples that failed to amplify at two or more loci. The STRUCTURE programme found that K = 4 was the most likely number of genetic clusters in the population. Using the Evanno et al. (2005) method implemented in STRUCTURE HARVESTER (Earl and van Holt 2012), there was not a clear divide between K = 3 and K = 4 as the most appropriate number of genetic clusters in the red squirrel population (Supp. Material 2). However, K = 4 also showed lowest deviance from the mean, and appeared more uniform in comparison to the other K values in Fig. 2 (K = 2, 3 and 4) suggesting that this was the most reliable estimate.

The PCoA was used to visualise the clustering of individuals. This analysis accounted for 51.57% of the overall genetic variation, and clustered the Waterford and Cork samples closely together. The Wexford and Northern Irish samples clustered as groups, while the Galway samples were more dispersed. All geographically divided groups exhibited some level of overlap (Fig. 3). The results from the population assignment based PCoA were in agreement with the STRUCTURE output for $K = 3$ and $K = 4$.

Identification of the MtDNA haplotypes from hair-tubes

DNA sequenced for haplotype analysis revealed a total of six mtDNA haplotypes in Co. Waterford including the four haplotypes previously published by Finnegan et al. (2008) (IE16, IE18, IEGB1 and IEGB2), and two novel haplotypes (IE23 and IE24 Genbank Accession no:) (Fig. 1).

MtDNA analysis from across Ireland

The mtDNA haplotypes varied across the sampling area which was reflected by a high level of haplotype diversity. A table of all haplotypes from this study and those previously recorded by Finnegan et al. (2008) are provided in Supp. Material 3. Across the 263-bp of sequence, 226 sites were monomorphic, 37 polymorphic (including a total of 46 mutations), 11 sites included singletons and 26 sites were parsimony informative with an overall nucleotide diversity of 0.029 ± 0.095 and gene diversity of 0.995 ± 0.00007. A total of six haplotypes were unique to this study.

The red squirrel haplotypes from this study were compared to those previously recorded in Ireland by Finnegan et al. (2008) and visualised using a network diagram in Fig. 4. The haplotype network reflects a high level of genetic variation within red squirrels across Ireland, but also within the study area in Co. Waterford. As in Finnegan et al. (2008), the majority of the haplotypes found in this study were similar to IEGB haplotypes which were previously recorded in museum specimens in Britain and contemporary Irish haplotypes. However, IEGB2 was previously only recorded in museum samples in Ireland, but was found to be present in this study in Co. Waterford. Finnegan et al. (2008) described IE17 and IE14 as being of a Continental European and British origin, and IE24 (newly discovered in this study), also appeared to group closely to this haplotype, suggesting a Central European

origin. Samples from Antrim in Northern Ireland, included two haplotypes, (IE25 and IE26) both similar to IE7, a haplotype previously recorded in the area by Finnegan et al. (2008). Samples from Cork contained the haplotypes IE18 and IE27, again both closely related. IE18 was previously recorded in Cork and West Waterford, and this haplotype showed close similarity to a haplotype observed in Northern Ireland, possibly representing a similar colonization history, or introductions from the same source. Samples from Co. Dublin contained the haplotype IE2.

Finally, the European-wide network diagram showed low levels of geographic clustering across Europe with many samples from Continental Europe overlapping with those from Great Britain, and a small number of Irish haplotypes (Fig. 5). A large number of the Continental European haplotypes were identified as identical haplotypes once the DNA sequences were truncated, and these are represented by larger vortexes in the diagram. A group of Irish and British museum haplotypes branched out from the main European samples indicating a degree of separation from the main European cluster. The other separated branch included samples from Southern Italy (Calabria), Russia and China.

Discussion

Non-invasive genetic sampling of red squirrels

The techniques presented in this paper offer a novel way to remotely obtain a source of DNA from red squirrels. The accuracy and sensitivity of the real-time PCR species identification and nDNA screening offer an efficient and robust technique to select samples for genotyping of non-invasive samples. As in O'Neill et al. (2013), the Ct values obtained from real-time PCR offer an insight into the quality and quantity of DNA in each sample, allowing only good quality samples to be used for genotyping. 89% of samples collected in this study using hair-tubes were identified to species level using the red squirrel, grey squirrel and pine marten real-time PCR assays. The samples that were not identified as red squirrel, grey squirrel or pine marten either had non-detectable quantities of DNA or may have originated from species that we did not test for. Of the positively identified red squirrel samples, 68.5% of samples were deemed suitable for microsatellite genotyping by using a real-time PCR assay that assessed the quantity and quality of the nDNA in each DNA extract. As a result of this screening method, a high level of genotyping success was achieved in this

study, with 98.3% of the samples that passed the screening threshold producing reliable genotyping data, thus reducing the amount of time and resources required for this analysis and substantially reducing the risk of introducing genotyping errors, an inherent problem in some non-invasive genetic studies (Broquet et al. 2007).

Population Genetics

For the non-invasively collected samples from Co. Waterford, average levels of heterozygosity were 0.48 and homozygosity were 0.53, with an average number of alleles of 3.6. These values are comparable to populations previously reported from Italy, Northern England and Jersey but higher than the island of Anglesey, although the panel of microsatellites used in those studies varied (Ogden et al. 2005; Trizio et al. 2005; Simpson et al. 2013). Three loci showed significant deviations from Hardy-Weinberg equilibrium in this study which might be attributed to genetic admixture caused by divergent origins of the population (Simpson et al. 2013). When the Waterford sample size was reduced and compared to the wider Irish population, there was no evidence of deviation from Hardy-Weinberg equilibrium, again indicating that genetic structuring was occurring within the county.

The mtDNA haplotype analysis of the hair-tube samples from Co. Waterford revealed a high level of genetic diversity within the county. The haplotypes found in the Nire Valley included some that were not found elsewhere in the county, providing further indication of genetic structure. This area is somewhat isolated as it is surrounded by the River Suir and the Comeragh Mountains, which may inhibit migration within Co. Waterford (Fig. 1). It is likely that multiple colonisations and introductions took place due to the mixed heritage seen in this study, with some of the haplotypes being of an Irish/British origin, and IE24 appearing to be more related to Continental European haplotypes. This is consistent with the mixed-origin red squirrels introduced into Ireland in the late 1800s (Barrington 1880; Shorten 1954).

Both the microsatellite DNA and mtDNA analysis showed high levels of genetic diversity and differentiation in the Irish red squirrel population. The genetic assignment assessment methods showed that the geographically divided populations sampled in this study were genetically differentiated from one another. However, this interpretation is complicated due

to the sampling approach used in this study due to difficulties implementing a systematic sampling approach across the country, and this was evident in the isolation-by-distance analysis which showed that populations were isolated by distance which may have influenced the STRUCTURE results (Frantz et al. 2009). The results may be further impacted by the history of the red squirrel population in Ireland which has clearly originated from multiple sources within Great Britain and Continental Europe, making population differentiation more likely.

At a national level, the Irish red squirrel is genetically quite diverse. At a local level however, genetic diversity is relatively low as only small numbers of mtDNA haplotypes were observed per woodland and the haplotype composition varied from site to site. This suggests a lack of continuous gene flow across sites and small populations of squirrels may be at particular risk of local extinction. The lack of contemporary gene flow between isolated populations has been recognised as a conservation risk in other species including the caribou (*Rangifer tarandus*) in western North America and the Indian tiger (*Panthera tigris*) (Weckworth et al. 2012; Mondol et al. 2013). Possible mitigation measures should include the protection and maintenance of hedgerows as they are important wildlife corridors in Ireland (Hickie 2004). Clear felling and over thinning of woodlands should be avoided and continuous forest cover practices should be encouraged where possible.

Simpson et al. (2013) showed evidence of a similar 120-year-old genetic footprint that has remained on the island of Jersey. There was also evidence of a spatial pattern that revealed a higher incidence of the disease amyloidosis where divergent haplotypes of red squirrels met in the centre of the island, possibly linked to genetic outbreeding. Such results have important implications in Ireland where many reintroductions have taken place and there are high numbers of divergent mtDNA haplotypes present leading to the possibility of the occurrence of an outbreeding depression.

The establishment of the invasive grey squirrel in Britain and Ireland has many parallels to the introduction of the red squirrel in Ireland. For instance, the grey squirrel establishment in Britain was facilitated by people moving squirrels to new areas in the late 19th and early 20th centuries. With the aid of microsatellite analysis, Signorile et al. (2016) was able to trace the genetic footprint of the 1911 introduction in Ireland back to Woburn in Bedforshire. The

Woburn population had been imported from New Jersey, USA. However, multiple introductions from different sources within the US also took place into Great Britain, and Signorile et al. (2016) found that this divergent stock did not mix as much as expected. In fact, the genetic diversity of the species is quite low and their invasion success was linked to their intentional movement by people rather than their ecological ability to invade.

Given the potential risks of localised extinction of isolated populations in Ireland and their history of human-mediated introductions and current lack of conservation management, population enhancement projects should be considered. Population reinforcements should be considered on a site-by-site basis selecting animals with similar haplotypes to the resident population rather than choosing animals by geographic proximity that may have highly divergent haplotypes. For instance, if a population reinforcement project was to be considered in the Nire Valley, Co Waterford where the haplotype IE24 (a continental European type haplotype) was found, animals with similar haplotypes such as IE14 (found in Wexford in this study) and IE17 (found in Down and Wicklow by Finnegan et al. (2008)) could be considered. Indeed, some of the Irish populations may also be considered as potential reintroduction/reinforcement candidates for conservation efforts in Great Britain, particularly in areas in England where red squirrels are now extinct. Haplotypes such as the IEGBs and those closely related would be worth considering.

However, feasibility studies would firstly need to take place to ensure that the source populations within Ireland were viable and could sustain removal as per IUCN Guidelines (IUCN/SSC 2013). The non-invasive genetic toolset described in this study can be used to assess contemporary and historical diversity to aid in the consideration of any translocation or population restoration projects.

Irish red squirrels in relation to their European counterparts

Red squirrels are known to have low levels of phylogeographic structure across Europe (Grill et al. 2009). This was also evident from our comparative analysis where little geographic structure was evident across much of Europe. Whilst it was clear that some of the Irish mtDNA haplotypes held affinities to continental European samples, a branch of Irish and

British museum mtDNA haplotypes showed differentiation from the rest of Europe (Fig. 5). The results from this study show the importance of the Irish population in a European context as they include mtDNA haplotypes that are now extinct or have not been recorded in contemporary Great Britain or elsewhere in Europe. This mirrors the situation with stoats (*Mustela erminea*) and pygmy shrews (*Sorex minutus*) whereby Ireland houses lineages/haplogroups which have either disappeared or are rare in Britain and other parts of Europe (Martinkova et al. 2007; McDevitt et al. 2011).

Finnegan et al. (2009) morphologically examined red squirrels in Ireland and found that 59% of the samples examined exhibited a light or blonde tail and ear tufts, a trait also seen in Cumbria, Scotland and Wales, but not commonly recorded elsewhere in Europe. Other species, including the Irish stoat, (*Mustela erminea*) also have unique morphological features when compared to stoats elsewhere in Europe (Miller 1912). More research is needed to investigate if the outlying mtDNA haplotypes seen in this study (Fig. 5) also represent individuals with the blonde colour trait. Future work could involve the DNA sequencing of a longer fragment of mtDNA and additional microsatellite data from squirrels across Ireland, Great Britain and Europe to test the likelihood of different historical scenarios that may have caused this outlying group.

Conclusion

It is imperative to actively conserve the Irish red squirrel population given the role people have previously played in their current distribution, habitat availability and connectivity which will need appropriate management going forward to prevent populations from becoming further genetically isolated and potentially becoming extinct. The Irish red squirrel population has a unique genetic heritage in a European context, but more importantly represents a gene pool that is now potentially extinct in some of Great Britain, and could potentially be used as a source population should translocation or reinforcement projects be considered there as well as in Ireland. The Irish red squirrel population therefore represents an important population that is worthy of conservation management both from an Irish and British perspective and could be considered as a joint responsibility given the importance to both islands.

Acknowledgements

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List of Tables

Table 1

Average descriptive statistics for red squirrel populations in Ireland. Abbreviations are number of samples amplified per loci (N), number of alleles per loci (A), observed heterozygosity (H_O), expected heterozygosity (H_E), (A_R) allelic richness, (F_{IS}) inbreeding coefficient, (WAT) Waterford, (GAL) Galway, (WX) Wexford, and (NI) Northern Ireland with values in bold indicating significant deviation from Hardy-Weinberg Equilibrium at $P = 0.05$.

Table 2

Pairwise F_{ST} values generated using the microsatellite data between (WAT) Waterford, (GAL) Galway, (WX) Wexford, and (NI) Northern Ireland, and corresponding significance values in bold after Bonferroni correction ($P = 0.002$).

List of Figures

Figure 1

Upper inset: Map of Ireland with counties mentioned and sampled in this study. Abbreviations: Antrim (ANT), Down (DOW), Dublin (DUB), Wicklow (WK), Wexford (WX), Waterford (WAT), Kilkenny (KK), Tipperary (TIP), Limerick (LK), Cork (CK), Kerry (KY), Offaly (OFF), Laois (LAO) and Galway (GAL). Map of survey sampling locations in Co. Waterford. *Lower inset:* Distribution of mtDNA haplotypes found in Co. Waterford. County border (red) and rivers (blue) also shown. Abbreviations for survey sites: Gardenmorris (GM), Ballyscanlon (BS), Brownswood (BW), Guilcagh (GC), Kildalton (KD), Nire Valley (NV), Faithlegg (FL), Mahon Bridge (MB), Boola Bridge (BO) and Ballymacabry (BC).

Figure

Graphical output from Bayesian analysis in STRUCTURE showing assignment probabilities of individuals to clusters for $K = 2$, $K = 3$ and $K = 4$. Clusters were divided into the following groups: Dublin (DUB), Waterford (WAT), Cork (CK), Galway (GAL), Wexford (WX), Midlands (MDS), and Northern Ireland (NI).

Figure 3

PCoA of red squirrel individuals. Populations were divided into the following groups: Dublin (DUB), Waterford (WAT), Cork (CK), Galway (GAL), Wexford (WX), Midlands (MDS), and Northern Ireland (NI).

Figure 4

Median-joining network of mtDNA sequences from Irish individuals from this study and Finnegan et al. (2008).

Figure 5

Median-joining network of mtDNA sequences divided into groups from Great Britain (GB), museum samples from Great Britain (mGB), Jersey and the Isle of Wight (CI), Ireland (IE), Europe (EU) and Russia and China (RC).

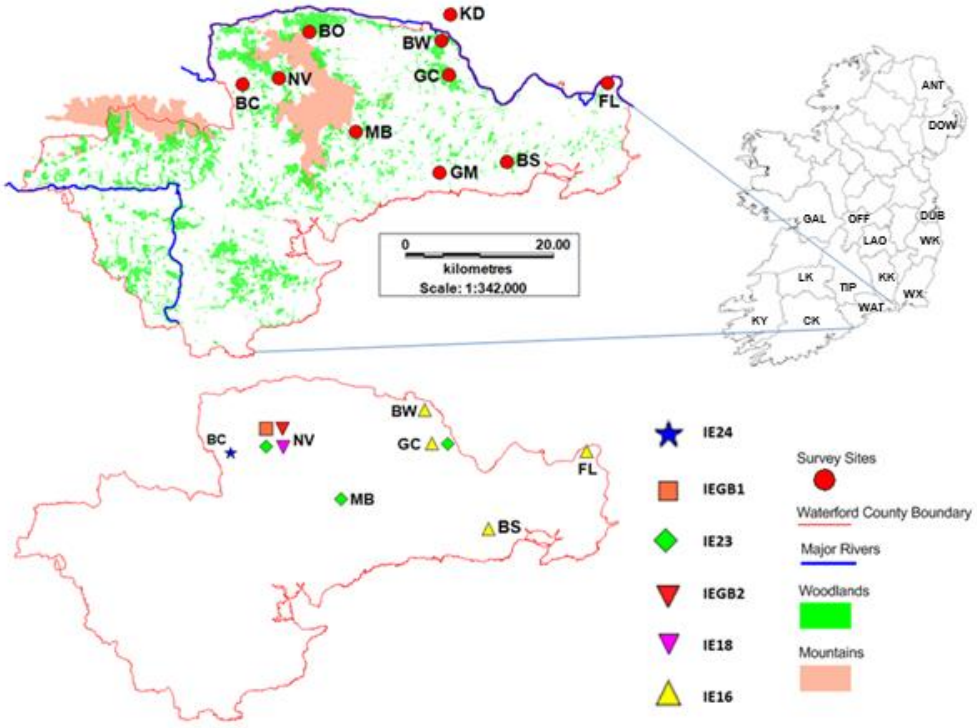
Table 1

	N	N_A	A_R	H_O	H_E	F_{IS}
WAT	16.7	2.7	1.8	0.38	0.4	0.009
GAL	10.1	3.2	2.2	0.48	0.56	**0.153**
WX	13.7	3.2	1.9	0.56	0.44	**-0.346**
NI	10.8	3.2	2.1	0.6	0.52	0.001

Table 2

	WAT	CK	GAL	WX	MDS	NI
DUB	0.228	0.257	0.06	0.297	0.041	0.189
WAT		**0.257**	**0.216**	**0.343**	0.182	**0.287**
CK			**0.174**	**0.397**	**0.258**	0.334
GAL				0.199	0.105	0.215
WX					**0.234**	**0.260**
MDS						0.076

Figure 1


KD
BO
BW
GC
NV
BC
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kilometres
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ANT
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DUB
LAO
WK
LK
KK
TIP
WX
WAT
KY
CK
IE24
IEGB1
IE23
IEGB2
IE18
IE16
Survey Sites
Waterford County Boundary
Major Rivers
Woodlands
Mountains

Figure 2

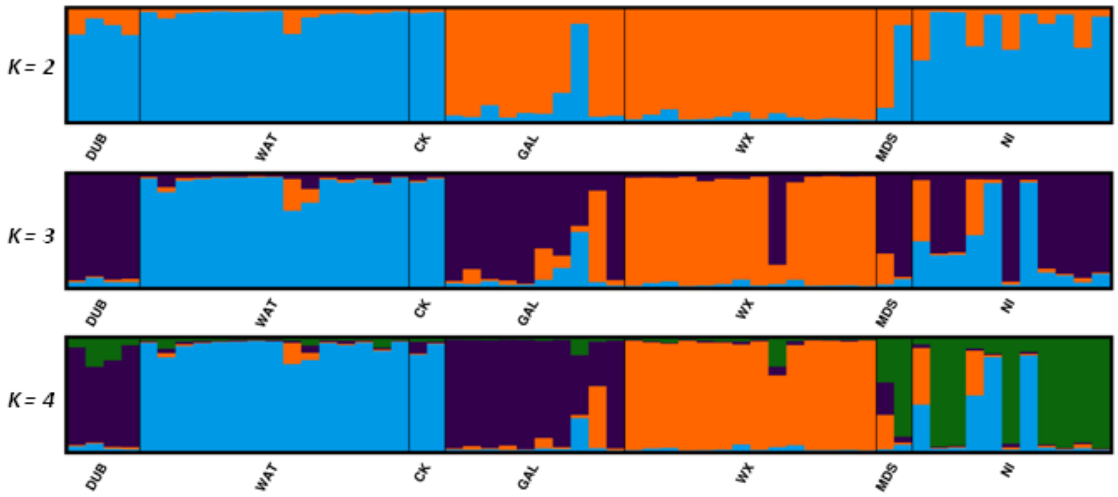

K = 2
DUB
WAT
CK
GAL
WX
MDS
NI
K = 3
DUB
WAT
CK
GAL
WX
MDS
NI
K = 4
DUB
WAT
CK
GAL
WX
MDS
NI

Figure 3

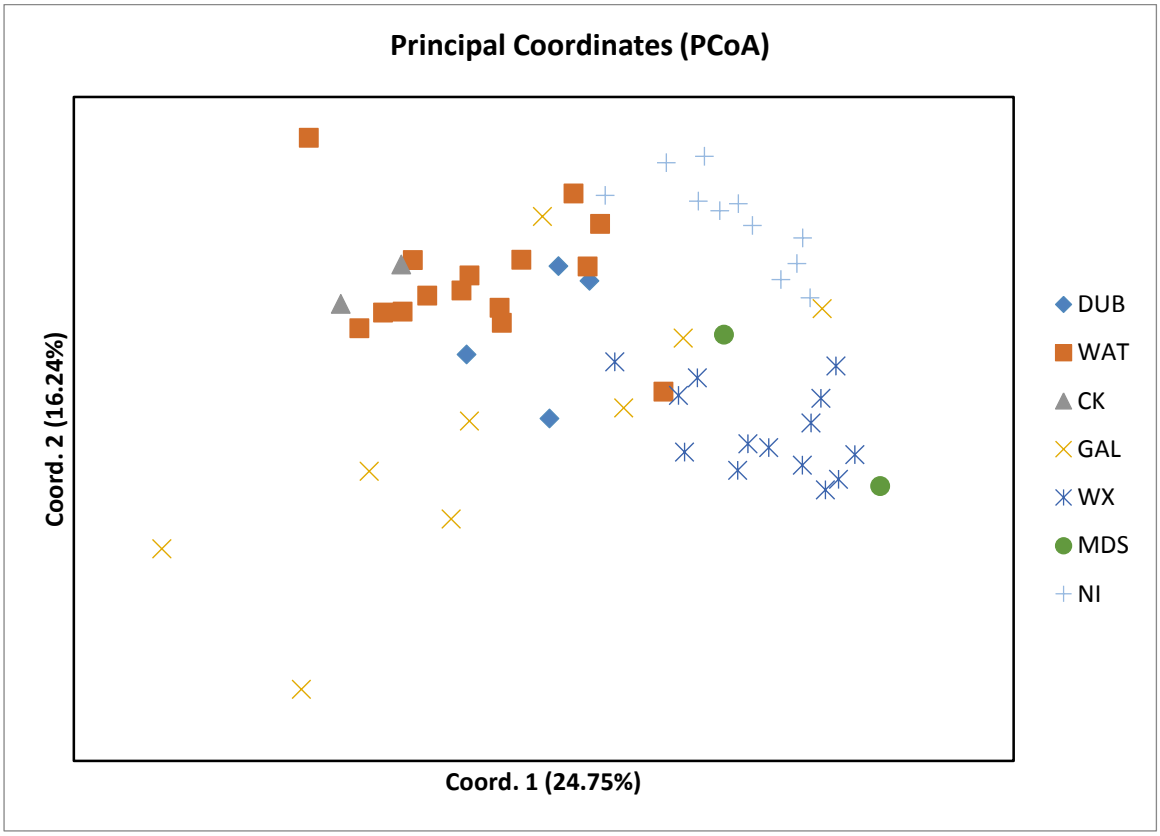

Principal Coordinates (PCoA)
Coord. 2 (16.24%)
Coord. 1 (24.75%)
DUB
WAT
CK
GAL
WX
MDS
NI

Figure 4

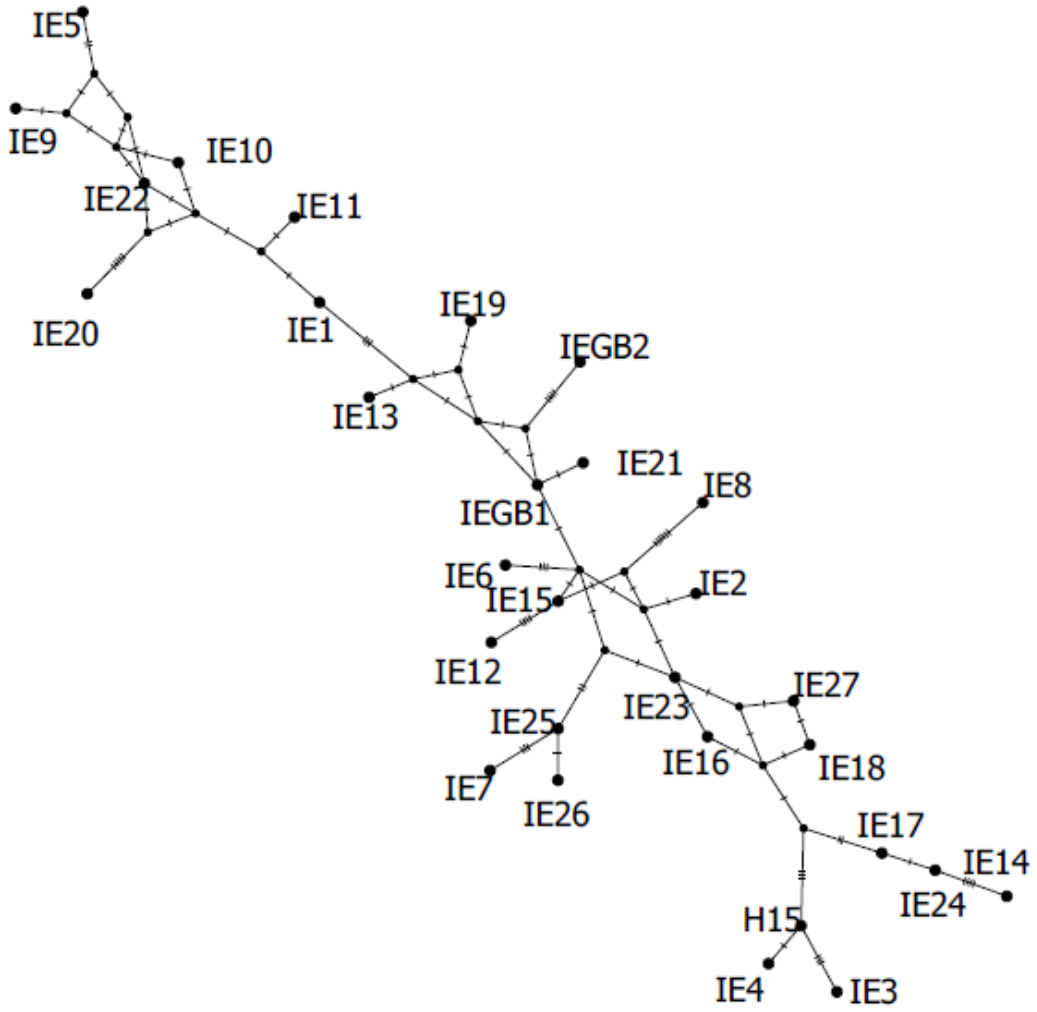
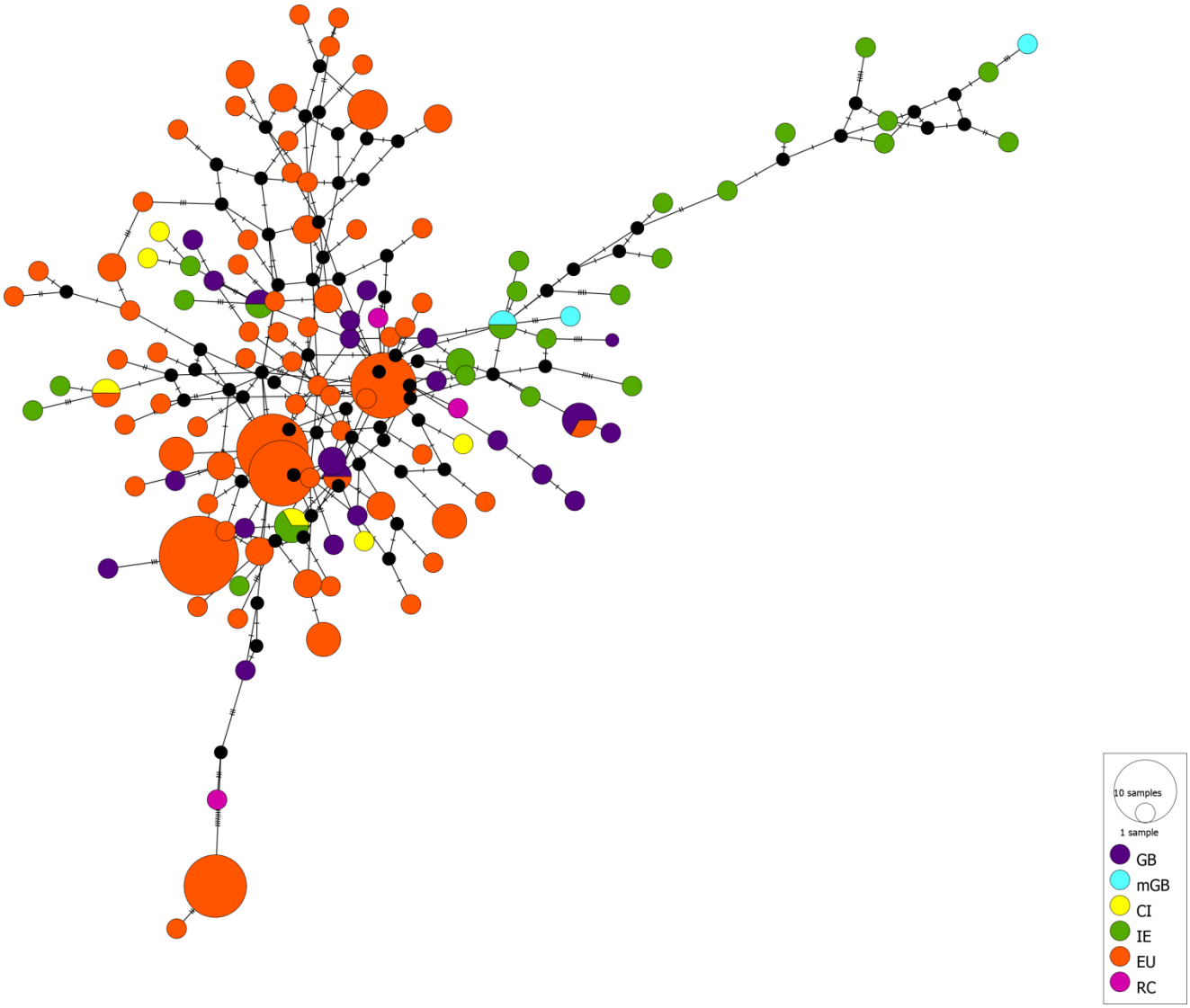

IE5
IE9
IE10
IE22
IE11
IE20
IE1
IE19
IEGB2
IE13
IE21
IE8
IEGB1
IE6
IE15
IE2
IE12
IE27
IE23
IE25
IE16
IE18
IE7
IE26
IE17
IE14
IE24
H15
IE4
IE3

Figure 5



Supplementary material 1:

Geographic locations of samples, the collectors involved and the number of samples from each area.

Location	Collectors	No
Midlands (Laois + Offaly)	E. Sheehy	3
Wexford (The Raven)	W. Carr & F. Marnell	14
Galway (Derryclare)	C. Waters & C. Lawton	11
Northern Ireland (Antrim)	D. Tosh	11
Dublin (Carrickgollogan)	S. Rubalcava	4
Cork (Glengarriff)	P. Sleeman	4

Supplementary material 2:

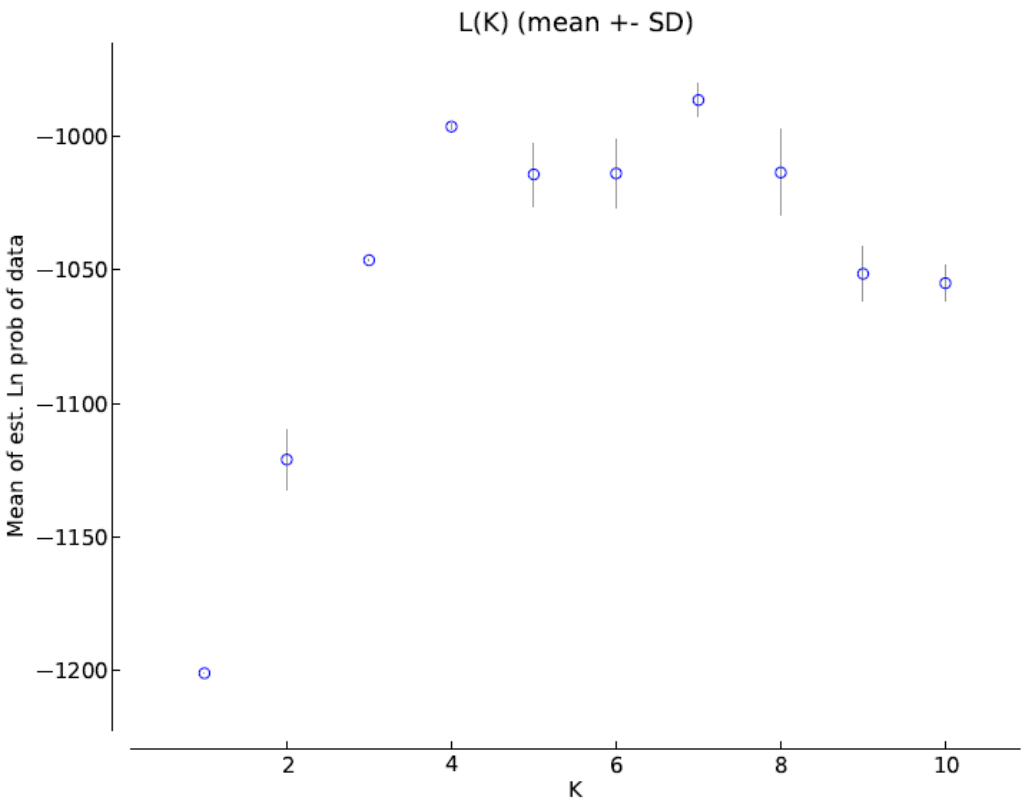
Descriptive statistics for non-invasively identified red squirrels identified in Co. Waterford. Size range (SR), number of samples amplified per loci (N), number of alleles per loci (A), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) values in bold indicating values that significantly indicating significant deviation from Hardy-Weinberg Equilibrium at P = 0.05, probability of identity per locus (PI) and probability of identity for siblings (PIsib).

	Rsu5	Lis12	Scv3	miniScv4	miniScv31	miniScv8	Scv6	Scv20	Lis3	Average
SR	185 -189	203 -209	136 -138	114 -118	167 -173	162 -168	172 -180	213 -219	171 -175	
N	29	28	29	27	22	28	30	29	30	28
A	3	4	2	3	4	4	4	4	4	3.6
HO	0.45	0.29	0.59	0.48	0.36	0.64	0.8	0.48	0.7	0.53
HE	0.41	0.31	0.49	0.44	0.45	0.67	0.59	0.43	0.55	0.48
Fis	**-0.40**	**-0.42**	-0.24	-0.17	-0.16	-0.13	-0.23	-0.34	**-0.23**	**-0.25**
PI	4. x 10-1	4.9 x 10-1	3.8 x 10-1	4.0 x 10-1	3.4 x 10-1	1.6 x 10-1	2.6 x 10-1	3.9 x 10-1	2.8 x 10-1	3.5 x 10-1
PIsib	6.5 x 10-1	7.2 x 10-1	6.0 x 10-1	6.4 x 10-1	6.1 x 10-1	4.6 x 10-1	5.2 x 10-1	6.3 x 10-1	5.4 x 10-1	6.0 x 10-1

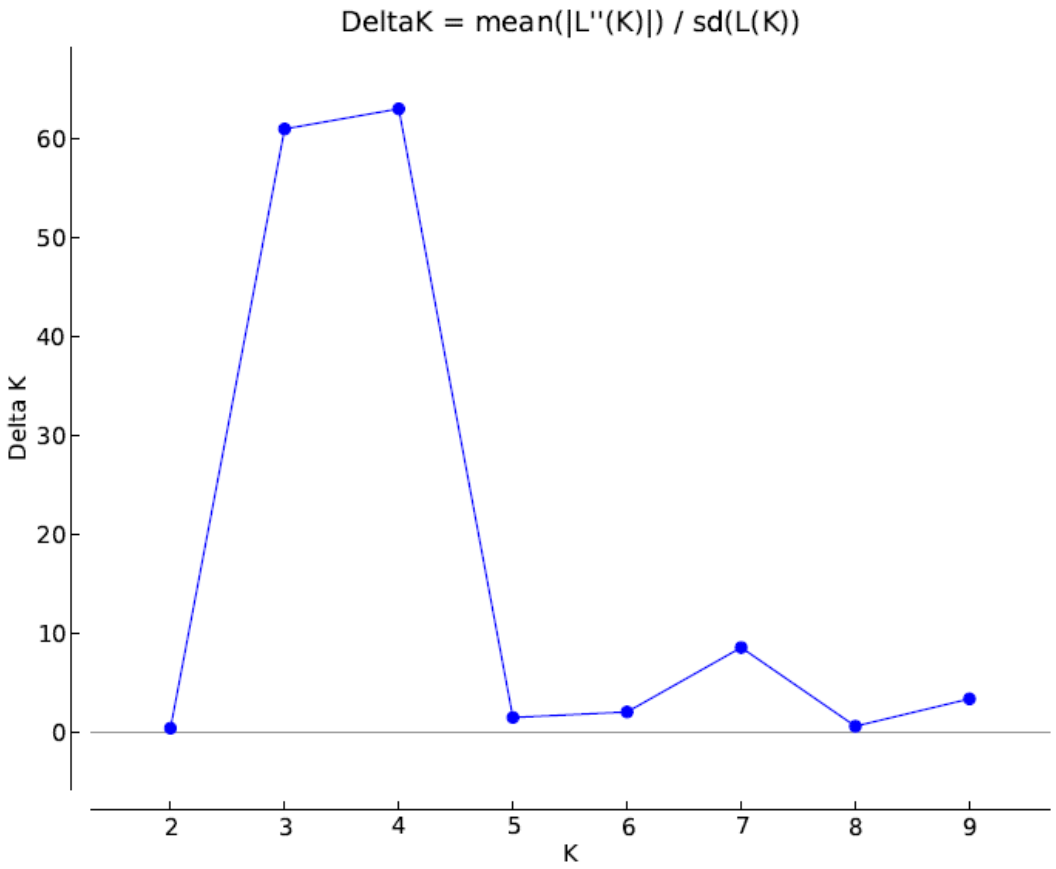
Supplementary material 2:

(a) Ln (probability of data) for K ranging from 1 to 10. (b) Estimation of number of subpopulations using delta K values for K ranging from 1 to 10 using the method developed by Evanno et al. (2005).

(a)



(b)



Supplementary material 3:

Locations of recorded mtDNA haplotypes in Ireland. Haplotypes recorded in this study are displayed in bold. Locations mapped in Fig. 1.

Haplotype	Location	Source
IE1	Dublin	Finnegan et al. (2008)
IE2	**Wicklow, Dublin**	**Finnegan et al. (2008), this study**
IE3	Wicklow	Finnegan et al. (2008)
IE4	Wicklow	Finnegan et al. (2008)
IE5	Wicklow	Finnegan et al. (2008)
IE6	Wicklow	Finnegan et al. (2008)
IE7	Antrim	Finnegan et al. (2008)
IE8	Kerry	Finnegan et al. (2008)
IE9	Kerry	Finnegan et al. (2008)
IE10	Galway	Finnegan et al. (2008)
IE11	Galway	Finnegan et al. (2008)
IE12	Galway	Finnegan et al. (2008)
IE13	Galway	Finnegan et al. (2008)
IE14	**Wexford**	**Finnegan et al. (2008), this study**
IE15	Wicklow, Kerry	Finnegan et al. (2008)
IE16	**Limerick, Galway, Waterford**	**Finnegan et al. (2008), this study**
IE17	Down, Wicklow	Finnegan et al. (2008)
IE18	**Waterford, Cork**	**Finnegan et al. (2008), this study**
IE19	Dublin	Finnegan et al. (2008)
IE20	Kilkenny	Finnegan et al. (2008)
IE21	Wicklow	Finnegan et al. (2008)
IE22	Dublin, Galway	Finnegan et al. (2008)
IE23	**Waterford**	**This study**
IE24	**Waterford**	**This study**
IE25	**Antrim, Northern Ireland**	**This study**
IE26	**Antrim, Northern Ireland**	**This study**
IE27	**Cork**	**This study**
IEGB1	Cork, Kerry, Galway, Wicklow, Tipperary, England	Finnegan et al. (2008)
IEGB2	Kilkenny, Laois, Wicklow, England	Finnegan et al. (2008)
H15	Wicklow, the Netherlands	Hale et al. (2004), Finnegan et al. (2008)