Invasive genetic rescue: dispersal following repeated culling reinforces the genetic diversity of an invasive mammal

Invasive genetic rescue: Dispersal following repeated culling reinforces the genetic diversity of an invasive mammal

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Abstract

Since its introduction from the United States in 1876, the invasive North American Eastern grey squirrel (*Sciurus carolinensis*) has contributed to the decline of the native Eurasian red squirrel (*Sciurus vulgaris*) in Britain. The aim of this study was to assess the overall impact of repeated control efforts carried out between 2011 and 2020 on the genetic diversity of the grey squirrel population in north Wales. This information can be used to inform future adaptive management plans, increasing the success of invasive species control efforts and enhancing red squirrel conservation efforts. Using a combination of mitochondrial DNA (mtDNA) and microsatellite DNA analysis, we found high genetic diversity in both marker types, with six diverse mtDNA haplotypes found and relatively high levels of nuclear genetic diversity, even after repeated culling efforts. We also found that repeated introductions from multiple locations in North America have generated a genetically diverse population in Britain today, compounding the management of this invasive species. Our results suggest that ongoing grey squirrel control efforts may not adequately reduce genetic diversity to a level where it contributes to a long-term population decline, and highlights the need to gather all available information, including historical and contemporary, to effectively create a plan for control efforts of invasive species.

Introduction

Prolonged invasive species control efforts can lead to a loss of genetic diversity amongst residual invasive populations, an effect that can accelerate population decline and ultimately increase the success of an eradication programme (Roman & Darling, 2007; Zalewski et al. 2016; Browett et al. 2020).

Unlike recent discoveries of invasive species in Britain such as the greater white toothed shrew (*Crocidura russula*) (Bond et al. 2022) which was likely an accidental or unintentional introduction; the presence of grey squirrel in Britain is the result of at least eight documented introductions including a minimum of 135 recorded individuals from the USA and Canada in the late 19th and early 20th centuries (Shorten 1954). These founding individuals introduced for ornamental purposes, quickly established, and subsequently spread to further locations or were chosen for some of the additional 31 documented translocations that took place across the country (Middleton 1931; Shorten 1954). Despite ecological and climatic differences between North America and Britain, the founding individuals often led to the establishment of large, viable populations (Gurnell et al. 2004; Stevenson-Holt & Sinclair 2015). Mitochondrial DNA (mtDNA) studies show that there is a high diversity of haplotypes found in the grey squirrel population in Britain today, reflecting the large number of original introductions from a broad geographic area, and confirming that the contemporary population has been derived from multiple sources (David-Gray et al. 1998; Stevenson et al. 2013; Stevenson-Holt & Sinclair 2015). Nuclear or microsatellite DNA has shown that high levels of genetic differentiation coupled with low levels of migration and regional gene flow occur among the grey squirrel populations in Britain (Signorile et al. 2016).

At the time of the grey squirrel introduction, the native Eurasian red squirrel (*S. vulgaris*) population had been in a state of decline in Britain since the 1700s due to harsh winters, deforestation associated with industrialization and shipbuilding, hunting and the trade of wild animals, with red squirrels being particularly popular. By the late 19th Century, there were over 20,000 red squirrels sold annually in London markets, and such was the demand that red squirrels were imported from throughout Europe due to local shortages (Shorten 1954; O'Meara et al. 2018). Grey squirrels contributed to further red squirrel population declines through resource competition and the spread of squirrelpox, a viral infection, which is asymptomatic in grey squirrels but invariably pathogenic in red squirrels (Chantrey et al. 2014; McInnes et al. 2020). As a result of regional population extinction, the red squirrel is of

conservation concern within Britain, and is listed as least concern under the latest global assessment in 2016 with a declining population trend (Shar et al. 2017), and is protected under schedule 5 of the Wildlife and Countryside Act (1981 as amended. Conservation efforts to restore and enhance populations are widespread (Shuttleworth, Lurz & Robinson 2021).The island of Anglesey off the coast of north Wales, which is connected to the mainland via two man-made bridge links has been the focus of red squirrel conservation efforts since 1998 (Shuttleworth et al. 2002, et al..

Complete eradication of grey squirrels from Anglesey occurred in the summer of 2013 (Schuchert et al. 2014; Shuttleworth et al. 2015), but two years later, several grey squirrels were reported by local people, requiring reactive management by the local conservation team. Nuclear DNA was used to identify genetic similarities between the grey squirrel population on Anglesey and those on the adjacent mainland indicating that animals had naturally colonised Anglesey, although some genetically differentiated animals were also found in the southwest of the island, suggesting a separate colonisation event had also occurred. Reinvasion was likely to have occurred from the mainland by grey squirrels swimming the sea-channel, crossing one of the bridges, or inadvertently/intentionally transported in vehicles and subsequently released (Signorile & Shuttleworth 2016; Shuttleworth 2021). The long-term maintenance of red squirrels on Anglesey is reliant on the continued absence of grey squirrels from the island, requiring continuous mainland control efforts (Shuttleworth & Halliwell 2016; Shuttleworth et al. 2020).

The aim of this study was to genetically assess culled grey squirrels in north Wales over a period of nine years, with a view to understanding how the eradication efforts have impacted the contemporary genetic diversity of the species over time. Using mtDNA and microsatellite data, we also aimed to investigate the reinvasion of the species following eradication while also considering the genetic ancestry and legacy of the species' original introductions from North America. Based on our findings, derived from the monitoring of genetic diversity following population suppression, we make suggestions regarding the long-term adaptive management of an invasive mammal population with a view to maintaining protected native species.

Materials and Methods

Ethical statement

Grey squirrels were culled by trained practitioners as part of ongoing efforts to manage and reduce the invasive population. Animals were trapped and humanely dispatched as per Schedule 9 of the Wildlife and Countryside Act (Wildlife and Countryside Act 1981, 2017), which makes it illegal to release or allow invasive species to escape into the wild. UK Best Practice was adopted and included high welfare standards (Gill et al. 2019). Animals were not specifically killed for this study, but samples were opportunistically retained over the course of trapping efforts in case potential studies may arise where such samples might be of interest.

Sample collection

A total of 410 samples, (341 tissue and 69 hair) were collected between 2011 and 2020 by the Red Squirrel Trust Wales (RSTW). Grey squirrel trapping was conducted in woodland areas on Anglesey (ANG), in Gwynedd (GWY) and Clocaenog (CL) Forest (Fig. 1). See Schuchert et al. (2014) and Shuttleworth & Halliwell, (2016) for more detailed descriptions of the study sites. A tissue or hair sample (30-40 tail hairs with follicles) was taken from each culled individual and stored at -20 °C.

DNA Extraction

Genomic DNA (gDNA) was extracted from tissue samples using the QIAGEN DNeasy Blood and Tissue® kits as per the manufacturer's instructions. The hair samples were extracted using the Macherey-Nagel™ NucleoSpin™ Tissue kit following the instructions provided for hair samples. The quality and quantity of the extracted tissue DNA was determined using the Nanodrop™ 8000 (Thermo Fisher Scientific Inc., USA). Purified DNA preparations were stored at -20 °C.

Mitochondrial DNA analysis

MtDNA analysis was performed on all samples extracted as part of this study. D-loop mtDNA primers published by Stevenson-Holt et al. (2013) were used to amplify a 329-bp region using the follow primer pair: Dloop forward primer 5'-GCCACCCCCAAGTTAAATGG-3' and Dloop reverse primer 5'-ATTCGTGCATTAATGCACTATCC-3'. A 10 µl PCR reaction mix consisted of 5 µl GoTaq® Hot Start Green Master Mix (Promega), 1 µl of a primer mix containing 5 µm each of the forward and reverse primers, 3 µl of molecular grade water and 1 µl of the DNA extract. Thermocycling conditions consisted of denaturation step of 1 min at 94 °C followed by 40 cycles of 30 s at 94°C, annealing 30 s at 52 °C, extension 1 min at 72 °C, final extension of 5 min at 72 °C. PCR products were visualised using gel electrophoresis and

positive PCR products were cleaned using the *micro*Clean (Clent Life Science, Stourbridge, UK) and sequenced using the forward primer on the 3500 Genetic Analyzer (Applied Biosystems).

MtDNA data analysis

MtDNA sequences generated in this study were compared by multiple alignments using the CLUSTALW method in MEGA, version 7 (Tamura et al. 2011). Haplotypes were identified using ARLEQUIN, version 3.5 (Excoffier and Lischer 2010). MtDNA haplotypes from this study were compared to those previously recorded in Britain (N = 12) by Stevenson-Holt & Sinclair (2015). An additional 50 mtDNA grey squirrel sequences (486-bp) sampled in North America and published by Moncrief et al. (2008) (accession numbers JX104415–JX104465) were compared to the British samples by multiple alignments using the CLUSTALW method as previously outlined. Data included the haplotypes previously identified by Stevenson-Holt & Sinclair (2015) and were combined with the data from this study and grouped as follows: Eastern USA (Virginia and Maryland) (N = 20), Mid-Western USA (Indiana) (N = 3) and Southern USA (Alabama, Georgia, Louisiana, Mississippi and Tennessee) (N = 27). Using 285-bp of the d-loop, a TCS network was constructed using a parsimony approach (Clement et al. 2002) in the programme POPART v.1.7.1 (Leigh & Byrant 2015), and haplotypes were colour coded by region of origin. DNASP version 5.10.01 (Librado and Rozas 2009) was used to estimate nucleotide diversity across the pooled 285-bp fragment mitochondrial DNA dataset (N = 66) and within the following three groupings: North America (N = 51), Britain (N = 15), Wales (N = 6) under the following parameters: C = conserved sites; V = variable sites; Pi = parsimony informative; S = singleton variable sites. DNASP version 5.10.01 was also used to assess genetic diversity by defining the number of haplotypes (n), haplotype diversity (Hd), nucleotide diversity (Pi), and the nucleotide average difference (K). Tajima's D and Fu's Fs values were estimated to test demographic expansion (Tajima, 1989; Fu, 1997), and significance of the data was calculated using 1,000 permutations across Wales and Britain and North America. We used the programme ARLEQUIN, version 3.5 to test for the presence of genetic structure by conducting an analysis of molecular variance (AMOVA), based on genetic distances within and among the groupings. Percentages of variation and F_{ST} were calculated, and their significance tested with 1,000 permutations of individuals among populations. In addition, F_{ST} was calculated for each pairwise comparison of the populations, with significance again tested using 1,000 permutations. Finally, all the above mtDNA analysis was also attempted on the north Wales population by defining the populations by year of cull and locality

to assess if any changes in nucleotide diversity and genetic structure occurred as a result of culling effort, but as haplotype composition remained largely consistent throughout the culling effort, little insight could be gain from further analysis within north Wales.

Microsatellite DNA analysis

A subset of the samples that were successfully haplotyped were subsequently genotyped (N = 256) using the following microsatellite loci: SCV3, SCV4 SCV6, SCV9, SCV15 (Hale et al. 2001), LIS3 (Shibata et al. 2003) GR05, GR11 (Fike et al. 2013), FO11 (Fike & Rhodes 2009) (Table 1). Samples were genotyped in duplicate using three multiplex reactions.

The PCR reaction contained 5 µl GoTaq® Hot Start Green Master Mix (Promega), 1 µl of the DNA, primer mix containing 5 µm each of the forward and reverse primers (concentration specified in Table 1) and molecular grade water in a 10 µl reaction. Thermocycling conditions consisted of an initial step of 95 °C for 10 min, followed by 20 cycles of 95 °C for 30 sec and a touchdown from 65 to 55 °C for 1 min decreasing by 0.5 °C per cycle, and then 72 °C for 1.5 min. This was followed by 20 cycles of 95 °C for 30 sec, 55 °C for 1 min, 72 °C for 1.5 min and a final extension of 72 °C for 10 min. Fragment analysis was completed using the 3500 Genetic Analyzer (Applied Biosystems). Alleles were scored using the GeneMapper software Version 5 (Applied Biosystems). All samples were amplified in duplicate and failed or inconsistent scores across both replicates were independently repeated from the PCR stage.

Microsatellite data analysis

The dataset was assessed for the presence of errors, including scoring error due to stuttering, allele dropout and the presence of null alleles, using the program MICRO-CHECKER v.2.2.3 (van Oosterhout et al. 2004). As samples consisted of a mixture of tissue and hair sourced DNA, the dataset was assessed to ensure it consisted of unique individuals and that the microsatellite panel used was sufficient to identify animals at an individual level. GENALEX v.6.5b (Peakall & Smouse 2006) was also used to assess probability of identity (PI) and probability of sibship (PIsib), that is, to assess how many microsatellites were required to identify unique, unrelated individuals, and related individuals.

Descriptive statistics were calculated for the overall sample set, and repeated by year and cull area to establish the genetic consequences of repeated culling. GENALEX was used to calculate observed (H_O) and expected (H_E) heterozygosity, the number of alleles (N_A). Allelic

Richness (A_R) and the inbreeding coefficient (F_{IS}) was calculated using FSTAT version 2.9.3 (Goudet 1995) 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. Tests for linkage disequilibrium were performed between pairs of loci using GENEPOP v.4.7 (Raymond & Rousset 1995; Rousset 2008).

The calculations for genetic diversity and genetic bottlenecks were repeated by dividing the data by locality and year of cull (Tab. 3). The number of animals culled in Anglesey ranged from one in 2016 and 2020 to three animals in 2014. Due to the small population sizes, further calculations were not completed for this site. Culling took place in Gwynedd and Clocaenog between 2011 and 2019 with sampling sizes ranging between 15 and 68.

The program BOTTLENECK v.1.2.02 (Piry et al. 1999) was used to assess the dataset for evidence of a genetic bottleneck. Bottlenecks are detected when there is an excess of heterozygosity in comparison to the number of alleles, as the number of alleles declines in a population before there is an impact on the level of heterozygosity (Luikart & Cornuet 1998). Expected heterozygosity at equilibrium (H_{eq}) was also calculated for each loci. This test was carried out using the two-phase model (TPM), which is recommended for use with microsatellite data. The TPM was used with the following settings: 80% single-step mutations, a variance among multiple steps of 12, and 5,000 iterations. The probability of significant heterozygosity excess was subsequently determined using Wilcoxon's signed rank test and the mode-shift indicator test which can detect the presence of a recent genetic bottleneck. A mode found outside of this normal L-shaped distribution is transient and detectable if the bottleneck has occurred in the last few dozen generations (Luikart et al. 1998).

STRUCTURE 2.3.4. (Pritchard et al.., 2000) was used to assign individuals to clusters using a Bayesian approach. A burn-in period of 250,000 Markov chain Monte Carlo steps was selected, followed by length of 750,000. An admixture model was chosen with LOCIPRIOR. The K values that were selected were 1 – 10 with the number of iterations set to 5. The results file generated was also entered into STRUCTURE Harvester (Earl & vonHoldt 2012) to assess and visualise all likelihood values across all values of K. The web server CLUMPAK was used to summarise and visualise the STRUCTURE results (Kopelman et al. 2015). To further investigate the presence of genetic structure, a principal coordinate analysis (PCoA) was

generated in GENALEX and visualised using ggplot2 (Wickham et al. 2016) in R studio (RStudio Team 2020). This was performed to further examine the genetic relationship among the individuals.

Genetic differentiation was evaluated with the software FSTAT. The data was separated out by cull year and location excluding the Anglesey data as there were not enough individuals. Pairwise F_{ST} values were generated under 1,000 permutations and a Bonferroni correction for multiple tests was applied.

Results

MtDNA

Six mtDNA haplotypes were recorded from 340 successfully sequenced individuals sampled across north Wales and Anglesey, three of which were new to this study, (H13, H14 and H15), following on from the 12 haplotypes described by Stevenson-Holt & Sinclair (2015) (Fig. 2). Three of the haplotypes H7, H9 and H12 were previously recorded in northern England (Stevenson-Holt & Sinclair 2015). All six haplotypes occurred in Clocaenog, five in Gwynedd (excluding H15) and three in Anglesey (H9, H12 and H14). When the haplotypes recorded in north Wales were compared to the haplotypes previously recorded in Britain by Stevenson-Holt & Sinclair (2015), H7, H9 and H12 had been previously recorded in northern England.

The network analysis of haplotypes from Britain and North America (Moncrief et al. 2012) and our study, split haplotypes into two clusters (Fig 3). The first cluster included haplotypes unique to this study (H13, H14 and H15), haplotypes from Britain, and haplotypes from the USA. The second cluster included haplotypes from the USA, and two haplotypes from Britain (H8, H10). The entire dataset included 56 polymorphic sites, 15 singleton variable sites and 41 parsimony informative sites. Within Britain, there was 24 variable sites, almost half of the total occurring in North America where 53 variable sites occurred. Interestingly, 16 variable sites occurred within our study site in north Wales which contained 16 variable sites, representing almost 29% of all variable sites, but only 10% of the total number of haplotypes detected throughout Britain and North America. Nucleotide diversity, a measure of relative age of the population, showed little difference between the USA (0.03617), the original population source of grey squirrels, and the invasive populations in Britain (0.02583) and Wales (0.02222).

Haplotype and nucleotide diversity remained consistent across the dataset, but the average number of nucleotide differences detected within our study site in North Wales ($K = 6.3$), was only slightly lower than that detected overall in Britain ($K = 7.4$), and not far behind that of North America ($K = 10.3$). Negative Tajima's D can suggest the presence of an excess of low frequency polymorphisms relative to expectation and can be indicative of a selection sweep or population expansion which was the case in the populations in in this study, however, these values were all non-significant for Tajima's D, but Fu's F statistic significantly deviated positively from 0 in Britain (-9.282), USA (-44.934) ($P < 0.001$). The detection of negative deviations of Fu's F indicates a potential recent recovery from a population bottleneck, and this was seen both in the species' native range in both North America and in its invasive range in Britain and may be caused by a deficiency of alleles as the populations are expanding. An

AMOVA, using genetic distances within and among the populations in Britain, Wales and the USA, showed that most of the variation was attributable to the divergence within the populations, 95.1% and overall F_{ST} = 0.049, bootstrap 0.05/ 99.95 percentile range -0.009 – 0.102. Pairwise fixation indices for combinations of the three populations also lend support to the identified genetic structure with values ranged from -0.077 between Wales and Britain, 0.05 between Britain and the USA and 0.07 between Wales and the USA, but only the pairwise comparison between Britain and the USA was significant $P < 0.05$.

Microsatellite DNA

Of the 256 successfully genotyped individuals, the success averaged 98% across all loci, ranging from 95.6% at GR05 to 100% at LIS3. No evidence of large allele dropout was detected at any loci, but evidence of homozygote excess was detected at the following loci: SCV9, SCV15, GR11, SCV6 and LIS3 ($P < 0.05$). Although an excess of homozygotes can be caused by stuttering or the presence of null alleles, we carefully re-examined all data which had been analysed in duplicate from the PCR stage for consistency and found no evidence of human error. Four animals were found to have two matching genotypes; C15166 and G1653 were found to be identical to each other. As were C1217 and C15137 identical to one another. In both cases animals were successfully genotyped across all loci. These matching samples were removed to reflect the actual number of 254 individuals when assessing individuals by cull year and location.

Average probability of identity was 2.9×10^{-1} across all loci, with a cumulative PI of 4.5×10^{-7}. Average probability of sibship was 5.4×10^{-1} across all loci, with a cumulative PIsib of 2.4×10^{-3}. The number of alleles averaged 6.7, with similar levels of allelic richness observed at 6.6. Average observed heterozygosity was 0.35, while average expected heterozygosity was 0.56. Average F_{IS} values were 0.35 and all loci deviated significantly from Hardy–Weinberg equilibrium at the 5% significance level. SCV3 showed no variability across the dataset. Linkage disequilibrium was detected in eight of the 36 possible pairwise comparisons at the 5% significance level, three of which remained significant following a Bonferroni correction ($P = 0.0014$). This included the following pairs: SCV6 and GR05; SCV4 and SCV15 and SCV4 and FO11.

There was no evidence of a genetic bottleneck in the dataset. The probability value for a one-tailed Wilcoxon's signed rank test for an excess of heterozygosity was $P = 0.84$. The mode-

shift test also showed a normal L-shaped distribution providing further evidence for a lack of a genetic bottleneck. The data showed that while some loci exhibited differences, both in H_E and H_{EQ}, there was not a significant excess of heterozygosity relative to the number of alleles due to differences between the levels of expected heterozygosity (e) relative to heterozygosity estimated under mutation–drift equilibrium (H_{EQ}) (Table. 2).

Overall, the number of alleles and allelic richness remained similar across all years and localities, although a slight decrease over time was observed in Gwynedd (A_R = 4.59 - 4.37). Levels of expected and observed heterozygosity also remained relatively consistent in Clocaenog, with a slight increase over time in both statistics occurring throughout the cull period (H_O = 0.494 -0.551; H_E = 0.494 – 0.551), while in Gwynedd, a slight overall decrease occurred (H_O = 0.410 - 0.353; H_E = 0.612 – 0.609). F_{IS} values were positive and significant at the 5% level for all populations indicated high levels of inbreeding. No significant evidence of population bottlenecks occurred.

Genetic structure

A total of 254 individuals were analysed for population-based assignment. The delta K showed that K = 2 was the most likely number of genetic clusters within the population (Fig. 4A) (Evanno et al. 2005). The plot of the mean likelihood, L(K), established from combining each replicate per K value and associated standard deviation from STRUCTURE HARVESTER showed a gradual plateau occurring in the dataset from K = 3 to K = 5, but this also coincided with an increase in variance suggesting the true K value lay between K = 2 or K = 5 (Fig. 4B). At K = 2, the majority of animals clustered in one population, with a second cluster consisting of animals culled in Gwynedd in 2014 and one animal culled in Anglesey in 2020 (Fig 4c). Overall, the STRUCTURE analysis indicated that there was genetic differentiation between Clocaenog and Gwynedd, and genetic differentiation within the Gwynedd population, particularly after the 2014 cull. There was also some evidence of genetic clustering in the PCOA (Fig. 5(A) and (B), which accounted for 20% of the genetic variation within the data. However, the genetic structure identified in Fig. 4, is not as clearly defined in Fig. 5, although this may be as result of the relatively low level of genetic variation presented in the PCOA. Pairwise F_{ST} values ranged from -0.004 between GW_2016 and GW_2019 indicating no significant genetic differentiation between those years, and 0.0125 between GW_2014 and CL_2011 indicating relatively strong and significant levels of genetic differentiation between

those culls (P = 0.002; Table 4). Overall, the population culled in Gwynedd in 2014 exhibited the highest levels of differentiation between all years and localities.

Discussion

In this study, we demonstrated that genetic monitoring can be used as a method to evaluate the effectiveness of a long-term culling operation to reduce an invasive species population. We used a combination of mtDNA to gain historical insights into the introduction and establishment of the grey squirrel in north Wales, and contemporary microsatellite DNA data to assess if culling had altered the genetic diversity. Previous studies have shown that culling is an effective management strategy as it shows a reduction in population size (Signorile & Shuttleworth 2016). This can trigger an inbreeding depression within the population which can reduce the chances of survival of the population (Charlesworth & Willis 2008). However, our study suggests a scenario of relative population stability despite intensive control efforts. This is likely a consequence of the high number of historical introductions of genetically diverse grey squirrels coming from a broad geographical area in North America, and continued gene flow from neighbouring populations within north Wales containing genetically differentiated populations as demonstrated through our mtDNA analysis.

mtDNA

A total of six mtDNA haplotypes were found within 340 individuals in the north Wales population, three haplotypes which were new to this study. Twelve haplotypes were previously recorded throughout Britain, but these haplotypes were taken from a relatively small sample sizes; 14 from Stevenson et al. (2013) and a further 73 from Stevenson-Holt & Sinclair (2015), and were representative of the populations in England and Scotland. However, given the large number of haplotypes found within north Wales alone, it is likely that the mtDNA diversity of the grey squirrel population is under recorded in Britain, and there are likely many other undocumented haplotypes present. Our mtDNA analysis showed that the haplotypes within north Wales are genetically diverse, and only two haplotypes, H9 and H15 positioned relatively close to each other on the network diagram (Fig. 3). Even the three haplotypes found on the island of Anglesey, H9, H12 and H14 are distinct from one another and likely originated from different introductions from North America.

When comparing the British haplotypes to those recorded in North America (Moncrief et al. 2012), the contemporary British population is highly diverse, with haplotypes from Britain found throughout the network which contained animals from its eastern and southern US distribution, an area including over 887 km^2 in comparison to approximately 209 km^2 in

Britain. Moncrief et al. (2012) only sampled 69 animals from throughout this region, which again suggests that mtDNA within North America is under recorded, but despite the relatively low intensity of sampling, we found three haplotypes from North America in the contemporary British population, H8, H11 and H12. Haplotype H8 was previously recorded in Cumbria in Northwest England, while H11 and H12 were recorded in Henbury in Northern England, a well-documented introduction site in Britain, from which other translocations took place (Stevenson-Holt & Sinclair 2015). Our analysis showed that both nucleotide and haplotype diversity were high in both Britain and North America, and there was some evidence of population expansion, which was to be expected in Britain due to the relatively recent history of the species but, was somewhat surprising in the species' native range in North America. However, some evidence of population expansion has also been recorded in native *S. vulgaris* populations in Europe (Grill et al.. 2009; Dozières et al.. 2012), which suggests that sciurids are capable of rapid population expansion, and this may be trait that leads to the invasive success of the grey squirrel, and warrants further research.

The high mtDNA diversity found within Britain today is testament to the large numbers of repeated introductions and translocations that took place over a 60-year period. This is further confounded by the species' naturally high level of genetic diversity owing to its evolutionary history in North America where its genetic legacy appears not to have been imprinted by glacial history, limiting interpretation of its phylogeographic history (Moncrief et al. 2012). Other *Sciurus* species have similar phylogeographic legacies including *S. niger* in North America and *S. vulgaris* in Eurasia (Grill et al. 2009; Moncrief et al. 2012; O'Meara et al. 2018). All have a high number of mtDNA haplotypes that cannot generally be traced to commonly recognised glacial refugia. While this legacy may on the surface appear to be a contributing factor to the overall success of the grey squirrel in terms of its colonisation ability following repeated introductions, the same cannot be said for the red squirrel, who has also been reintroduced and translocated multiple times in parts of Britain and Ireland and retains a similarly diverse genetic history (O'Meara et al. 2018).

Microsatellite DNA

In relation to the microsatellite data, we found the grey squirrel population was genetically diverse which was higher than some previous research on grey squirrels in Britain as seen in

Signorile et al. (2014). The levels of allelic richness recorded in north Wales are higher than previously reported for Northumberland in northeast England (A_R = 4.3) and in East Anglia in the east of England (A_R = 3.44). Levels of expected and observed heterozygosity were also higher for both populations in Northumberland (H_E = 0.66; H_O = 0.64) and East Anglia (H_E = 0.71; H_O = 0.77), and less of a difference between both expected and observed values of heterozygosity were observed (Signorile et al. 2014). F_{IS} values were also lower in both the Northumberland (0.04) and East Anglia (-0.15) populations (Signorile et al. 2014) suggested that these populations are less inbred than populations in this current study. In comparison to red squirrel samples studied in Ireland by O'Meara et al. (2018) where populations from Galway, Waterford and Wexford and from Northern Ireland were analysed and allelic richness levels ranged from 1.8 to 2.2 and average heterozygosity levels were recorded at Galway (H_O = 0.48, H_E = 0.56), Waterford (H_O = 0.38, H_E = 0.4), Wexford (H_O = 0.56, H_E = 0.44) and Northern Ireland (H_O = 0.6, H_E = 0.52). The grey squirrel population in this study in north Wales exhibit much higher levels of allelic richness, higher or similar levels of H_E and lower levels of H_O.

Given the high levels of inbreeding and the lower levels of observed heterozygosity in comparison to expected heterozygosity, it was anticipated that the population might currently or previously have experienced a genetic bottleneck (Luikart & Cornuet 1998). However, there was no evidence of a recent genetic bottleneck in our dataset as evidenced by a normal 'L' shaped distribution of mode-shift test and non-significant heterozygote excess. It may be the case that the high levels of allelic richness are countering or protecting the population from entering a bottleneck. Bottlenecks are detected when levels of allelic richness decrease before a decrease in heterozygosity has occurred resulting in higher levels of heterozygosity than expected by the number of alleles (Luikart et al. 1998). The high levels of inbreeding in absence of a genetic bottleneck is somewhat of a paradox, as invasive species are often expected to have overcome a genetic bottleneck during the invasion process (Frankham 2005). In the case of the grey squirrel in North Wales, it appears to have avoided a bottleneck, despite high levels of inbreeding, perhaps due to the high numbers of genetically diverse founders. In comparison to this, the grey squirrel populations in Ireland do appear to have gone through a bottleneck event according to Dominguez McLaughlin et al. (2022) in which they suggest that there were only two introduction events due to the genetic clustering observed in their study. A study with more comparable introduction events shows, similar genetic legacies in the invasive brown anole

lizard (*Anolis sagrei*), as multiple introductions from different areas, resulted in higher genetic diversity in invasion sites than in its original native range (Kolbe et al. 2004).

The impact of repeated culling over time appeared to illicit mixed results in relation to genetic diversity. For instance, observed and expected levels of heterozygosity generally decreased over time in the Gwynedd population between 2014 (H_O = 0.410; H_E = 0.612 and 2019 (H_O = 0.353; H_E 0.609), but increased in Clocaenog between 2011 (H_O = 0.336; H_E = 0.494) and 2015 (H_O = 0.372; H_E 0.551). However, variation also occurred within the sampling years, which is likely a consequence of geneflow and new animals entering the culled area, particularly in Clocaenog which is geographically closer to England and as was said earlier, is likely to have greater colonisation potential. This is further corroborated by an increase in the number of alleles and levels of allelic richness in the area, which increased during the culling period. Inbreeding levels were high and significant across all years sampled, and again no evidence of genetic bottlenecks was found. Indeed, the STRUCTURE and PCoA results from this study have shown that the cull that took place in Gwynedd in 2014 contained a genetically distinct population, with further evidence of population differentiation occurring between later culls in Gwynedd and Cloceanog, suggesting a mechanism for how migrants can introduce new diversity into a population upon arrival. These results were further supported by our analysis of population differentiation, which showed some significant support for population structure supporting our theory of inward geneflow following a culling event. It seems likely that the removal of animals creates a space whereby animals from nearby areas migrate to fill, and while Signorile et al. (2016) found that grey squirrels exhibited relatively low levels of dispersal, it seems that the removal or reduction of adjacent populations, encourages inward dispersal. In a conservation context, this mechanism is called a genetic rescue, whereby even a small number of new immigrants can contribute additional genetic variation to the remnant population reducing the risk of extinction (Whiteley et al. 2014). In the case of the invasive grey squirrel, it may be a mechanism which supports and protects the genetic diversity of the species following intensive control efforts.

The animals that dispersed onto and were culled in Anglesey, although small in number, did not differ from the populations in Gwynedd or Clocaenog, and likely originated from both areas. The most recent animal culled in Anglesey in 2020 clustered with the animals culled from Gwynedd in 2014, suggesting that while this cluster appeared to have been removed by earlier culls, it is likely that it was not fully removed and may be recovering.

Overall implications and recommendations

The genetic assessment of invasive species is important to inform ongoing management, particularly when invasive species are being actively controlled, which require significant resources such as finances and human effort. By assessing genetic diversity of grey squirrel samples collected from control efforts, we found that despite nine years of control efforts, the population is not likely to be exterminated in the near future without sustained regular culling efforts. The likelihood of new animals moving into a culled area is also high, as we demonstrated in our microsatellite data analysis, that once an area was cleared of a population, a new and genetically distinct population moved in to occupy that empty space. The high levels of mtDNA diversity, even within a small area of north Wales adds further genetic variability to the population. The implications of this means that control efforts have to take place at regular intervals to ensure that new squirrels do not invade a cleared area, requiring further financial and labour supports. A limitation of this study is that the samples were collected during the cull period, and we do not have representative prior samples prior to this period. This could have provided valuable insights into the initial levels of genetic diversity and genetic structure within the population which might have provided a better understanding of the potential impact regarding eradication and recolonisation, as recommended by Zalewski et al. (2016). Future studies could include molecular ecologists at the planning stage and should be incorporated into funding plans by state agencies considering long-term eradication programmes (Browett et al. 2020).

Despite the broad ecological similarity and complete niche overlap between the invasive grey squirrel and the native red squirrels, it is notable that both having been introduced, reintroduced, and translocated multiple times in Britain, the red squirrel continues to be much more susceptible to the negative effects of a decrease in population size than the grey squirrel. However, the grey squirrel appears to be able to recover from a reduction in population size as it increases dispersal and gene flow. This ability appears to be one of the biological factors that makes the grey squirrel such a successful invasive species (Shuttleworth et al. 2020).

One of the ways invasive species are thought to be so successful is because they often do not encounter stress to the same degree that they do in their native environment and may be released from predatory pressures. This can result in an increase in fitness which can counter other eco-evolutionary processes normally experienced by species in their native environments such as inbreeding (Colautti et al. 2017). Intriguingly, recent studies in Britain and Ireland have shown

that the presence of a predator, the pine marten (*Martes martes*) (Sheehy & Lawton 2014; Twining et al. 2020, 2021) is associated with a reduction of grey squirrel populations and thus facilitates a natural recovery of the red squirrel. As a result, several pine marten reinforcement projects have taken place in Britain (Sheehy et al. 2018; McNicol et al. 2020a, 2020b). Captive-bred pine martens have been released in the Gwynedd area of our study (Bamber et al. 2020). A combination of increased predator presence and an improvement in habitat may provide the best circumstances to maintain the Welsh red squirrel population, while actively reducing the grey squirrel. The investment in the restoration of predatory species like the pine marten to the north Wales countryside may provide benefits not only to the conservation and management of native and invasive species but will provide further increases in biodiversity benefiting the wider ecosystem and society in general.

Author Contributions

RS performed lab work. RS and DOM performed the data analysis and wrote the manuscript with contributions from AM, CS, DE, COR and CH. DOM, AM and CS conceived and designed the study. Survey, monitoring and eradication was performed by CS in Wales, and samples were provided by CS and DE. CH provided sequence data from a previous study.

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Data accessibility

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. Sequence data will be made publicly available upon publication at Genbank.

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Tables

Table 1. Microsatellite loci used to assess genetics of grey squirrels culled in Gwynedd, the Cloacaenog Forest, and Anglesey Island, north Wales, between 2011 and 2020 - SCV3, SCV4 SCV6, SCV9, SCV15 (Hale et al. 2001), LIS3 (Shibata et al. 2003) GR05, GR11 (Fike et al. 2013), and FO11 (Fike and Rhodes 2009). Microsatellites were amplified in three PCR multiplexes (Mix 1, Mix 2, and Mix 3). The fluorescent dye, expected size range, and concentration of each primer added for each microsatellite to the multiplex reaction are also shown.

	Dye	Size range	Conc (µM)	Forward 5'-3'	Reverse 5'-3'
Mix 1					
LIS3	6 - FAM	140 – 160	0.6	CCAACAGTTGCTGTCGCTCC	GTTTCTTCAAATCCTGAACCGGCCCC
SCV6	ATTO550	185 - 201	1	GCAATCCTTGTCCTTGCATT	TGAGTCATTGGATGAAAACCA
SCV4*	ATTO550	100 - 115	1	CTGGAGATGGAGTGAGTGAGG	CCAGGAATCCTCTTGAATGC
Mix 2					
SCV3	6 - Fam	134 - 183	0.5	TTGGCTCATGGTTTCAGAGA	CCCCTCACTTCCTCCATTTC
GR05	VIC	200 - 218	1	TCTATATAACCTCTGTTTCATTGTCAC	CATGAATCTTGTTGAGATCCTG
GR11	ATTO550	178 - 204	0.3	CGGTGTCTTCCTCAAGTTCC	GAATCATCACGCGAACTGC
Mix 3					
SCV9	VIC	190 - 218	1	TCATGCTAATGCCAATCACAA	TGGAAGTTCTGTGGACGTTTC
SCV15	6 - FAM	258 - 296	0.8	TCCCATGATGAAATGTGTCC	AGACAGTCGAACCCAGCGTA
FO11	6 - FAM	174 - 184	0.8	CCATTTATGAGGGAGGTAGGG	TTGAATCTGTAGATTGGGTAGTATGG

** Mini microsatellite for SCV4 (miniSCV4R) was designed by O'Meara et al. (2018). The SCV4 reverse primer was also modified following Brownstein et al. (1996) to include 5'-GTTTCTT 3' sequences on the reverse primers to promote non-templated nucleotide addition.*

Table 2: Descriptive statistics for grey squirrels in north Wales across nine microsatellite loci. Abbreviations are as follows: number of samples amplified per loci (N), number of alleles per loci (A), Allelic Richness (A_R) observed heterozygosity (H_O), expected heterozygosity (H_E), expected heterozygosity at equilibrium (H_{EQ}), inbreeding coefficient (F_{IS}).

	SCV4	LIS3	SCV6	SCV3	GR11	GR05	SCV15	SCV9	FO11	Average	STDEV	%DEV
N	255	256	255	252	250	240	255	255	241	251	6.245	40.192
N_a	7	6	7	1	8	4	11	7	9	6.67	2.872	2.322
A_R	7	5.9	7	1	7.9	4	10.8	6.9	9	6.61	2.831	2.335
H_O	0.706	0.102	0.443	0	0.4	0.171	0.549	0.431	0.382	0.35	0.224	1.563
H_E	0.767	0.496	0.794	0	0.694	0.266	0.757	0.677	0.606	0.56	0.268	2.090
H_{eq}	0.677	0.627	0.679	0	0.717	0.477	0.796	0.677	0.677	0.59	0.238	2.484
F_{IS}	**0.085**	**0.795**	**0.44**	0	**0.422**	**0.36**	**0.272**	**0.372**	**0.37**	0.35	0.227	1.544

Table 3: Average descriptive statistics for the grey squirrel populations in north Wales across eight loci. Abbreviations are as follows: number of samples amplified per loci (N), number of alleles per loci (A), Allelic Richness (A_R) observed heterozygosity (H_O), expected heterozygosity (H_E), expected heterozygosity at equilibrium (H_{EQ}), inbreeding coefficient (F_{IS}). Values in bold indicate significant deviation from Hardy-Weinberg Equilibrium at P = 0.05, and after Bonferroni correction P = 0.00078.

SITE	YEAR	N	A	A_R	H_O	H_E	H_{EQ}	F_{IS}
GWYNEDD	2014	68	5.9	4.59	0.410	0.612	0.645	**0.337**
	2016	26	5.1	4.43	0.409	0.589	0.646	**0.325**
	2017	30	5	4.44	0.513	0.624	0.622	**0.193**
	2019	28	4.9	4.37	0.353	0.609	0.610	**0.436**
CLOCAENOG FOREST	2011	15	4	3.90	0.336	0.494	0.573	**0.350**
	2012	23	4	3.75	0.366	0.533	0.520	**0.334**
	2014	18	4.3	4.01	0.382	0.595	0.580	**0.382**
	2015	39	5	4.07	0.372	0.551	0.620	**0.336**

Table 4: Results of pairwise FST values between culling locations and years using eight microsatellite loci (i.e. omitting loci SCV3, which was affected by null alleles). Bold values indicate statistical significance after Bonferroni correction (P = 0.002).

	CL_2012	CL_2014	CL_2015	GWY_2014	GWY_2016	GWY_2017	GWY_2019
CL_2011	-0.017	0.011	0.006	**0.125**	0.033	**0.085**	0.037
CL_2012		0.009	-0.003	**0.123**	**0.038**	**0.091**	**0.044**
CL_2014			0	**0.089**	**0.034**	**0.053**	0.026
CL_2015				**0.115**	**0.045**	**0.086**	**0.051**
GWY_2014					**0.111**	**0.089**	**0.091**
GWY_2016						**0.03**	-0.004
GWY_2017							**0.022**

Figures

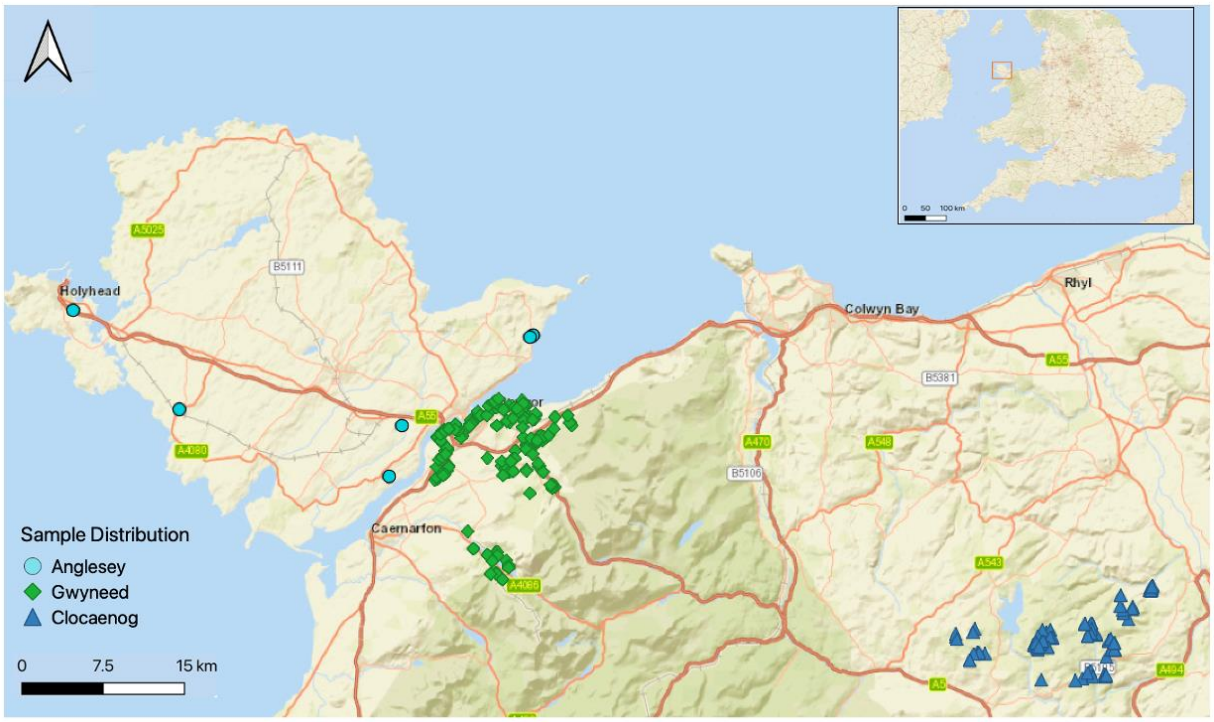

Figure 1. The distribution of grey squirrel samples collected from three areas (Anglesey (blue circles), Gwynedd (green diamonds) and Clocaenog (blue triangles)) across north Wales. The map also sows the A4, A5 and B4 road networks. The shaded green area between the Gwynedd and Clocaenog sample sites shows the mountainous region of Snowdonia. Top right inset: the location of north Wales, highlighted by the red square, relative to southern Britain, the east of Ireland and the north of France.

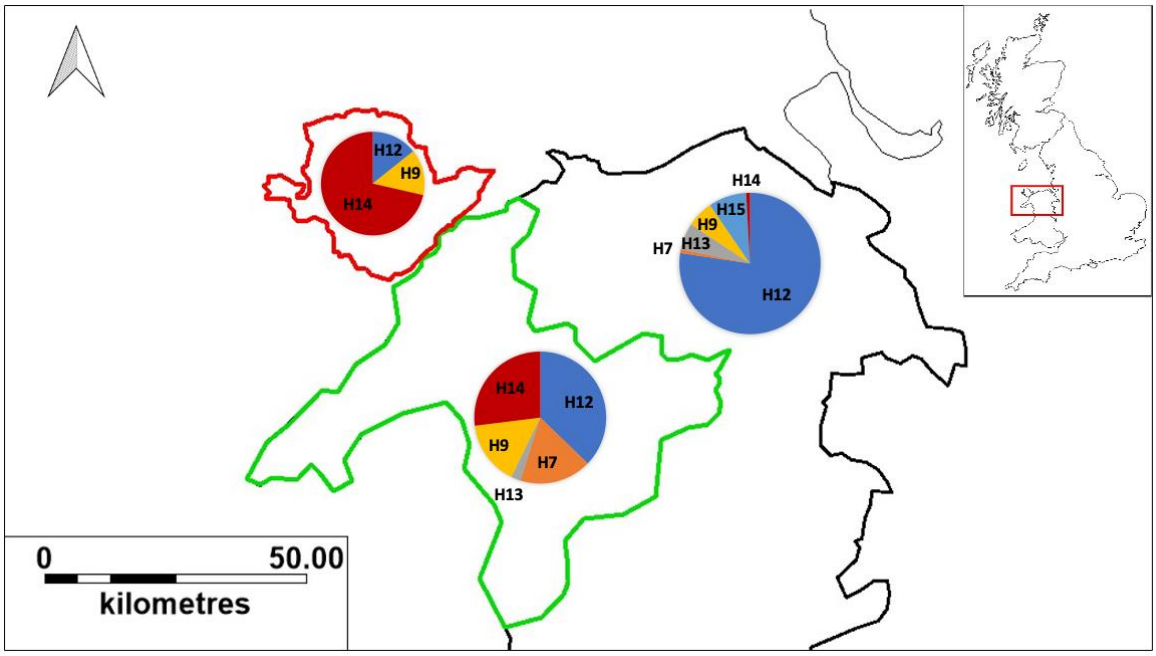

Figure 2. Distribution of mtDNA haplotypes found in Anglesey (N = 7) (location border in red), Gwynedd (N = 217) (border in green) and Clocaenog (N = 116) (border in black). Top right inset: the location of north Wales, highlighted by the red rectangle, relative to Britain,

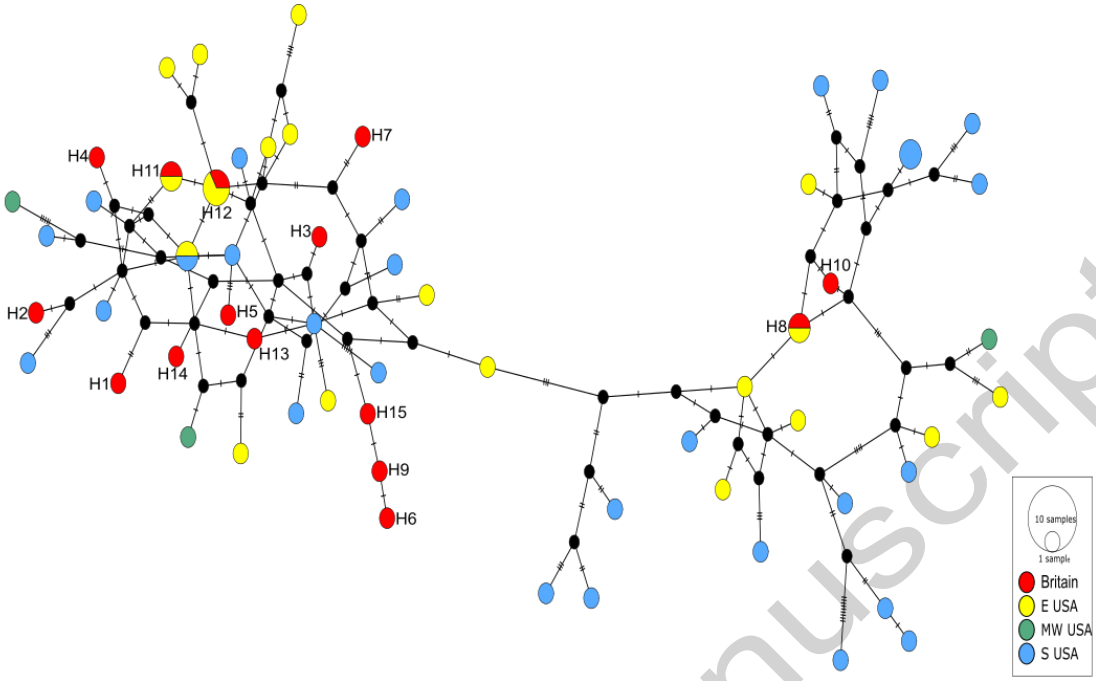

Figure 3. TCS network diagram generated using 285 bp D-loop mitochondrial DNA combing the new sequences generated from this study (H13, H14 and H15) with those previously published from Stevenson-holt & Sinclair (2015) and Moncrief et al. (2012). The British haplotypes H1 – H15 have been labelled for clarity.

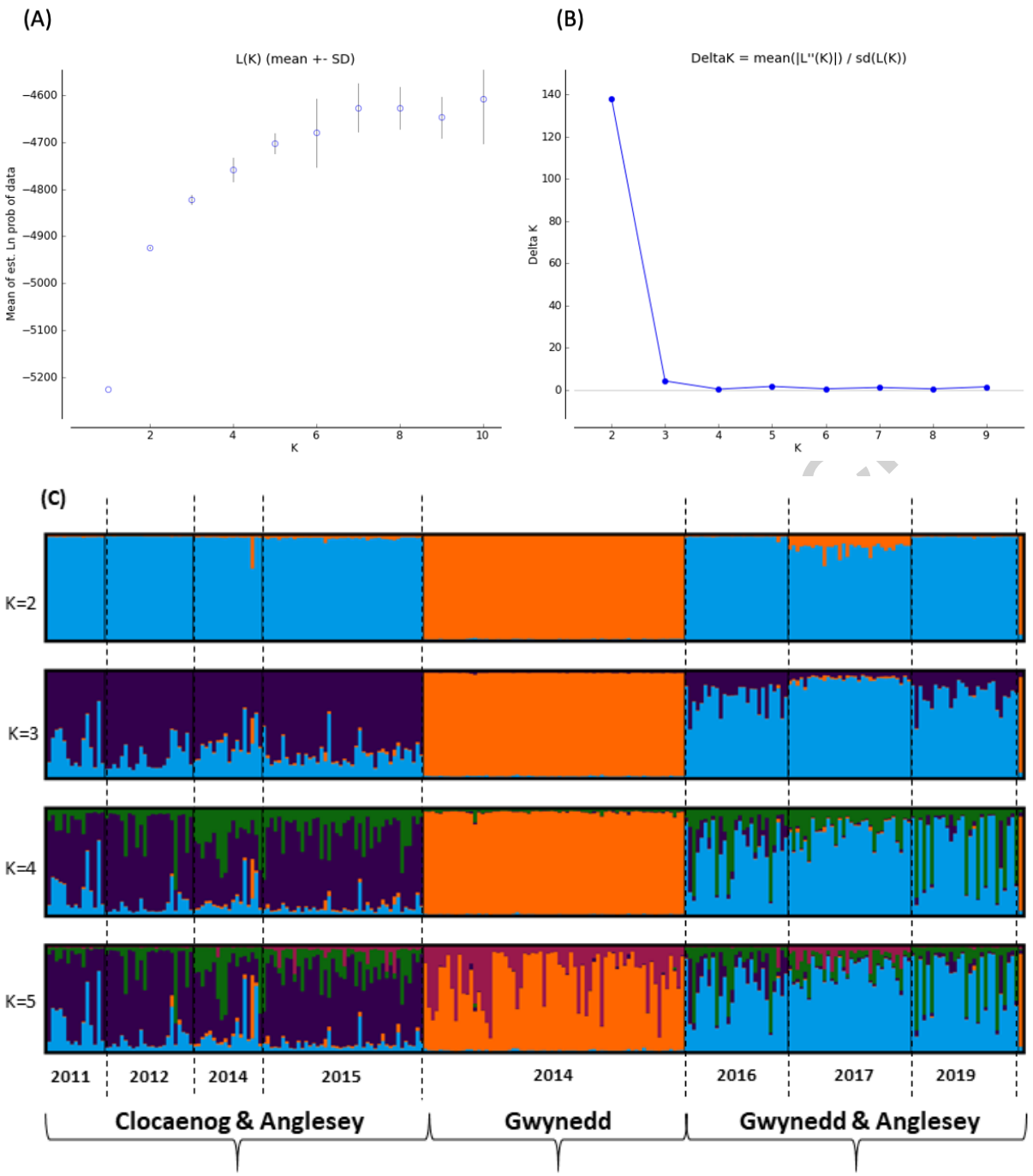

Figure 4: (A) plot of mean likelihood L (K) and standard deviation per *K* value of 254 individual grey squirrels genotyped at eight microsatellite loci. (B) Plot of Evanno's ΔK method (Evanno et al. 2005). (C) Membership of individual grey squirrels to K = 2, 3, 4 and 5 as inferred by STRUCTURE analysis. Each grey squirrel is represented by a single vertical bar. The geographic region and year of capture is indicated below the structure plot.

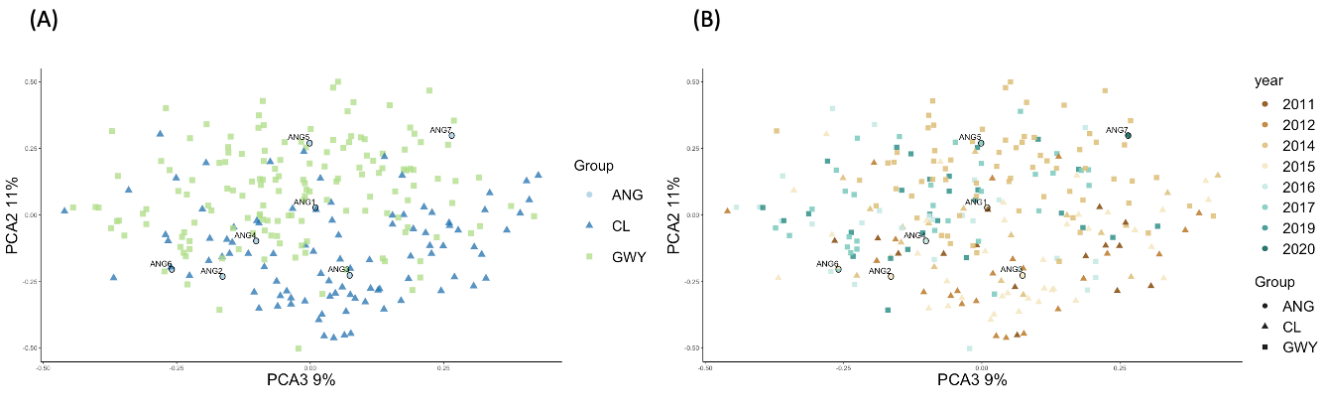

Figure 5. Principal coordinate analysis of individual grey squirrels. (A) 20% of genetic variation of the data grouped by location, (B) 20% of genetic variation of the data grouped by location and year sampled (N = 254).