A genetic analysis of grey squirrel (Sciurus carolinensis) populations in Ireland

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Abstract The grey squirrel (Sciurus carolinensis) is an invasive rodent in Ireland that has had detrimental impacts on the native Irish red squirrel (S. vulgaris) as well as on silviculture. This invasive species spread rapidly throughout Ireland, but in recent years appears to be declining in certain areas of the country. This study analysed the genetic profile of grey squirrel populations in Ireland to gain insight into their introduction, evolutionary history in Ireland, and vulnerability to management strategies. The genetic diversity and population structure of eight grey squirrel populations in Ireland was assessed using 11 species-specific microsatellite loci, and was compared to a small population from Tennessee, U.S.A., part of the native range of the grey squirrel. This is the first time these microsatellite markers developed specifically for grey squirrels have been used to study the species in Ireland. We found low to moderate genetic diversity overall across Irish populations as well as the presence of inbreeding. One population in particular, (in Co. Kildare), was differentiated from all other populations, which could indicate genetic isolation between Irish populations or a secondary introduction of S. carolinensis to Ireland.

Keywords Grey squirrel · Red squirrel · Genetic diversity · Population structure · Isolation · Introduction

Introduction

The grey squirrel (Sciurus carolinensis, Gmelin 1788) is a rodent native to eastern North America that has been introduced into South Africa, Australia, and several regions in Europe (Bertolino 2008). The grey squirrel was first brought into England from the (U.S.A) in the late 1800s (Middleton 1931), and then into Ireland in 1911 from an established population in Bedfordshire, England (Watt 1923). The origin of this introduction into Ireland was confirmed via genetic analyses (Signorile et al. 2016a). This is the first documented introduction of grey squirrels into Ireland and reports the release of 12 individuals in County Longford. There are two other reports of possible introductions in Ireland, however the validity of these reports is unconfirmed (O’Teangana et al. 2000).
The grey squirrel became established in 22 of the 32 counties in Ireland by 1999 (O’Teangana et al. 2000). The spread of grey squirrels into the west of Ireland has been impeded by the River Shannon (O’Teangana et al. 2000) and by fragmented landscape without enough suitable habitat or connecting woodlands to support migration (Carey et al. 2007; Flaherty and Lawton 2019). Populations of grey squirrels have now declined in many rural regions, although they persist in urban areas (Lawton et al. 2015, 2020). The grey squirrel’s success at colonising new areas has been associated with their dispersal habits and ability to overcome physical barriers (Hennessy et al. 2018; Koprowski 2005), their adaptation to a varied and versatile diet (Palmer et al. 2007), as well as their ability to prosper in urban-suburban habitats, in the presence of humans (Parker and Nilon 2008).

The grey squirrel is a highly invasive species in Ireland and its introduction has historically had a detrimental impact on the native European red squirrel (S. vulgaris, Linnaeus 1758). The introduction and spread of the grey squirrels led to a decline in red squirrel numbers in Ireland (Carey et al. 2007). Similar patterns of reduction in range and population size, and in some cases complete displacement of red squirrel populations have been reported in England and Italy (Gurnell and Pepper 1993; Wauters et al. 1997). This is due to competition between the red and grey squirrels and spread of disease. Exploitative competition for food resources between the two species has been an important factor in the reduction of red squirrel populations (Gurnell et al. 2004; Wauters et al. 2005). The grey squirrel has also introduced squirrel pox virus (SQPV) to red squirrel populations, and while it does not negatively affect grey squirrels, it is lethal to red squirrels (Tompkins et al. 2003; Sainsbury et al. 2000; Thomas et al. 2003; McInnes et al. 2013). Furthermore, grey squirrels cause significant amounts of damage to trees through barkstripping activity. In 2011, the annual economic cost of this forestry damage was estimated at €4.5million between the Republic of Ireland and Northern Ireland (Kelly et al. 2013).

The genetic profiles of introduced populations of grey squirrel in England, Italy, and Ireland were compared by Signorile et al. (2016b). This study indicated that the Irish populations of grey squirrels had the lowest genetic diversity, followed by the Italian populations. This could be explained by the smaller number of individuals that established populations in Italy and on the island of Ireland, which could in turn result in more effective control efforts for this invasive species. Another study investigated the genetic profile of grey squirrels in England with comparison to a population from the U.S.A. (Signorile et al. 2016a). Their results indicated low genetic diversity and presence of population structure for grey squirrel across populations in England compared to the U.S.A. population. In their analyses, Signorile et al. (2016a) included a collection site from Co. Louth in Ireland that had lower genetic diversity than the U.S.A. and England populations. Genetic analyses have also been utilised to determine the likely origin as well as the point of introduction of different populations of grey squirrels into regions in Europe and the U.K. (Signorile et al. 2016b; Stevenson-Holt and Sinclair 2015).

A study of seven grey squirrel populations across 349 individuals in Ireland using five microsatellites found no evidence of inbreeding in any population (McGoldrick 2011). The collection of samples used in the study by McGoldrick (2011) was subsampled for our study in addition to further samples collected specifically for our study.

Genetic information of invasive species can also be important for control and eradication plans. Analysing the genetics of invasive species allows the identification of a population size at which control/eradication becomes feasible in order to prevent the re-colonisation and spread of the target species (Robertson and Gemmell 2004). Genetic diversity of grey squirrels following eradication efforts on the Isle of Anglesey, England was lower than on the mainland, where similar eradication efforts were made (Signorile and Shuttleworth 2016), and demonstrated that genetic analyses could be a useful tool to incorporate into eradication plans of the invasive species. Squirrel samples from Ireland have been used in several European studies (Signorile et al. 2014, 2016b), and McGoldrick (2011) also investigated the genetics of grey squirrels in a broad ecological study of the species in Ireland. However, there has been no published study investigating the population genetics of different grey squirrel collection sites in Ireland and Northern Ireland, compared to a U.S.A. population to gain insight into the evolutionary history and introduction pathways of grey squirrels in Ireland. Additionally, no study has investigated the population genetics of grey squirrels in Ireland using markers developed...
specifically for the grey squirrel. Here we used 11 species-specific microsatellite markers (Fike et al. 2013) to assess the genetic diversity, population structure, and evolutionary history of eight grey squirrel collection sites throughout Ireland and compare them to a small sample of squirrels from Knoxville, Tennessee U.S.A. This is the first time these species-specific markers have been used to study the genetics of grey squirrels in Ireland. We further aimed to examine potential evolutionary scenarios using the microsatellite data to see if there is evidence of more than one introduction of grey squirrels to Ireland or genetic isolation between population groups.

Materials and methods

Sample collection and DNA extraction

One hundred grey squirrel samples from nine collection sites, (eight collection sites in Ireland, and one in the state of Tennessee, U.S.A.) were obtained via a variety of methods (Table 1). Of these 100 samples, 96 were obtained from Ireland, and four from the U.S.A. All samples were obtained opportunistically from deceased individuals. Some of the Irish samples were donated to the study by culling programmes in Northern Ireland co-ordinated by ‘Ulster Wildlife’, Belfast, whereas the remaining Irish samples were obtained from specimens that had been collected and used for another study (McGoldrick 2011) at Trinity College Dublin (TCD), Ireland. A random subsample of 40 grey squirrels from four different sites from the McGoldrick collection were used in addition to the 56 samples from four additional Irish sites collected specifically for this study. The Tennessee (TN) samples were collected from roadkill individuals in Knoxville, TN, U.S.A., and included in the study for comparison to the Irish samples.

Approximately 50 mg of tissue from the ear pinna or thorax muscle was used for DNA extraction. DNA was extracted using the ‘E.Z.N.A Tissue DNA kit’ (OMEGA Bio-Tek, Norcross, Georgia, U.S.A.), following the manufacturer’s protocol but scaled-up (doubling) the tissue sample size and quantity of reagents to ensure a large quantity of DNA was obtained from each sample. Quantity and purity of isolated DNA was assessed using a spectrophotometer (SimpliNano; Biochrom, Holliston, Massachusetts, U.S.A.).

PCR and genotyping

Eleven polymorphic dinucleotide repeat motif microsatellite loci were amplified using primers developed for grey squirrels (Fike et al. 2013). PCR was

| Sampling site          | County (Ireland)/State (USA) | Number of individuals | Source of specimens | Assigned group | GPS co-ordinates          |
|------------------------|------------------------------|-----------------------|---------------------|----------------|---------------------------|
| Clongowes Wood         | Co. Kildare                  | 5                     | Trinity College Dublin study | Northern Group | 53.313944, −6.682928 |
| Beauparc Estate        | Co. Meath                    | 14                    | Trinity College Dublin study | Northern Group | 53.687843, −6.575360 |
| Castle Leslie          | Co. Monaghan                 | 15                    | Trinity College Dublin study | Northern Group | 54.316063, −6.485917 |
| Portglenone Forest     | Co. Antrim (south)           | 6                     | Trinity College Dublin study | Centre Group  | 54.861770, −6.471334 |
| Glens of Antrim/Braid Valley | Co. Antrim (north)   | 15                    | Culling Programme, N.I. | Centre Group  | 55.033119, −6.148094 |
| Mourne Mountains       | Co. Down (south)             | 19                    | Culling Programme, N.I. | Centre Group  | 54.186147, −5.999012 |
| Hollywood/Stricklands Glen, Belfast | Co. Down (north) | 15                    | Culling Programme, N.I. | Centre Group  | 54.626158, −5.832750 |
| Muff Glen/Millgrove, Eglington | Co. Derry                  | 7                     | Culling Programme, N.I. | Southern Group | 55.005514, −7.182902 |
| Knoxville              | Tennessee                    | 4                     | Roadkill            | N/A            | 35.953067, −83.926594 |
carried-out in 10 µL reaction mixtures consisting of 2 µL of DNA sample (2 ng/µL), 0.5 µL dimethyl sulphide (DMSO), 5 µL of Accustart II PCR SuperMix (QuantaBio, Beverly, Massachusetts, U.S.A.), 0.5 µL of both reverse and forward primer (5 µM each), and 1.5 µL sterile, distilled water. A positive and negative control for each primer was added, with the positive control consisting of a DNA sample that was amplified successfully across all 11 loci, and the negative control consisting of a reaction mixture with water in place of a DNA sample. A touchdown cycling process was used for all reactions (Korbie and Mattick 2008). The thermal profile used for all PCR was as follows: 94 °C for 3 min for initial DNA denaturation, 15 cycles of further denaturation at 94 °C for 40 s, annealing step beginning at 63 °C and decreasing by −0.5 °C each cycle for 15 s, and extension at 72 °C for 30 s. This was followed by 20 cycles of 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 30 s, with final extension at 72 °C for 4 min. PCR products were visualised and sized using a capillary electrophoresis system (QIAxcel; QIAGEN, Valencia, California, U.S.A.) with an internal alignment marker of 15–600 bp. A 25–500 bp DNA size marker (QIAxcel; QIAGEN, Venlo, The Netherlands) was added to each PCR plate to provide amplified allele size standards. Any failed or weak PCR reactions were repeated twice. Due to amplification failure across three or more loci, one squirrel DNA sample (from the Co. Down, South collection site) was removed from the dataset. This resulted in 99 samples used for further analysis. 

Data analyses

Raw allelic data was binned to allelic categories according to fragment length using the Excel macro FLEXIBIN (Amos et al. 2007), which also allows manual checking for possible errors. Software program R version 3.6.3 (R Core Team 2020) was used for all statistical analyses. To ensure the number of microsatellite loci used in the study was sufficient to discriminate between tested individuals and capture their genetic variation, genotype accumulation curve was generated using the poppr package (Kamvar et al. 2015).

Genetic diversity

Genetic diversity indices were calculated using the poppr package (Kamvar et al. 2015). Genetic diversity was assessed for 11 microsatellite loci across all nine collection sites (eight Irish sampled sites and one TN sampled site) for comparison. Number of alleles per locus and per collection site were calculated. Shannon–Wiener Diversity index (H) was calculated, as well as $H_o$, the number of the observed heterozygotes observed per locus, and $H_e$, Nei’s gene diversity (Nei 1978) or expected heterozygosity. The Shannon–Wiener diversity index is more sensitive to rare alleles and less weighted on more dominant alleles than other indices such as 1-D (Simpson’s diversity index) (Hill 1973). Unique alleles per locus and per collection site as well as evenness of allele distribution were calculated. Allelic richness ($A_r$) which is the rarefied number of alleles per locus that can imply the potential of a population to persist or adapt to different environmental conditions, was also calculated using poppr.

Population structure

We analysed the population structure across the eight Irish collection sites, and then reanalyzed with the inclusion of the TN population for comparison. We additionally performed these analyses on the eight Irish collection sites assembled into larger groups (Centre, Northern, and Southern Group) (Table 1). These groupings were based on initial genetic differentiation results.

HIERFSTAT package (Goudet 2005) was used to calculate the following Wright’s F-statistics: $F_{ST}$ (Fixation index)—a measure of allele fixation or genetic differentiation in a population; $F_{IS}$ (inbreeding coefficient), a measure of heterozygote deficiency; and pairwise $F_{ST}$ to analyse the genetic differentiation between pairs of collection sites.

STRUCTURE (version 2.3.4) (Pritchard et al. 2000), a program that identifies population clusters or groupings, was used to analyse the population structure of the grey squirrel collection sites. STRUCTURE was run with 100,000 burn-in period and 300,000 iterations, with 30 runs of each K value (K = 1–10). STRUCTURE Harvester version 06.94 (Earl 2012) was then used to visualize the results and to infer the
number of genetic clusters present (K) by calculating the optimum K value using the Evanno method (Evanno et al. 2005). These results were visualised as a bar graph using POPHELPER (version 2.2.6) (Francis 2017).

Nei’s genetic distance was used to create neighbour-joining (NJ) phylogenetic trees to visualise the genetic relatedness of samples in the collection sites. NJ trees were produced with 1000 bootstrap permutations to estimate the support value of the nodes within the NJ trees. We obtained NJ trees for all the collection sites. We also constructed an NJ tree for the eight Irish collection sites aggregated into three groups to investigate relationships between these groups of sampled sites.

Discriminant Analysis of Principal Components (DAPC), a multivariate analysis used to visualise the number of groups of genetically related individuals (Jombart et al. 2010), was calculated using the ADEGENET (Jombart 2008) package. DAPC combines Principal Component Analysis (PCA) and Discriminant Analysis (DA) analyses to determine the principle components that visualise the main patterns of variation and to differentiate between groups (Jombart et al. 2010). Two DAPC analyses were carried out: one analysis on all of the collection sites (Ireland and U.S.A.), and the second on the eight Irish collection sites divided into three groups.

Poppr was used for Analysis of Molecular Variance (AMOVA) to estimate the population structure and genetic differentiation among collection sites (Excoffier et al. 1992). AMOVA was carried out using 999 permutations for significance testing. Three hierarchical AMOVA analyses were performed: (a) across all nine collection sites (Ireland and U.S.A.), (b) among eight Irish collection sites, and (c) among the eight Irish sampled sites divided into three groups based on initial genetic differentiation results and geographical proximity. AMOVA was calculated by grouping individuals into hierarchical groups (individuals, subpopulations, and total population) to evaluate overall contributions to genetic variance.

The Mantel test was used to test for the presence of correlation among the physical geographical distances and the genetic distance of the Irish grey squirrel sampled sites (Diniz-Filho et al. 2013). This statistical test was completed using the MASS (version 7.3-51.4) package (Venables and Ripley 2002).

Evolutionary history construction

Approximate Bayesian Computations (ABC) were used to investigate the population history of grey squirrels in Ireland using the program DIYABC version 2.0 (Cornuet et al. 2014). DIYABC was used to compare different possible evolutionary scenarios from the microsatellite data to best explain the present-day diversity and population structure of the grey squirrel collection sites in Ireland and their relationship to the U.S.A. collection site. This method identifies the most likely scenario of the ones provided and could explain the colonisation pathways of the grey squirrel in Ireland. Here, we investigated the four most probable evolutionary scenarios that can explain evolutionary history of grey squirrels in Ireland, based on current knowledge and our initial genetic diversity and structure results.

In scenario 1, an ancestral population of grey squirrels gave rise to two genetically differentiated clusters in the U.S.A. One of these is the TN sample population, while the other group was introduced to England where the group separated into two different clusters which then underwent further bottlenecks as they were each introduced into Ireland. In scenario 2, an ancestral population gave rise to the TN population sampled in this study. From this TN population a bottleneck occurred as the squirrels were introduced to England from the U.S.A. In England, the introduced squirrels diverged into two genetic clusters, which were separately introduced to Ireland, each undergoing a bottleneck event from their small introduction to Ireland and giving rise to the Southern Irish group of grey squirrels (Co. Kildare collection site) and the Northern/Centre group in Ireland. In the third scenario, two distinct groups arose in the U.S.A. from an ancestral population. One of these was the TN population, and the other genetic group was introduced to England and underwent a bottleneck event, followed by a consequent bottleneck event when the squirrels were introduced to Ireland. In Ireland, they separated into two separate genetic clusters. In the final, fourth scenario we investigated, an ancestral population gave rise to two distinct groups in the U.S.A.—the TN population and another U.S.A cluster. Squirrels from this second U.S.A. group were introduced to England, and later Ireland, giving rise to the Centre/Northern
genetic cluster in Ireland, and the TN group was also introduced to England and then Ireland separately, eventually giving rise to the Southern genetic cluster in Ireland.

Results

Genetic diversity

Genetic analyses of 11 microsatellite loci was carried-out across 99 grey squirrel samples in the nine collection sites. Analyses of diversity per locus were performed both across the 96 samples from the eight Irish sites only (Table 2) and again across all nine sampled populations (including the TN population) for comparison (Table 3).

Analysis per locus across the eight Irish collection sites showed an average allelic richness (Ar) of 2.3. The overall average observed heterozygosity (Ho) was 0.20, whereas the calculated expected heterozygosity, or Nei’s gene diversity (He) was 0.49 indicating the diversity at these sites was lower than expected under Hardy Weinberg equilibrium. Mean Shannon–Wiener

Table 2 Genetic diversity indices and F statistics of 11 microsatellite loci across eight Irish collection sites

| Locus | Motif | No. of alleles | Ar | Ho  | He  | H   | Evenness | Pa | FST  | FST P-value | FIS |
|-------|-------|----------------|----|-----|-----|-----|----------|----|------|-------------|-----|
| GR05  | (AC)26| 4              | 2.10| 0.36| 0.71| 0.64| 1        | 0.21| 0.23 |             | 0.1 |
| GR09  | (AG)9 | 3              | 1.85| 0    | 0.29| 0.57| 0.54     | 0   | 0.18 | 0.2         | 1   |
| GR10  | (AC)11| 4              | 1.24| 0.12| 0.3 | 0.39| 0        | 3   | 0.6 | 0.63        |     |
| GR11  | (AG)12| 8              | 3.23| 0.65| 0.68| 1.39| 0.7      | 3   | 0.09 | 0.11        | 0   |
| GR16  | (AG)12| 5              | 2.05| 0.3  | 0.51| 0.96| 0.65     | 1   | 0.19 | 0.21        | 0.26|
| GR17  | (AC)19| 4              | 1.79| 0.18| 0.53| 0.84| 0.84     | 1   | 0.51 | 0.54        | 0.32|
| GR22  | (GT)12(GA)9| 6 | 2.22| 0.03| 0.41| 0.84| 0.53     | 3   | 0.12 | 0.13        | 0.92|
| GR36  | (AG)10(AT)13| 8 | 3.24| 0.3 | 0.72| 1.55| 0.68     | 2   | 0.13 | 0.14        | 0.55|
| GR48  | (AG)19| 5              | 2.42| 0.1  | 0.61| 1.05| 0.83     | 1   | 0.08 | 0.09        | 0.81|
| GR51  | (GT)20| 4              | 2.21| 0.06| 0.46| 0.81| 0.68     | 1   | 0    | 0           | 0.87|
| GR53  | (CT)28| 5.1            | 2.63| 0.18| 0.63| 1.13| 0.82     | 1   | 0.06 | 0.07        | 0.69|
| Average|      | 5.09           | 2.27| 0.2  | 0.49| 0.92| 0.66     | 1.55| 0.17 | 0.19        | 0.55|

Ar allelic richness, Ho observed heterozygosity, He Nei’s gene diversity, or expected heterozygosity, H Shannon–Wiener diversity index, FST genetic differentiation (negative FST values are taken as 0), FIS inbreeding coefficient, Pa private alleles at a locus across collection sites

Table 3 Genetic diversity indices and F-stats of 11 microsatellite loci across all nine sample sites

| Locus | Motif | Number of alleles | Ar | Ho  | He  | H   | Evenness | Pa | FST  | FST P-value | FIS |
|-------|-------|-------------------|----|-----|-----|-----|----------|----|------|-------------|-----|
| GR05  | (AC)26| 4                 | 1.98| 0.32| 0.39| 0.69| 0.63     | 1  | 0.23 | 0.25        | 0.11|
| GR09  | (AG)9 | 3                 | 1.76| 0    | 0.28| 0.55| 0.53     | 0  | 0.19 | 0.21        | 1   |
| GR10  | (AC)11| 6                 | 1.43| 0.08| 0.14| 0.38| 0.37     | 5  | 0.49 | 0.52        | 0.47|
| GR11  | (AG)12| 9                 | 3.87| 0.69| 0.69| 1.42| 0.69     | 4  | 0.08 | 0.09        | -0.02|
| GR16  | (AG)12| 5                 | 2.04| 0.29| 0.51| 0.94| 0.65     | 1  | 0.18 | 0.2         | 0.24|
| GR17  | (AC)19| 5                 | 2.03| 0.24| 0.55| 0.93| 0.78     | 1  | 0.47 | 0.5         | 0.23|
| GR22  | (GT)12(GA)9| 6 | 2.31| 0.06| 0.43| 0.88| 0.52     | 1  | 0.11 | 0.13        | 0.88|
| GR36  | (AG)10(AT)13| 11 | 3.44| 0.32| 0.74| 1.68| 0.63     | 5  | 0.13 | 0.15        | 0.53|
| GR48  | (AG)19| 6                 | 2.60| 0.12| 0.62| 1.09| 0.82     | 2  | 0.06 | 0.06        | 0.79|
| GR51  | (GT)20| 7                 | 2.52| 0.11| 0.48| 0.90| 0.63     | 4  | 0    | 0           | 0.78|
| GR53  | (CT)28| 5                 | 2.78| 0.22| 0.64| 1.14| 0.82     | 1  | 0.04 | 0.04        | 0.65|
| Average|      | 6.1               | 2.43| 0.22| 0.5 | 0.96| 0.64     | 2.27| 0.18 | 0.19        | 0.51|

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diversity index per locus was 0.92, indicating low diversity at these loci in Irish collection sites. Average number of private alleles per locus was 1.55.

These results of diversity in Irish sampled sites were compared to the results of all nine sampled sites (the eight Irish sites and the TN site together). The results that included the additional TN samples were comparable to Irish samples alone. The addition of the TN population to the analysis resulted in mean allelic richness of 2.43, mean observed heterozygosity of 0.22, mean Shannon–Wiener diversity index of 0.96, and average number of private alleles of 2.27.

Population structure

F-statistics were calculated for the eight Irish collection sites, and all nine sampled sites. In the Irish collection sites, $F_{ST}$ results ranged from 0 with no genetic variation (locus GR51), to 0.6 (locus GR10) showing high levels of genetic variation (Table 2). Mean $F_{ST}$ value was calculated to be 0.17 across the 11 loci of eight Irish collection sites indicating the presence of high genetic differentiation across the Irish collection sites. $F_{IS}$ values were found to range from $-0.01$ to 1, with mean $F_{IS}$ of 0.55 indicating the presence of inbreeding across the Irish collection sites at these 11 loci.

The same analyses were also carried out on all nine collection sites. The addition of the TN, population again yielded comparable results to the analysis of Irish sites only with an average $F_{ST}$ of 0.18, and an average $F_{IS}$ of 0.51. The addition of the TN, U.S.A site to this analysis appears to have slightly inflated genetic diversity and reduced inbreeding despite the small sample size.

Pairwise $F_{ST}$ of the nine collection sites (Table 4) showed presence of population structure with moderate to high levels of genetic differentiation between all collection sites ($F_{ST} = 0.05$ or higher) with the exception of the Co. Monaghan and Co. Down, North collection sites which showed low levels of genetic differentiation between them ($F_{ST} = 0.03$). Pairwise $F_{ST}$ values had a range between 0.03 (between Co. Monaghan and Co. Down, North sampled sites), to 0.26 (between populations in Co. Antrim, South and Co. Kildare). The Co. Kildare collection site was highly differentiated from all other sampled sites with pairwise $F_{ST}$ values of 0.19 or more. The TN collection site was moderately to highly differentiated from all Irish sampled sites, but did not show as high a level of differentiation from the Irish collection sites as the Co. Kildare collection site did.

Pairwise $F_{ST}$ analysis of the three groups of Irish collection sites showed low to moderate genetic differentiation between the Centre and Northern groups ($F_{ST} = 0.05$). There was higher genetic differentiation between the Centre and Southern groups ($F_{ST} = 0.08$), and the highest level of differentiation was between the Northern and Southern groups ($F_{ST} = 0.12$).

### Table 4 Pairwise $F_{ST}$ matrix of nine *Sciurus carolinensis* collection sites

| Collection sites | Co. Antrim (south) | Co. Antrim (north) | Co. Down (south) | Co. Down (north) | Co. Meath | Co. Monaghan | Co. Derry | Co. Kildare |
|------------------|--------------------|--------------------|-----------------|------------------|-----------|--------------|-----------|------------|
| Co. Antrim (north) | 0.065               |                    |                 |                  |           |              |           |            |
| Co. Down (south)  | 0.113               | **0.055**          | 0.055           | 0.153            | 0.158     | 0.159        | 0.059     |            |
| Co. Down (north)  | 0.153               | 0.143              | 0.083           |                  |           |              |           |            |
| Co. Meath         | **0.158**           | 0.159              | 0.091           | 0.059            |           |              |           |            |
| Co. Monaghan      | 0.112               | **0.091**          | **0.051**       | 0.030            |           |              |           |            |
| Co. Derry         | 0.177               | **0.102**          | 0.084           | 0.079            |           |              |           |            |
| Co. Kildare       | **0.263**           | **0.204**          | **0.216**       | 0.200            | **0.250** | **0.197**    | **0.198** |            |
| Knoxville, TN, USA | 0.155               | **0.079**          | 0.065           | 0.081            | 0.101     | **0.080**    | **0.130** | **0.212** |

Statistically significant values are shown in bold. Significance tests were performed with 10,000 permutations.
The Neighbour-joining (NJ) tree of all nine grey squirrel sampled sites showed the grey squirrel collection sites were divided into two main clusters (Fig. 1). One cluster consisted of the Co. Kildare group, and the second major cluster grouped together the remaining eight collection sites along with the TN, samples. Bootstrap value exceeds 95% with the nodes well-supported. The NJ tree produced for the Irish sampled sites grouped into three groups (Fig. 2) showed the Southern group to be more distant genetically from the Centre and Northern groups, which appeared to be genetically closer. Bootstrap values exceeding 99% showed nodes are very well supported.

DAPC results of all nine collection sites (Fig. 3) showed similar major groupings as the NJ tree with the sampled sites divided into two major clusters. One cluster consisted of the Co. Kildare collection site, whereas the other main cluster showed the remaining eight collection sites were all grouped closer together as in the NJ tree. DAPC results of the grouped Irish collection sites (Northern, Centre, and Southern groups) were also in agreement with the respective NJ tree, with the Southern group separated further from the Centre and Northern groups (Fig. 4).

Moreover, results from STRUCTURE (Fig. 5) inferred the presence of two genetic clusters. STRUCTURE was completed for $k = 2–4$, with STRUCTURE HARVESTER identifying two clusters (optimum $K = 2$) as being the most likely across the nine grey squirrel collection sites. This showed one Irish collection site (Co. Kildare) and theTN, population as one cluster, and the remaining seven Irish sampled sites as another cluster. The number of genetic clusters here is in agreement with the NJ and DAPC results, however the STRUCTURE results differ slightly from these as the TN, collection site was assigned to the same cluster as the Co. Kildare collection site, whereas the remainder of the Irish collection sites were assigned to the second cluster.

AMOVA partitioned the total genetic variation of the eight Irish collection sites across the 11 microsatellite loci (Table 5). The AMOVA of all eight Irish collection sites of grey squirrel showed that the majority of the genetic variation lies between individuals within collection sites, contributing 44.5% to total variation ($P < 0.001$). Variation within individuals accounted for 39.5% of the total variation ($P < 0.001$), and variation between collection sites contributed 16.0% ($P < 0.001$) of total variation. AMOVA was also carried out on the eight Irish sampled sites grouped into three groups (Northern, Centre, and Southern groups as before) (Table 5). The main source of variation was between individuals within a collection site, which accounted for 41.3% of the total variation ($P < 0.001$). Variation within individuals contributed 36.7% ($P < 0.001$) to the total variation, variation between groups (here meaning each
of the three groups of collection sites) contributed 17.7% ($P < 0.001$), and variation between collection sites within a group contributed 4.5% ($P < 0.001$) of the total variation. In both AMOVA analyses, variation between individuals within collection sites was the main source of variation, and this contributed more to total variation than variation within individuals, which indicates some level of population structure in Ireland. AMOVA analyses of all nine sites (including the TN, site) and all nine populations divided into four groups (the three Irish groups as before and the additional TN site as a group) had comparable results (Table 6).

The Mantel Test showed a positive correlative relationship between the geographical distance and the genetic distance of the eight Irish grey squirrel collection sites (Fig. 6). The relationship was weak but significant ($r = 0.22; P < 0.001$).

Evolutionary history construction

ABC analyses of four hypothesised evolutionary scenarios for the grey squirrel collection sites in Ireland and TN, strongly suggested a secondary introduction of grey squirrels from England to Ireland (Fig. 7). Scenario 2 (in which squirrels from the U.S.A were introduced to England where they diverged into two distinct clusters, which were then each separately introduced into Ireland), was shown to be the most likely scenario with a very high posterior probability of 85%, whereas each of the other scenarios had very low probability (scenario 1 = 2%, scenario 3 = 12%, and scenario 4 = < 1%). Scenario 2 regards the TN, collection site as having arisen from an ancestral population with a small introduction of this TN group to England where they then diverged into two separate clusters, from which two separate introductions to Ireland occurred. The ABC analysis showed that Scenario 2 is the most probable evolutionary
scenario to explain the current population structure of sampled Irish populations and the TN, collection site.

**Discussion**

This study provided novel insights into the population genetics of grey squirrels and suggested the possible occurrence of a secondary introduction of the species into Ireland. We found limited genetic diversity, presence of population structure, and inbreeding among Irish grey squirrels. Our results indicated two distinct genetic clusters of grey squirrels in Ireland, which were highly genetically differentiated from one another. There are two possible explanations for the genetic differentiation and diversity revealed in this study. These two distinct clusters could have arisen due to extensive separation and differentiation of populations following the one historic introduction, or due to a second introduction of grey squirrels into Ireland, which seems more likely given the limited time that the animals have been in Ireland. Here we will discuss both of these explanations in relation to our results. However, it is also important to note the limited sample size in this study, and further studies with greater sample sizes would be beneficial to corroborate our findings.

This study revealed overall low genetic diversity in Irish grey squirrels across 11 microsatellites. However, moderate genetic diversity was detected across some of the sample sites. Inbreeding at Irish collection sites was also indicated. This reduced genetic diversity and presence of inbreeding are in agreement with the history of the introduction of grey squirrels to Ireland via one small introduction in 1911. Inbreeding and limited genetic diversity as a result of small isolated introductions of grey squirrel was
also determined in a previous study that found Northern Ireland and Italian populations had lower genetic diversity than populations in the U.K. as they were founded by the introduction of a smaller number of individuals (Signorile et al. 2014).

Compared to the eight Irish collection sites, the TN, site had a higher number of alleles and number of private alleles than any of the Irish sampled sites despite small sample size. Although results from the eight Irish sites and from the combined nine sites (including the TN, site) were comparable, a very small difference was observed when the TN, sample site was included in the diversity analyses, which appeared to indicate higher genetic diversity. However, a test including more samples would be required to confirm that this difference is statistically significant. Contrast between native range and introduced populations has been seen in other animals with the diversity generally higher in the native range (Gilg et al. 2013; Tsutsui and Case 2001). Our results are consistent with the findings of a similar study that compared the genetic diversity of grey squirrel populations in the U.K. and one sample from Ireland to the U.S.A (Signorile et al. 2016a). The study showed the U.K. populations had lower genetic diversity and heterozygosity than the U.S.A population, whereas the Irish sample had lower diversity than both the U.K. and the U.S.A. population. The population in the native range in the (U.S.A.) had the highest diversity, followed by those in the U.K. where several introductions of grey squirrel took place, in contrast with Ireland, which had a smaller founder size (Signorile et al. 2016a).

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(2011), which indicated that no inbreeding occurred in Irish populations. McGoldrick (2011) also reported moderate genetic differentiation among their study sites but did not find the populations to be as highly differentiated as this study. However, McGoldrick (2011) carried out analysis using five moderately polymorphic microsatellites that were developed originally for red squirrels, whereas the

Table 5 AMOVA across 11 loci of (a) all eight Irish collection sites, and (b) the eight Irish collection sites divided into three groups (northern, central, and southern)

| Form of variation                     | Df | Sum Sq. | Variance | % of variation | P value |
|---------------------------------------|----|---------|----------|----------------|---------|
| (a) Eight Irish sites                 |    |         |          |                |         |
| Variation amongst populations         | 7  | 159.50  | 0.87     | 16.01          | 0.001   |
| Variation between samples within populations | 67 | 467.12  | 2.42     | 44.49          | 0.001   |
| Variation within individuals          | 75 | 161.00  | 2.15     | 39.51          | 0.001   |
| Total variation                       | 149| 788.21  | 5.43     | 100.00         |         |
| FST = 0.16. FIS = 0.53. FIT = 0.60    |    |         |          |                |         |
| (b) Eight Irish sites divided into three groups |    |         |          |                |         |
| Variation between populations         | 2  | 100.01  | 1.03     | 17.69          | 0.001   |
| Variation between subpopulations within a population | 5  | 59.49   | 0.26     | 4.46           | 0.001   |
| Variation between samples within a subpopulation | 67 | 467.72  | 2.42     | 41.27          | 0.001   |
| Variation within individuals          | 75 | 161.00  | 2.15     | 36.65          | 0.001   |
| Total variation                       | 149| 788.21  | 5.86     | 100            |         |
| FST = 0.18. FCT = 0.05. FSC = 0.53. FIT = 0.63 |    |         |          |                |         |

Df degrees of freedom, Sum Sq. sum of squared differences of an observation from the mean, Variance amount of variance at each level. % of variation = Percentage of variation of the total variation

(a) FST: heterozygosity among sampled sites in regards to the total heterozygosity of across all collection sites, FIS: inbreeding coefficient among individuals in relation to heterozygosity among collection sites, and FIT: observed heterozygosity among individuals in relation to the total heterozygosity of all collection sites

(b) FST: heterozygosity among the three groups in regards to the total heterozygosity of across all sample sites, FCT: heterozygosity among collection sites with respect to the heterozygosity of the groups, FSC: heterozygosity among individuals within sample sites, and FIT: observed heterozygosity among individuals in relation to the total heterozygosity

Table 6 Analysis of Molecular Variance (AMOVA) across 11 microsatellite loci of (a) nine Sciurus carolinensis sites (Ireland and TN), and (b) the nine S. carolinensis sites divided in four groups (northern, central, and southern Irish, and TN)

| Form of variation                     | Df | Sum Sq. | Variance | % of variation | P value |
|---------------------------------------|----|---------|----------|----------------|---------|
| (a) Nine sites (Ireland and TN)       |    |         |          |                |         |
| Variation amongst populations         | 8  | 173.54  | 0.88     | 15.87          | 0.001   |
| Variation between samples within populations | 69 | 485.38  | 2.39     | 43.27          | 0.001   |
| Variation within individuals          | 78 | 176     | 2.26     | 40.87          | 0.001   |
| Total variation                       | 155| 834.92  | 5.52     | 100            |         |
| FST = 0.16. FIS = 0.51. FIT = 0.59    |    |         |          |                |         |
| (b) Nine sites divided into four groups |    |         |          |                |         |
| Variation between populations         | 3  | 114.05  | 0.99     | 16.78          | 0.001   |
| Variation between subpopulations within a population | 5  | 59.49   | 0.26     | 4.39           | 0.001   |
| Variation between samples within a subpopulation | 69 | 485.38  | 2.39     | 40.55          | 0.001   |
| Variation within individuals          | 78 | 176.00  | 2.26     | 38.29          | 0.001   |
| Total variation                       | 155| 834.92  | 5.89     | 100            |         |
| FST = 0.17. FCT = 0.05. FSC = 0.51. FIT = 0.62 |    |         |          |                |         |
present study was completed using 11 highly polymorphic markers specific to grey squirrels. This difference in microsatellite choice could possibly explain the disagreement between results. The use of more highly polymorphic markers allows detection of more recent population variation as they can rapidly accumulate recent mutations (Guichoux et al. 2011). As well as this, using multiple different markers allows variations across more loci to be revealed.

Our results suggest that in Ireland, grey squirrels are differentiated into at least two subpopulations. McGoldrick (2011) also found significant population differentiation at a national scale in Ireland with differentiated subpopulations rather than a panmictic Irish population. This finding supports previous studies (Signorile et al. 2016a) that revealed high levels of population structure across introduced grey squirrel populations in the U.K. Our results from DAPC analysis and NJ tree implied that the sites we studied in Ireland were collected in at least two if not three Irish clusters. In this study, the samples in one collection site (Co. Kildare), were highly genetically differentiated from samples from all other Irish collection sites. This site in Co. Kildare, which is the most southern of the sampled Irish sites, was highly genetically differentiated from the other collection sites, and made up one genetic cluster (or subpopulation) in Ireland. Along with this one highly differentiated cluster, there were two more genetically similar clusters. Therefore, we suggest the sites studied here represent two distinct subpopulations in Ireland—the Co. Kildare cluster and the Northern/Centre cluster. It would be interesting to include more southern Irish collection sites as well as using larger sample sizes in a future study to determine whether or not grey squirrels in this region are highly differentiated from more northerly groups.

The highly differentiated collection site in Co. Kildare could potentially be a result of differentiation over time, and could have occurred after geographic separation due to some barrier that hinders the movement of individuals. This hypothesis also accounts well for the presence of slight inbreeding found across Irish populations due to all Irish squirrels originating from the introduction of a limited number of individuals, followed by subsequent restriction of gene flow between regions. Factors that may impede gene flow between collection sites could include habitat fragmentation or barriers, such as rivers and roads (Brunke et al. 2019; Hennessy et al. 2018; Selonen et al. 2018). There appears to be no major physical barriers that could impede dispersal of squirrels from the Co. Kildare site, but Ireland’s lack of forests could be an important factor. Forests are crucial habitats for squirrels, however, Ireland has the lowest forest cover in Europe at less than 11% of the total area (Irish Forest Service 2013). An investigation of possible dispersal routes of grey squirrels in Ireland (Flaherty 2016) evaluated the ‘least-cost pathways’ of squirrel movement, taking into account land cover and connectivity. The least-cost pathways found connective corridors that allowed grey squirrel dispersal throughout most of the country. The map produced in this study showed lower connectivity or unsuitable habitat in the general Co. Kildare area. Although there are corridors connecting the midlands and south to the north, there are also some large expanses with low connectivity among these regions, and the corridors do not appear to be direct. This lower connectivity and reduced suitable habitat (such as forested areas) could potentially be the cause of the reduced gene flow between the Co. Kildare collection site and the more northerly sampled populations. This may explain the positive correlation between genetic distance and geographic distance with the Mantel test results. The strength of the positive relationship between genetic distance and geographic distance in the Mantel test was weak, but significant. The Mantel
test would further support the hypothesis of differentiation due to some level of geographic separation.

Although geographical barriers to gene flow are a possible explanation for the high levels of genetic differentiation of the Co. Kildare site from all the other collection sites, it could also be a result of two separate introductions of grey squirrels into Ireland. The results of this study suggest that the Co. Kildare population could have a different source of origin than the other Irish sites. Though there has only been one documented introduction of the species to Ireland, there were reports alluding to possible further introductions of grey squirrels to Ireland though these appeared to originate from unreliable anecdotal sources (O’Teangana et al. 2000). ABC analyses have been used previously to determine the colonisation of regions by invasive species and indicate gene flow between genetic clusters of these invasive animals (Drygala et al. 2016). The origins of isolated populations of other rodents, as well as the origins of other mammals introduced from England to Ireland have also been identified using ABC analyses (Allen

Fig. 7 ABC analysis showing possible evolutionary pathways that could describe the genetic diversity and population structure found at the collection sites in present day. NA = occurrence of bottleneck, Na1-5 = unknown ancestral populations, Pop 1 = Northern/Central Ireland, Pop2 = Southern Ireland (Co. Kildare), Pop3 = TN
et al. 2020; Ingram et al. 2015). Our ABC analysis strongly suggested that the most likely evolutionary scenario to explain the genetic diversity and structure of the nine collection sites in this study was a second introduction into Ireland. This potential second introduction was shown by our ABC analysis to originate most likely from a second distinct genetic cluster in England. This analysis indicated the possibility that the grey squirrels that were introduced to England (and later Ireland) originated from a population genetically close to the TN, collection site. This does not necessarily indicate that the grey squirrel population in Ireland originated from Tennessee, but rather that of our sampled sites, the Tennessee population is the most likely to have been an ancestral population, which is in agreement with the actual history of the species. The grey squirrels that were introduced in England are then shown to have diverged into distinct genetic clusters. Individuals from two different clusters in England were then introduced to Ireland in two independent events.

A secondary introduction is very possible as small rodents can easily be introduced to new areas by anthropogenic movement (Brouat et al. 2014; Simberloff 2009). An additional introduction of genetically distinct grey squirrel individuals from England is also highly likely as several distinct genetic clusters in England have been identified (Signorile et al. 2016a). This could potentially explain the differentiation between the Co. Kildare collection site and the other Irish sites. Furthermore, it is a possibility that multiple introductions alongside natural migrations and spread have given rise to the two distinct grey squirrel Irish subpopulations suggested by these results. A secondary introduction combined with fragmented habitat with little connectivity could be the cause of such genetically differentiated groups of grey squirrels in Ireland.

**Implications**

The overall low diversity of grey squirrels as well as high genetic differentiation among some of the collection sites in Ireland has potential implications for the monitoring and control of grey squirrels in Ireland. The application of population genetics data has been used in the past as a tool to assess the genetic status of invasive species and develop control and eradication strategies for other rodents (Robertson and Gemmell 2004). In terms of controlling the invasive grey squirrel population in Ireland or continuing localised eradications, this low genetic diversity may offer opportunities to manage the invasive grey squirrel population or explain the recent regional eradications in parts of the country. Grey squirrel populations in Northern Ireland and Italy, which had low genetic diversity, were suggested to be more susceptible to control and eradication efforts (Signorile et al. 2016a). Low genetic diversity among some of the grey squirrel sites in Ireland could also prove beneficial to the survival of the native Irish red squirrel population. Deficiency in heterozygosity of grey squirrel populations could negatively affect their potential to adapt to changing conditions and lessen competition with the red squirrel. Poor genetic diversity could be implicated to some extent in the recent collapse in grey squirrel populations in the Midlands. Though this decline in grey squirrels in the Midlands area is associated mainly with increased pine marten Martes martes presence (Lawton et al. 2020; Sheehy and Lawton 2014), it could also be augmented by low genetic diversity in Irish grey squirrel populations, or the low genetic diversity may be hindering the grey squirrels from adapting to this recovering native predator.

The presence of moderate-to-high structure among populations and the private alleles found in the Irish squirrels implies that there is decreased gene flow between populations, which could also be advantageous in controlling grey squirrels. Although these results indicate that control of Irish grey squirrel populations could be feasible, more genetic analyses need to be completed alongside population ecology studies, as the ecology of squirrels themselves (for example their high reproductive rates), could allow them to persist after attempted control measures. Population numbers make a quick recovery after different control/eradication pressures (Goldstein et al. 2016; Lawton and Rochford 2007).

Population genetics has proven a useful tool in the monitoring and management of invasive species in the past (Abdelkrim et al. 2005; Mallez et al. 2015; Wuest et al. 2017) and could be a practical tool in monitoring populations of grey squirrels in Ireland. This is especially important with the recent change in grey squirrel distributions in Ireland and their uncertain future with the recent spread of the predatory Martes martes.
pine marten (Sheehy and Lawton 2014). However, a large-scale investigation with greater sample sizes and broader geographical scope to include more collection sites from the south of Ireland is recommended for future studies. It would also be very beneficial to include more U.S.A. collection sites with larger sample sizes for comparison.

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Data availability Our data will be placed on DRYAD repository once accepted for publication.

Declarations

Conflict of interest The authors have not disclosed any conflict of interest.

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