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Not out of the woods yet: genetic insights related to the recovery of the pine marten (*Martes martes*) in Ireland

CATHERINE O'REILLY[1], PETER TURNER[1], DECLAN T. O'MAHONY[2], JOSHUA P. TWINING[3], DAVID G. TOSH[4], CHRISTOPHER SMAL[5], KATE MCANEY[6], CIARA POWELL[1], JOHN POWER[1] and DENISE B. O'MEARA[1,*]

[1]*Molecular Ecology Research Group, Eco-innovation Research Centre, School of Science and Computing, Waterford Institute of Technology, Cork Road, Waterford, Ireland*
[2]*Agri-Food and Biosciences Institute, Belfast BT9 5PX, Northern Ireland, UK*
[3]*School of Biological Sciences, Queen's University of Belfast, 17 Chlorine Gardens, Belfast BT9 5AJ, Northern Ireland, UK*
[4]*National Museums Northern Ireland, 153 Bangor Road, Holywood BT18 0EU, Northern Ireland, UK*
[5]*Ecological Solutions, 64 The Grove, Rathdown Upper, Greystones, County Wicklow, Ireland*
[6]*Vincent Wildlife Trust, Donaghpatrick, Headford, County Galway, Ireland*

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In this study, the history of the pine marten (*Martes martes*) in Ireland is reviewed, revealing that the population has undergone several retractions and expansions over the last few hundred years. Here, we consider the genetic legacy of this flux in fortunes and its likely impacts upon the conservation and future recovery of the species. Using nuclear DNA markers (microsatellites), we found that the genetic diversity present in Ireland today is like that of other Irish carnivores, but there is evidence of a genetic bottleneck and low effective population size that might result in further reductions of diversity in the future. There is a lack of genetic structure, showing that the population has not been fragmented genetically, despite the low percentage of woodland in Ireland. We also reviewed the mitochondrial DNA diversity present in the Irish population and showed that there is only one contemporary and one extinct haplotype present; a reduced diversity relative to other Irish carnivores. The Irish haplotypes, both extant and extinct, are shared or are genetically similar to haplotypes commonly present in southern Europe today. We discuss the possibility of reinforcing the Irish population with animals from these sources to help supplement and maintain genetic diversity for future generations.

ADDITIONAL KEYWORDS: conservation – genetics – historical demography – mammals – phylogeography – population reinforcement – rewilding.

INTRODUCTION

Large, charismatic carnivores, including the wolf (*Canis lupus*), brown bear (*Ursus arctos*) and Eurasian lynx (*Lynx lynx*), are today recovering across human-dominated landscapes that formed part of their former range. One-third of Europe's mainland landmass now contains at least one of these large carnivores, and all species are said to have either stable or increasing population trends (Chapron *et al.*, 2014). This on-going re-establishment has been attributed to legislation granting protection to the species and their habitats, efforts to foster co-existence between carnivores and people, and overall improved public opinion (Chapron *et al.*, 2014). Large carnivores remain absent from Ireland (the island). It has been between almost 3000 and 9000 years since bears and lynx, respectively, roamed Ireland, whereas the wolf disappeared comparatively recently, a mere 300 years ago (Edwards, 2014; Montgomery *et al.*, 2014).

With increasing enthusiasm for rewilding, particularly for trophic restoration and the reintroduction of predators, an island such as Ireland, on the north-west periphery of Europe, is not going to experience a natural recovery of these species as has happened elsewhere in mainland Europe (Boitani & Linnell, 2015; Linnell & Jackson, 2019).

*Corresponding author. E-mail: domeara@wit.ie

Consequently, if Ireland is to host such species in the future, reintroductions will be required. However, given the problems associated with relatively recent reintroductions in Ireland of once extinct birds of prey, the golden eagle (*Aquila chrysaetos*) and the white-tailed eagle (*Haliaeetus albicilla*) (O'Toole *et al.*, 2002; O'Rourke, 2014; O'Donoghue, 2019), and the objections associated with reintroductions of a variety of species in Britain (the island) (Sandom & Wynne-Jones, 2019), much work would be required to undertake such efforts in Ireland. Indeed, in the case of mammal conservation, efforts might currently be best concentrated on understanding the fate and conservation requirements of extant resident species, including small- and medium-sized carnivores, such as the Irish stoat (*Mustela erminea hibernica*), otter (*Lutra lutra*), badger (*Meles meles*), red fox (*Vulpes vulpes*) and pine marten (*Martes martes*) (Lysaght & Marnell, 2016).

The past demography of the pine marten in Ireland is thought to be linked intrinsically to the history of woodland cover. Considered a woodland specialist, the earliest evidence of pine marten in Ireland dates to 2800 BP (Montgomery *et al.*, 2014). This corresponds to the late Bronze Age, a period when woodland cover was considered to have been high but beginning to decline (Mitchell & Ryan, 1997; Byrnes, 2007), and it is likely that the pine marten population thrived in such habitats. Ireland's woodland declined in the 16th and 17th centuries to 2% landcover and, despite moderate increases in the 18th century, it declined further to 1.5% at the beginning of the 20th century (OCarroll, 2004; Byrnes, 2007). Today, Ireland's forest cover stands at 11% total land cover, but only 1.2% is native woodland, with the majority consisting of non-native commercial conifer (Bullock & Hawe, 2013; Department of Agriculture, Food & the Marine (DAFM), 2019). Total forest cover in Ireland is 20% below the European average, but the highest it has been in Ireland for > 350 years (DAFM, 2019).

Over a 122-year period, from 1697 to 1819, > 230 000 skins of deer (*Cervus elaphus* and *Dama dama*), fox, rabbit (*Oryctolagus cuniculus*), mountain hare (*Lepus timidus*) and otter were exported from Ireland (Fairley, 1983). Commenting on the notable absence of both the red squirrel (*Sciurus vulgaris*) and the pine marten from these exports, Fairley (1983) suggested that the low numbers were attributable to the 'decay' of woodlands, and that the few furs that might have been available had a market demand within Ireland. It is likely that the pine marten population had crashed or that numbers were very low, a situation well documented and paralleled in the red squirrel, a species that requires similar woodland habitat, but was successfully reintroduced in the 19th century (Barrington, 1880; O'Meara *et al.*, 2018). There is no evidence that similar introductions of the pine marten took place during this period, and it is likely that the pine marten was subjected to persecution on Irish estates by gamekeepers and hunters, as commonly occurred in England and Wales (Strachan *et al.*, 1996; Birks, 2017; Sainsbury *et al.*, 2019). There is evidence that the pine marten still occurred in parts of Ireland during this time, including counties in the west, south-east and north-east (Patterson, 1894; Ruttledge, 1920). The sightings were most frequent in the south-east and parts of the west (Ruttledge, 1920; Gethin, 1936).

In the 1970s, owing to concerns regarding the status of the pine marten in Ireland, a national survey was conducted. O'Sullivan (1983) found that pine marten distribution was largely restricted to the mid-western part of Ireland. Subsequent legal protection at Irish [Wildlife Acts 1976 to 2012 and Wildlife (Northern Ireland) Order, 1985] and European level (European Habitats Directive, Annex V) and the banning of strychnine for use as a poison in the 1980s are all thought to have facilitated the recovery of the species. Recent island-wide surveys have shown that the species is now recovering and is found across parts of Ireland where it had been absent for many years (O'Mahony *et al.*, 2005, 2012, 2017a, b; Lawton *et al.*, 2020).

Previous genetic studies by Davison *et al.* (2001) and Jordan *et al.* (2012) revealed only one contemporary mitochondrial DNA haplotype present in the Irish pine marten population, hap *p*, whereas hap *a* was found to be the dominant haplotype in Britain. Hap *i* was historically present throughout both Ireland and Britain but is now absent from Ireland. Hap *i* is very rare in Britain but was found on an island off the west coast of Scotland in 2010 (Jordan *et al.*, 2012). In a wider phylogeographical study of pine marten by Ruiz-Gonzalez *et al.* (2013), hap *p* was incorporated as part of a larger DNA haplotype called Mm20. This haplotype was found to cluster with other haplotypes from southern and Mediterranean Europe, whereas the contemporary and dominant haplotype present in Scotland (Mm28, which incorporated hap *a*) grouped with haplotypes present in central and northern Europe, suggesting that the population within Britain had divergent origins. Efforts to examine the contemporary nuclear genetic diversity of the species have been limited to localized studies. Comparison of allele frequency of pine martens from south-east Ireland with French pine marten was lower per locus, as were levels of expected and observed heterozygosity (H_E and H_O) [$H_E = 0.35$, $H_O = 0.34$ (Ireland); $H_E = 0.59$, $H_O = 0.54$ (France); Mullins *et al.*, 2010]. In a similar small study from the midlands of Ireland, allele frequency, expected and observed heterozygosity ($H_E = 0.39$, $H_O = 0.46$) were also low when compared with European populations but slightly higher than south-east Ireland (Sheehy *et al.*, 2014).

The recovery of the pine marten population in Ireland and the accumulating evidence supporting its potential role in the suppression of an invasive species, the grey squirrel (*Sciurus carolinensis*), has been hailed as a conservation success story (Sheehy *et al.*, 2014, 2018; Sheehy & Lawton, 2014; McNicol *et al.*, 2020; Lawton *et al.*, 2020; Twining *et al.*, 2020a, b). However, the breeding population of pine martens remains low, estimated to be between 2330 and 3852 individuals (O'Mahony *et al.*, 2017a, b), and despite more ecological research having taken place into the species distribution, aided by the development of non-invasive genetic tools (O'Reilly *et al.*, 2007; Mullins *et al.*, 2010; O'Meara *et al.*, 2014; Sheehy *et al.*, 2014; O'Mahony *et al.*, 2012, 2017a, b), little is known about the contemporary genetic diversity of the species in Ireland. Furthermore, it is not known whether past population retractions and expansions have negatively impacted genetic diversity and how this might influence the ongoing recovery and maintenance of the population in the future.

The aim of this study was to assess the genetic diversity of the pine marten across Ireland, with a view to understanding how historical population retractions and expansions have shaped the contemporary diversity of the species, and to discuss the challenges faced by the pine marten as it continues to recover and expand. Based on the phylogeographical history of the species, we make suggestions regarding potential genetic sources to support the future conservation and reinforcement of the species in Ireland.

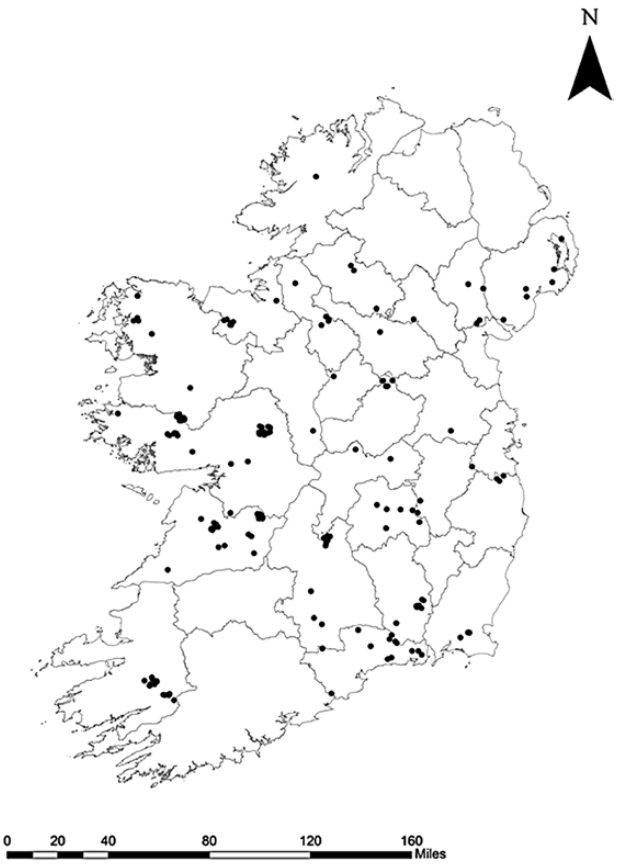

Figure 1. Distribution of Irish pine marten samples analysed in this study ($N = 249$).

MATERIAL AND METHODS

Sample collection

A total of 249 individual pine martens were collected from across Ireland, including Mullins *et al.* (2010), the National Pine Marten Survey from O'Mahony *et al.* (2017a, b), part of a PhD project in the north of Ireland (Twining, 2020) and from long-term collection of animals killed via road traffic accidents. Samples were collected from ~2008 to 2018. The distribution of the samples collected is mapped in Figure 1. The mitochondrial DNA (mtDNA) haplotypes from Davison *et al.* (2001) and Ruiz-Gonzalez *et al.* (2013) were also reanalysed with a view to understanding the phylogeographical origins of the Irish pine marten.

DNA analysis

Genomic DNA (gDNA) was extracted from hair and tissue samples using the ZR Genomic DNA-Tissue MicroPrep (Zymo Research, Irvine, CA, USA) according to the Solid Tissue and Hair protocol with Zymo-Spin II columns. Purified DNA preparations were stored at −20 °C. Samples were identified to both species and sex using the real-time PCR methods outlined by O'Reilly *et al.* (2008) and Mullins *et al.* (2010). Samples were genotyped in triplicate using the following 12 microsatellite markers: *Mel1*, *Ma2*, *Gg7*, *Mar21*, *Mvis1341* (as detailed by O'Mahony *et al.*, 2017a), *Mar53*, *Mar43*, *Mel105*, *Ma08*, *Mar64*, *Mer041* and *Mvis075* (Table 1). Genotyping was carried out as described by Croose *et al.* (2016).

Microsatellite analysis

The dataset was assessed for the presence of errors, including scoring error arributable to stuttering, allele dropout and the presence of null alleles, using the program MICRO-CHECKER v.2.2.3 (van Oosterhout *et al.* (2004), and unique individuals were verified via GENALEX v.6.5b (Peakall & Smouse, 2006). The number of alleles (A) and observed and expected heterozygosities were calculated via GENALEX v.6.5b (Peakall & Smouse, 2006). Allelic richness (A_R) and the inbreeding coefficient (F_{IS}) were calculated using FSTAT v.2.9.3 (Goudet, 1995). The significance levels

Table 1. Details of six additional microsatellite loci used in this study

Locus	Primer sequence 5′–3′	Source	Reference	Size range (bp)
Ma08	Forward: ***FAM***-GTTTTCTAATGTTTCGTGTG	Eurofins	Davis & Strobeck (1998)	102–108
	Reverse: CAGTGGTTGACTACAAGAAA	Eurofins	Davis & Strobeck (1998)	
Mel105	Forward: ***FAM***-GATATTCCCCTCCCACCACT	Eurofins	Carpenter *et al.* (2003)	194–196
	Reverse: ***GTTTCTT***AAGACAAAGTTCCCCTGTATTG	Eurofins	This study	
Mvis075	Forward: ***FAM***-GAAATTTGGGGAATGCACTC	Eurofins	Fleming *et al.* (1999)	151–153
	Reverse: ***GTTTCTT***GGCAGGATAGGATGTGAGCT	Eurofins	Fleming *et al.* (1999)	
Mer041	Forward: ***ATTO550***-TGTGTGATCTCTGGGAATTCTC	Eurofins	Fleming *et al.* (1999)	160–166
	Reverse: ***GTTTCTT***GCTCCCCAGATAAAAGC	Eurofins	Fleming *et al.* (1999)	
Mar43	Forward: ***FAM***-GTCACCCCAGGAGAGGGTG	Eurofins	Natali (2010); this study	114–132
	Reverse: GGTGCCAACTCAGCAGAAGG	Eurofins	This study	
Mar64	Forward: ***YAK***-GGCCCCAAAGTCTTACAGTTC	Eurofins	Natali (2010)	170–188
	Reverse: CGTTTTGAATCATGCTGTGG	Eurofins	Natali (2010)	

To facilitate multiplex reactions, the forward primer for *Mar43* and the reverse primers for *Mar43* and *Mel105* were redesigned. The fluorescent dyes (in bold italic text) used to label the forward primers were FAM, ATTO550 and Yakima Yellow (YAK). The reverse primers for *Mel105*, *Mvis075* and *Mer041* were modified with a 5′ sequence of GTTTCTT (in bold italic text) to promote non-templated nucleotide addition (Brownstein *et al.*, 1996).

for F_{IS} were calculated by randomizing the alleles within the population using 10 000 permutations and compared with the observed data to assess the presence of deviations from Hardy–Weinberg equilibrium. Tests for linkage disequilibrium were performed between pairs of loci using GENEPOP v.4.7 (Raymond & Rousset, 1995; Rousset, 2008).

The program BOTTLENECK v.1.2.02 (Piry *et al.*, 1999) was used to assess whether signatures of a genetic bottleneck were present in the Irish pine marten population. Genetic bottlenecks are evidenced by the presence of heterozygosity excess relative to the number of alleles. This occurs in a system impacted by a genetic bottleneck because the number of alleles declines in the population before there is an impact on the level of heterozygosity, pulling the two statistics out of their natural state of equilibrium (Luikart & Corneut, 1998). 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 5000 iterations. The probability of significant heterozygosity excess was subsequently determined using Wilcoxon's signed rank test. The mode-shift indicator test was also used to detect the presence of a recent genetic bottleneck. A non-bottlenecked population at near mutation–drift equilibrium is expected to have a high portion of alleles occurring at low frequency. The test groups the microsatellite alleles into ten frequency classes to test whether the distribution follows a normal L-shaped distribution where the least frequently occurring alleles are the most numerous. A mode-shift 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). The *M*-ratio statistic developed by Garza & Williamson, (2001) was also used to investigate a potential reduction in population size. During a reduction in population size, the number of alleles (k) is expected to decline faster than the range in allele size (r) because most of the alleles lost will fall within the range rather than at the edge, and as a result the *M*-ratio (k/r) is expected to be lower in a population that has been reduced. The *M*-ratio value in populations that have not experienced a reduction in population size is expected to be ≥ 0.8, and a value < 0.7 indicates a reduction in population size. The effective population size and associated 95% confidence intervals were estimated using the program NeEstimator v.2 (Do *et al.*, 2014), using the linkage disequilibrium method developed by Waples & Do, (2008) and applying a *P*Crit value of 0.02 to screen out rare alleles from the estimate.

The number of putative genetic clusters (K) present in the dataset was modelled using the program STRUCTURE v.2.3.1 (Pritchard *et al.*, 2000; Falush *et al.*, 2003). The Bayesian clustering algorithm implemented in the program was used to analyse the data with default settings and a burn-in period of 100 000, followed by 400 000 replicates with no prior population information. Values of K ranged from one to six, with each K value replicated five times to assess the most likely number of inferred populations. The most likely K was assessed by calculating the mean likelihood, L(K), and implementing the ΔK method (Evanno *et al.*, 2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The Web server CLUMPAK was used to summarize and visualize the STRUCTURE results (Kopelman *et al.*, 2015). To examine the presence of genetic structure further, a principal coordinates analysis in GENALEX v.6.5b (Peakall & Smouse, 2006) was used as a multivariate approach to supplement the STRUCTURE analysis.

Mitochondrial DNA analysis

The pine marten control region (CR) mtDNA haplotypes (N = 18) previously published by Davison *et al.* (2001) were downloaded from GenBank (accession numbers AF336949–AF336964 and AF336968–AF336969) and compared by multiple alignments using the CLUSTALW method implemented in MEGA v.7 (Tamura *et al.*, 2011). Using 322 bp of the CR, a median-joining network was constructed using the median algorithm of Bandelt *et al.* (1999) in POPART v.1.7.1 (Leigh & Byrant, 2015), and haplotypes were colour coded by country of origin. Data included haplotypes previously identified by Davison *et al.* (2001) and Jordan *et al.* (2012) in Ireland (one), Britain (Scotland) (two), Finland (two), Sweden (three), Latvia (three), Slovenia (two), Spain (one), The Netherlands (three), Germany (three), France (two), Italy (three) and the Czech Republic (two). Haplotypes originating from captive-bred animals or from *Martes zibellina* (sampled in Finland) were not included in this analysis.

The mtDNA analysis was repeated with the 69 ~1600 bp mtDNA pine marten sequences published by Ruiz-Gonzalez *et al*. (2013) (accession numbers HM025990–HM026058). The sequence region included part of the cytochrome *b* gene, tRNAPro, tRNAThr, CR, and part of the 12S rRNA. This dataset included haplotypes that occurred in Ireland (one), Britain (Scotland) (one), Italy (11), Spain (nine), Portugal (one), Croatia (one), France (six), Luxembourg (five), Austria (two), The Netherlands (three), Germany (nine), Czech Republic (one), Poland (seven), Hungary (four), Romania (one), Latvia (one), Estonia (three), Finland (three), Sweden (six), Norway (one) and Russia (17). Owing to the high number of countries sampled, the sequences were grouped by geographical region to aid visualization of the position of the Irish (IRE) and British (BRI) pine marten haplotypes. The Mediterranean group (MED) consisted of haplotypes from Italy, Croatia, Spain and Portugal; the central and eastern European group (CEE) consisted of haplotypes from France, Luxembourg, Austria, Germany, The Netherlands, Czech Republic, Poland, Hungary and Romania; and the north-east Europe and Russian group (NEE) consisted of haplotypes from Latvia, Estonia, Finland, Sweden, Norway and Russia.

RESULTS

The descriptive statistics for the 249 individual pine martens genotyped in this study are provided in Table 2. The majority of animals were genotyped successfully at all microsatellites, with success rates ranging from 97.6 to 99.2% at *Mar43* and *Mar6*, respectively, to 100% at the remaining ten loci. There was no significant evidence of scoring

Table 2. Descriptive statistics for pine martens in Ireland across 12 microsatellite loci

Parameter	*Mel1*	*Ma2*	*Mvi1341*	*Gg7*	*Mar21*	*Mar53*	*Mel105*	*MER041*	*Mvis075*	*Mar6*	*Ma08*	*Mar43*	Average
N	249	249	249	249	249	249	249	249	249	247	249	243	248.3
N_A	3	5	3	4	4	4	6	5	3	5	3	7	4.33
A_R	3.00	4.95	3.00	4.00	3.95	4.00	5.98	4.98	2.98	4.97	3.00	7.00	4.32
H_O	0.430	0.655	0.558	0.562	0.522	0.606	0.406	0.594	0.502	0.619	0.414	0.691	0.547
H_E	0.441	0.660	0.531	0.583	0.500	0.638	0.403	0.568	0.468	0.639	0.404	0.750	0.549
H_{EQ}	0.241	0.423	0.257	0.332	0.335	0.342	0.478	0.412	0.256	0.419	0.258	0.538	0.358
F_{IS}	0.027	0.01	–0.049	0.038	–0.042	0.052	–0.003	–0.044	–0.07	0.033	–0.022	*0.08*	0.001

Abbreviations are as follows: A, number of alleles per locus; A_R, allelic richness; F_{IS}, inbreeding coefficient, with values in italics indicating significant deviation from Hardy–Weinberg equilibrium at $P = 0.05$; H_E, expected heterozygosity; H_{EQ}, expected heterozygosity at equilibrium ($P = 0.02$); H_O, observed heterozygosity; N, number of samples amplified per locus;

error attributable to stuttering, allele dropout or the presence of null alleles in the dataset. There was some evidence of a homozygote excess at *Gg7* and *Mar3* ($P < 0.03$). The number of alleles per locus ranged from three to seven and averaged 4.3 across all loci. The average allelic richness value across all loci was also 4.3. Levels of observed heterozygosity ranged from 0.414 at *Ma08* to 0.691 at *Mar43* and averaged 0.547 across all loci. Levels of expected heterozygosity ranged from 0.403 at *Mel105* to 0.750 at *Mar43* and averaged 0.549. The average inbreeding coefficient value across all loci was 0.001. One locus, *Mar43*, deviated significantly from Hardy–Weinberg equilibrium at the 5% significance level, but no significant deviations were present after a Bonferroni correction ($P = 0.004$). High and significant F_{IS} values can indicate that inbreeding has occurred in the population and are also associated with the presence of genetic admixture, but in this study, the average F_{IS} value was almost zero, and many of the loci presented in this study with negative values. Linkage disequilibrium was detected in eight of the 66 possible pairwise comparisons at the 5% significance level but were not found to be significant after a Bonferroni correction ($P = 0.0007$).

The test used to detect the presence of a recent genetic bottleneck showed that there was an excess heterozygosity relative to the number of alleles attributable to differences between the levels of expected heterozygosity (H_E) relative to heterozygosity estimated under mutation–drift equilibrium (H_{EQ}) (Table 2). In this case, differences between the two sets of data were significant, indicating that the population might recently have undergone a genetic bottleneck. The probability value for a one-tailed Wilcoxon's signed rank test for an excess of heterozygosity was $P = 0.02$. Eleven out of the 12 loci had a higher than expected level of heterozygosity relative to the expected value under equilibrium, causing the excess heterozygosity. However, the mode-shift test resulted in a normal L-shaped distribution and did not confirm the presence of a recent bottleneck (Supporting Information, Fig. S1). The average *M*-ratio value across all loci was 0.47 (range 0.27–0.86), less than generally accepted threshold of 0.7 for a population that has not undergone a reduction in population size. In this case, ten of the 12 loci used in this study had an *M*-ratio < 0.7, suggestive of a reduction in population size (Supporting Information, Table S1). The effective population size (N_E) estimated in this study was 233.7 (95% confdence interval 153–413.7), below the guiding principle of 500 required for long-term viability of a population.

The STRUCTURE results showed no population substructure present in the Irish pine marten population. Furthermore, $K = 1$ is the most likely number of clusters present across the dataset, and $K = 2$ also demonstrated that there was no evidence of genetic structure across the dataset. The ΔK method suggested $K = 3$, but this is because the method of Evanno *et al.* (2005) cannot detect a $K = 1$ situation. However, the plot of the mean likelihood, L(K), of inferred K value, established from combining each replicate per K value and associated standard deviation from STRUCTURE HARVESTER, also showed $K = 1$ (Fig. 2B). There was also no evidence of genetic clustering in the principal coordinates analysis (Fig. 3), which accounted for 55.1% of the genetic variation within the data. The lack of genetic structure within the data supported the STRUCTURE data presented in Figure 2A.

Re-examination of 321 bp of the control region of the mtDNA sequences showed that the Irish haplotype, *p*, is most closely related to haplotype *h* found in Slovenia and haplotype *i* found in Britain, Latvia and Spain (Fig. 4). Haplotype *i* was formerly present in Ireland. The common British haplotype, hap *a*, is most closely related to haplotypes *d*, *c* and *b* found in Germany, France, Sweden, Finland and the Czech Republic (Fig. 4).

Repetition of the mtDNA network analysis using the 1561 bp sequences showed the sole Irish haplotype clustered within the MED group, whereas the only British haplotype in that dataset clustered with the CEE group (Fig. 5). A large portion of the NEE group segregated separately from these two clusters and appeared divergent; these were mostly animals from Russia. There was, however, some admixture present throughout. The haplotypes that clustered with the Irish haplotypes included Mm5, Mm6, Mm7 (Spain), Mm17 (Italy) and Mm19 (Croatia). This branch stemmed from a common haplotype within the European/Iberian group, which included Mm9 and Mm16. These two haplotypes overlapped to form a single haplotype in our analysis. Both these haplotypes are found in Spain, Portugal, Italy and Germany (Ruiz-Gonzalez *et al.*, 2013)).

DISCUSSION

In this study, we aimed to assess the genetic diversity of the contemporary Irish pine marten population using microsatellites to gain insights into how past declines have impacted the population today. We also aimed to review the phylogeographical history of the species using previously published mtDNA to consider the genetic history of the pine marten and how this information might be used to inform the future conservation management and sustain the on-going recovery of the species in Ireland.

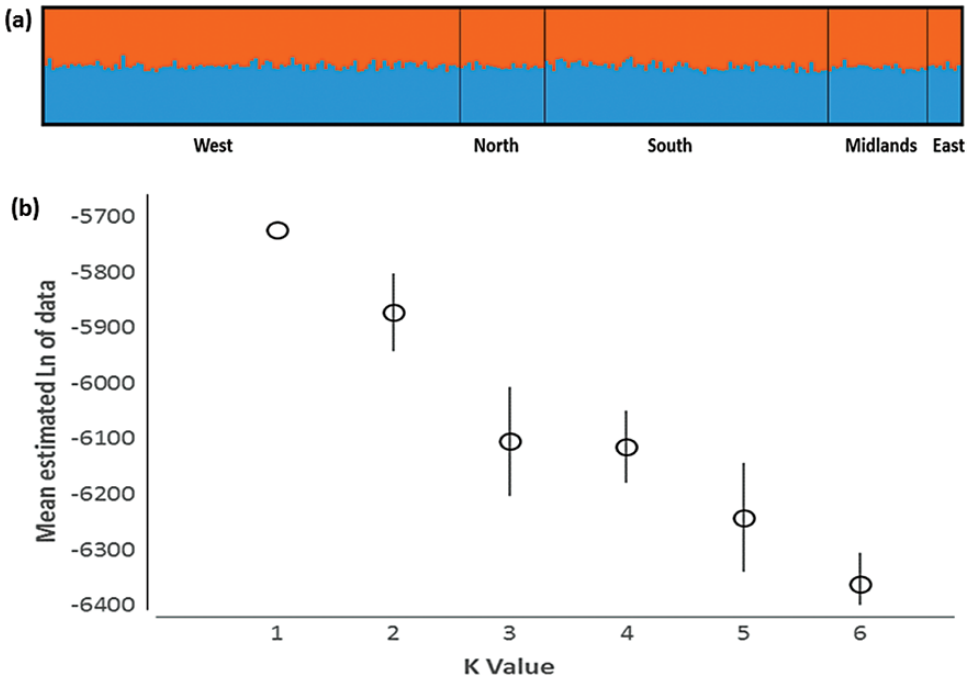

Figure 2. A, membership of individual Irish pine martens to $K = 2$ as inferred by STRUCTURE analysis. The animals were divided geographically by region into west, north, south, midlands and east of Ireland. B, plot of mean likelihood L(K) and standard deviation per K value from STRUCTURE HARVESTER on a dataset containing 249 individual pine martens genotyped at 12 microsatellite loci.

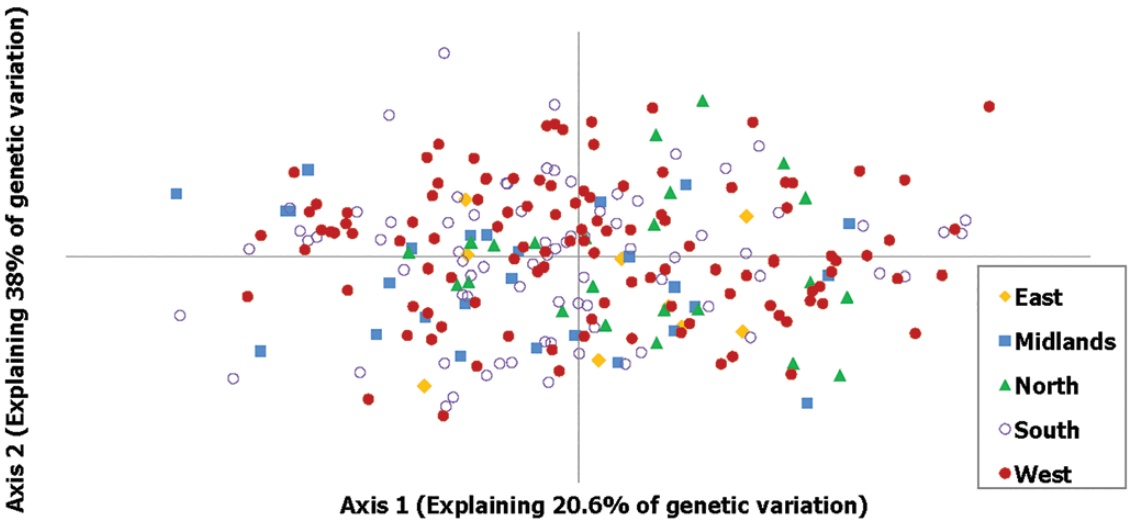

Figure 3. Principal coordinates analysis of individual pine martens. Animals were coded by geographical region. The animals were divided geographically by region into west, north, south, midlands and east of Ireland ($N = 249$).

In terms of nuclear genetic diversity, the number of alleles in the Irish pine marten population is low, averaging 4.3 per locus, but slightly higher than what was encountered by Mullins *et al.* (2010) and Sheehy *et al.* (2014), averaging 2.3 and 2.9 alleles per locus, respectively. The average levels of H_E (0.55) and H_O (0.58) in the present study were also higher than those reported earlier. This is likely to be attributable to the localized nature of those studies and the much larger sample size used in the present study. The results

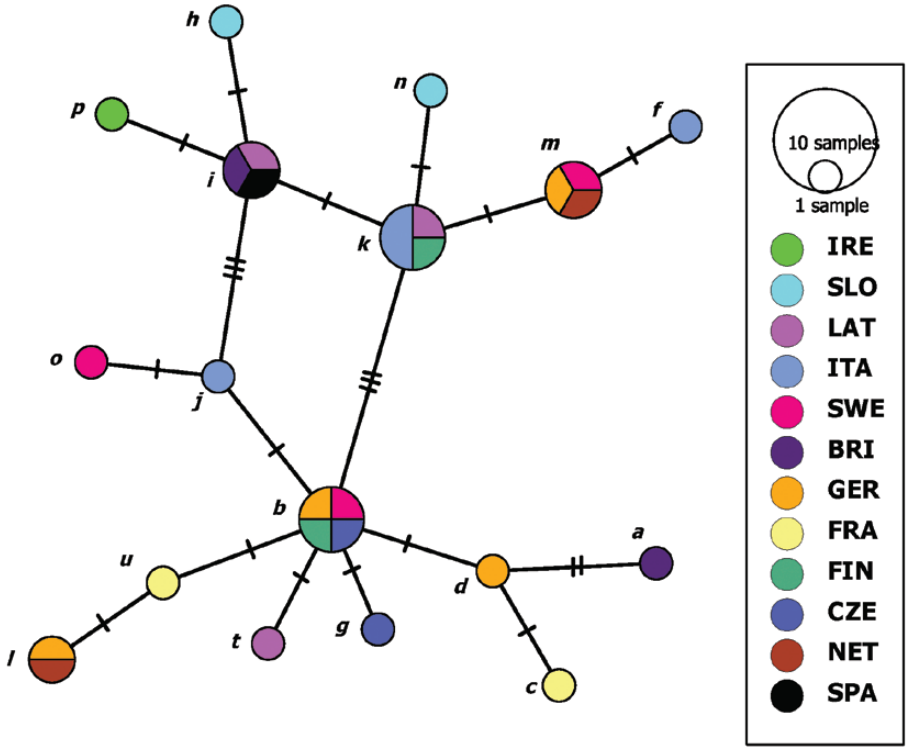

Figure 4. Median-joining network diagram generated using 321 bp of mitochondrial DNA haplotypes (control region) of pine marten samples from across Europe (Davison *et al.*, 2001) (N = 18). Only one haplotype, haplotype *p*, is found in Ireland today. Haplotype *i* was also present in Ireland historically but is now absent (Jordan *et al.*, 2012). Abbreviations are as follows: CZE, Czech Republic; FIN, Finland; FRA, France; GER, Germany; IRE, Ireland; ITA, Italy; LAT, Latvia; NET, The Netherlands; SLO, Slovenia; SPA, Spain; SWE, Sweden; BRI, Britain. The circle size reflects the number of haplotypes.

reported here are similar to those recorded in the pine marten population from the Basque Country, where the average number of alleles was 4.1 and average H_E and H_O values were 0.58 and 0.53, respectively (Ruiz-González *et al.*, 2014). Similar results were obtained in France, where an average allelic richness of 3.6 and an average H_E and H_O of 0.58 and 0.57, respectively, were found (Mergey *et al.*, 2012). Slightly higher levels of diversity were reported in Denmark, where contemporary allelic richness averaged 4.2 and expected levels of heterozygosity averaged 0.7 (Pertoldi *et al.*, 2008). However, all studies used different combinations of microsatellites; therefore, the values are not directly comparable but are useful to provide context.

Levels of genetic diversity in the Irish pine marten population were similar to those of other Irish carnivores. Badgers averaged 4.2 alleles per locus (O'Meara *et al.*, 2012), and red foxes had an average of 8.9 alleles across loci and an average allelic richness of 3.9 (Statham *et al.*, 2018). No national efforts have examined nuclear diversity of otter populations in Ireland, but one localized study in the south of Ireland showed an average of three alleles per locus (White *et al.*, 2013). Levels of observed and expected heterozygosity were also similar across these studies.

There was no evidence of inbreeding in the Irish pine marten population, but there was some evidence of a genetic bottleneck. In a non-bottlenecked population, ≤ 50% of the loci might be expected to exhibit evidence of heterozygosity excess or deficiency (Luikart & Corneut, 1998), but in this case 92% of the loci under the TPM supported the presence of a heterozygote excess. The *M*-ratio test showed that the population had evidence of a recent population retraction, a signature that has been found to remain for > 100 generations in other populations (Garza & Williamson, 2001). This suggests that the detection of a bottleneck signature is likely to reflects the history of the species in Ireland, where it has undergone multiple population retractions and expansions. Considering that expected levels of heterozygosity tend to decline after allele numbers have been reduced following a genetic bottleneck (Piry *et al.*, 1999), it would be a reasonable expectation that a similar result could manifest itself in the Irish population in future generations.

The Irish pine marten population has a low effective population size of 233.7. Effective population sizes

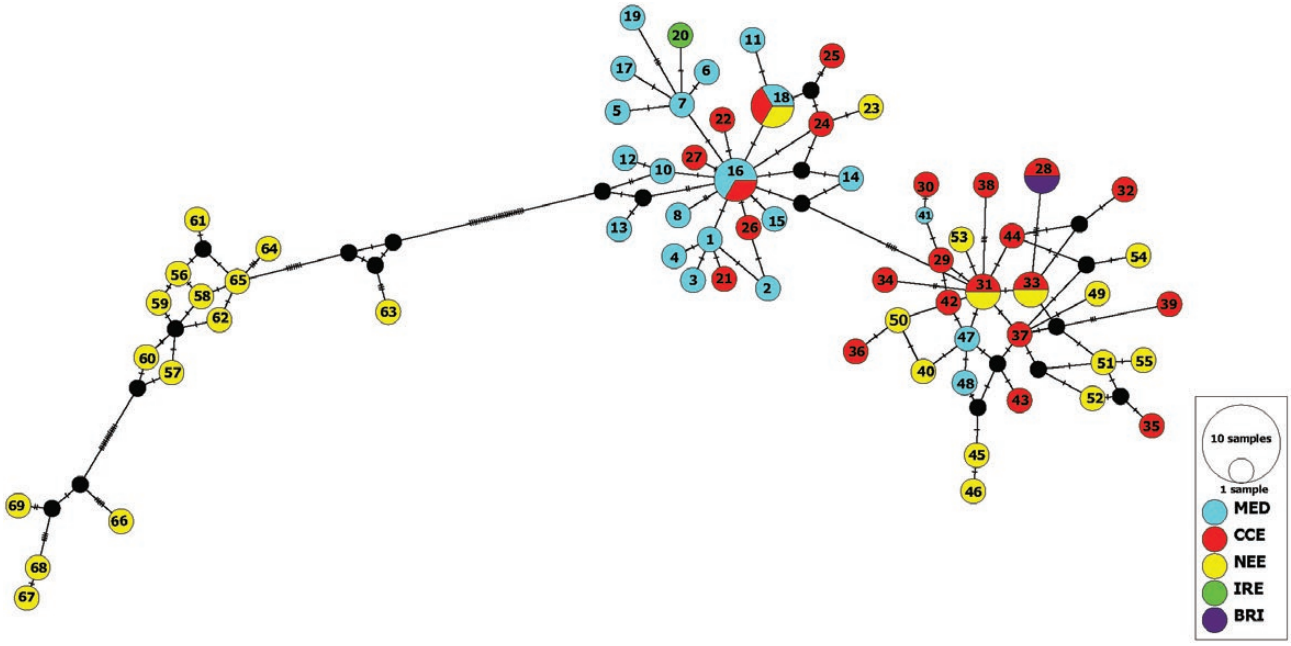

Figure 5. Median-joining network diagram generated using 1561 bp of mitochondrial DNA haplotypes (sequence includes part of the cytochrome *b* gene, tRNAPro, tRNAThr, control region and 12S rRNA; Ruiz-Gonzalez *et al*. 2013) ($N = 69$). Numbers 1–69 reflect haplotypes Mm1–Mm69 from Ruiz-Gonzalez *et al*. (2013). Abbreviations are as follows: CEE, central and eastern European group; IRE, Ireland; MED, Mediterranean group; NEE, north-eastern Europe and Russia; BRI, Britain. The circle size reflects the number of haplotypes.

for two contemporary pine marten populations in Denmark were estimated to be 802 and 505 (Pertoldi *et al.*, 2008). The 50/100 rule is based on evidence that where a population of $N_E \leq 50$, an inbreeding depression cannot be prevented in the short term, and $N_E > 500$ is required for long-term genetic viability (Jamieson & Allendorf, 2012). Other authors have argued that $N_E > 1000$ is required for long-term fitness (Frankham *et al.*, 2014). In situations where the N_E is < 500, the population remains at long-term risk for extinction, because genetic drift could cause a further loss of genetic variation, resulting in morphological developmental issues and reductions in fitness, potentially leading to extinction (Hoelzel *et al.*, 2002; Frankham *et al.*, 2014). The loss of genetic variation can also prevent a population from adapting to a change in environmental conditions, resulting in further contraction of the population that can also potentially result in extinction (Harmon & Braude, 2010; Weeks *et al.*, 2015; Kelly, 2019). In the case of some larger and critically endangered carnivores, such concerns have warranted the consideration of a genetic rescue, e.g. the Mexican wolf (*Canis lupus baileyi*) and the Florida panther (*Puma concolor coryi*) (Hedrick & Fredrickson, 2010). Similar attempts are underway in Spain to genetically enforce the European mink population (*Mustela lutreola*) via a captive breeding programme, after the detection of low genetic variability within the population (Michaux *et al.*, 2005). A European funded LIFE project project is attempting to breed Estonian and Spanish mink with a view to increasing the genetic viability of the Spanish population (LIFE13NAT/ES/1171). Few studies have successfully demonstrated the genetic success of such efforts, but Ranke *et al.* (2020) investigated the genetic implications of an experimental island reinforcement of house sparrows (*Passer domesticus*) and demonstrated that diversity could be increased, but required careful selection of individuals from donor sites and the improvement of habitat to reduce the negative impacts of genetic drift and the need for further introductions.

The pine marten population in the west, north-west and south-east of Ireland had been considered historical strongholds for the species (Ruttledge, 1920; O'Sullivan, 1983), and some evidence of genetic structure might have been expected as a result of animals expanding from these areas, but the population appears uniform (Fig. 2A). A study of pine martens in France, including discreet populations from around the country, found no evidence of a genetic bottleneck or inbreeding, and levels of genetic diversity were comparable to those of the Irish population (Mergey *et al.*, 2012). However, Mergey *et al.* (2012) did find evidence of genetic clustering and isolation by distance, although the structure detected might have been a consequence of not sampling outside of the studied populations. In

the present study, samples from across Ireland were used, which might have avoided these issues. It is also a possibility that the absence of the sympatric stone marten (*Martes foina*) from Ireland has resulted in the pine marten occupying a wider ecological niche than typically seen elsewhere in Europe, where both species coexist, which might have facilitated gene flow resulting in the lack of genetic structure as seen in the present study. Interspecific competition for resources between both marten species was one of the complex factors that was found to influence the occurrence of genetic structure within northern Spain (Ruiz-Gonzalez *et al.*, 2015).

A study of recovering fishers (*Martes pennanti*) in the north-eastern USA found evidence of genetic structure in a population that had expanded after heavy persecution (Hapeman *et al.*, 2011). The genetic structuring in that case was attributed to past reintroductions, possible migration from outside the study area, and the presence of hydrological features, particularly large lakes, which might have inhibited gene flow (Hapeman *et al.*, 2011). Natural range expansion of brown bears from Russia into Finland was also shown to have occurred by the presence of genetic structure attributable to the migration of genetically distinct animals into the country (Kopatz *et al.*, 2014; Hagen *et al.*, 2015). The lack of significant landscape features, such as lakes, and the inability of natural range expansion from other countries into Ireland might also help to explain the lack of structure in the present study. The large ranging behaviour of pine martens, as seen in recent reintroductions to Wales (McNicol *et al.*, 2020), shows that gene flow might have been maintained across an island the size of Ireland. The expansion of the species range in the face of low forest cover indicates that the species can use suboptimal habitat, and the adaptability and resilience of Irish pine martens to establish populations in habitat of low suitability during periods of range expansion should not be overlooked (O'Mahony, 2017) and supports the importance of marginal habitats, such as scrub and tree lines, to the pine marten in Ireland (Twining *et al.*, 2020c). Owing to a current lack of optimal pine marten habitat in Ireland, maintaining areas of scrub and increasing the level of native woodland cover should be encouraged and incentivized (Hickey *et al.*, 2020; Twining *et al.*, 2020c).

In contrast to our findings here, Allen *et al.* (2020) found evidence of genetic structure present in the Irish badger population. However, this was also reflected by differences in mtDNA haplotypes of divergent origins, which probably represented different colonization events or introductions. It should be noted that the Irish badger population has seven different mtDNA haplotypes, compared with one for the pine marten (O'Meara *et al.*, 2012; Allen *et al.*, 2020). The otter, another mustelid, has a rich mtDNA diversity in Ireland, with nine haplotypes found in the Irish population, five of which were considered novel to the island (Finnegan & Néill, 2010). The stoat (*Mustela erminea*) too has a rich mtDNA diversity and heritage in Ireland, with 11 uniquely Irish mtDNA haplotypes found on the island (Martínková *et al.*, 2007). In the case of the one pine marten mtDNA haplotype present in Ireland, hap *p* (Davison *et al.*, 2001), this forms part of the longer Mm20 sequence recorded by Ruiz-Gonzalez *et al.* (2013). Hap *i*, which is now extinct in Ireland and very rare in Britain, is still found in southern and mainland Europe (Davison *et al.*, 2001; Jordan *et al.*, 2012). The longer haplotype, Mm20, retains a genetic similarity to haplotypes found in north-west Spain (Asturias and Galicia), north-east Croatia (Zagreb) and north-west Italy (Turin and Cuneo). These haplotypes appear to have diverged from a common haplotype (Mm16/Mm9), which overlapped in our network diagram owing to genetic similarity and are common today in north-west Spain, north Portugal, north Italy and Sardinia. Interestingly, Mm9 also occurs in Germany and the Czech Republic. It is possible that other haplotypes that might formerly have been present in Ireland have gone extinct during periods of population decline. However, further investigation of museum collections, building on the work of Jordan *et al.* (2012), would be required to investigate this further.

It is not known how or when pine martens got to Ireland, but the Irish population retains a genetic heritage similar to that found in Iberia and southern Europe. Such a background within an Irish species is not unusual, and other Irish species retain this 'Lusitanian' heritage, sometimes as part of a wider genetic diversity. Some of the mtDNA haplotypes within the Irish badger population have links to Iberia and Scandinavia (O'Meara *et al.*, 2012; Allen *et al.*, 2020), whereas pygmy shrews (*Sorex minutus*) also share mtDNA haplotypes with pygmy shrews from the north of Spain (Vega *et al.*, 2020). Statham *et al.* (2018) found a unique Irish mtDNA haplotype in red foxes that grouped with red foxes from Spain. Statham *et al.* (2018) demonstrated that foxes in Ireland and northern Europe today retain a genetic heritage with Iberia.

Beyond the class Mammalia, similar patterns were also found in other taxonomic groups. Three haplotypes of land snails found in Ireland (*Cepaea nemoralis*) were found in the Pyrenean population (Grindon & Davison, 2013). Reich *et al.* (2015) examined the genetic diversity of the Kerry slug (*Geomalacus maculosus*), a species found only in south-west Ireland, north-west Spain and north

Portugal, and found that the population in Ireland was most genetically similar to those in Asturias and Cantabria in north Spain. Several plant species exhibit a Hiberno-Lusitanian distribution, in that they exist only in parts of Spain and Ireland. This includes the strawberry tree (*Arbutus unedo*), a heath species, *Daboecia cantabrica*, and a heather species, *Erica mackayana* (Praeger, 1932; Sheehy-Skeffington & Van Doorslaer, 2015). *Erica mackayana* occurs only along the west of Ireland and the north-west Cantabrian coast of Spain (Sheehy-Skeffington & Van Doorslaer, 2015). *Erica mackayana* does not seed in Ireland, which is one of the factors that led Sheehy-Skeffington & Van Doorslaer (2015) to conclude that it was introduced accidentally during periods of trade between north Spain and Ireland.

Regardless of how the pine marten came to Ireland and how well it appears to be recovering from population declines, it might be time to consider a genetic reinforcement. The detection of a genetic bottleneck, low effective population size and the low number of mtDNA haplotypes (only one extant) present in the population today suggests that the continued presence of the species in Ireland might be at risk in the future. It is not known how a genetic bottleneck and associations in the expected reduction of genetic diversity, in addition to the low effective population size, are likely to impact the population, but the values are below the accepted thresholds for long-term population viability, and further losses in genetic diversity could affect fitness and the ability to adapt to environmental change. Considering how genetically variable the pine marten is elsewhere in Europe, it is time to consider how the genetic diversity within the Irish population can be restored and, at the very least, maintained, while considering the unique heritage of the population. Given the phylogeographical history of the species in Ireland, diversity could be restored or gained via the translocation of genetically similar animals (IUCN, 2013). Populations that occur in north-west Spain (Galicia and Asturias) would be the ideal source, if such efforts were to be considered as part of a feasibility study that could compare levels of genetic diversity in both populations to assess the potential contribution a genetic reinforcement might have on the Irish population (Ranke *et al.*, 2020). Given that this phylogroup was also present historically throughout Britain, the genetic reinforcement of the Irish population might also be of future benefit to the conservation of the species there. It is possible that the contraction of the species range in Britain has resulted in similar reductions in genetic diversity and bottlenecks to those observed in Ireland, and this warrants further investigation based on the results seen here.

Conservation of genetic diversity is crucial for the adaptive capacity of populations facing environmental change, but genetic reinforcement is rarely proposed for populations not deemed currently to be in danger, such as the pine marten in Ireland. However, a genetic reinforcement of the Irish pine marten population based on the evidence presented here could increase its long-term viability. It is pertinent to consider the fate of extant wildlife populations, such as the pine marten, to understand fully how best they can be managed, restored and sustained for future generations before contemplating informed introductions of other extinct mammals as part of rewilding efforts.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Mode-shift test showing a normal 'L'-shaped distribution.
Table S1. *M*-ratio calculations (*k*/*r*), where *k* is the number of alleles and *r* is the range in allele size.