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A non-invasive approach to determining pine marten abundance and predation

Emma Sheehy · Denise B. O'Meara · Catherine O'Reilly · Anthony Smart · Colin Lawton

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Abstract A non-invasive approach was used to investigate variation in pine marten (*Martes martes*) abundance between the midlands and east of Ireland, and to determine the frequency of occurrence of squirrels and other small mammals in the diet. Remotely plucked hair samples were genotyped to differentiate between individual animals, and real-time polymerase chain reaction (PCR) was used to identify predator and prey DNA in scats. Macro analysis of prey remains was carried out on a sub sample of scats and the results from both methods are compared. Non-invasive techniques were successful in determining the presence and relative abundance of the pine marten at woodland level. As expected, abundance was found to be higher in the core population of the midlands than in the east. Pine martens were found to reach higher numbers per km^2 of forested habitat in Ireland than their British or European counterparts. Both traditional hard part analysis and molecular dietary analysis of mammalian prey yielded similar results. We provide the first evidence of the European pine marten predating upon the North American grey squirrel (*Sciurus carolinensis*) in its invasive range. While the grey squirrel was not available as a prey item in any of the midlands sites, it was available in the east, where it featured significantly more frequently in the diet than the native red squirrel. In both the midlands and the east the woodmouse is the most frequently occurring mammal in the diet.

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E. Sheehy (✉) · A. Smart · C. Lawton
Mammal Ecology Group, School of Natural Sciences, Ryan Institute, National University of Ireland, Galway, Ireland
e-mail: emmasheehy@gmail.com

D. B. O'Meara · C. O'Reilly
Molecular Ecology Research Group, Waterford Institute of Technology, Cork Road, Waterford, Ireland

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Introduction

In the nineteenth and twentieth centuries, the European pine marten (*Martes martes*) population in Ireland experienced widespread decline as a result of habitat loss (large-scale deforestation) and heavy persecution (O'Sullivan 1983). Population censuses in the 1980s (O'Sullivan 1983) and again in 2005 (O'Mahony D, O'Reilly C, Turner P 2006 National pine marten survey of Ireland 2005) revealed that pine marten distribution in Ireland is still mainly concentrated around core populations in the west and midlands, along with several smaller populations in the south west and south east of the country. However, the pine marten population in the west and midlands of Ireland has undergone a range expansion in recent decades, as a result of increased habitat availability and connectivity through afforestation, and importantly protection by law (O'Mahony D, O'Reilly C, Turner P 2006 National pine marten survey of Ireland 2005). The most recent population estimate for the island of Ireland is 3060 individuals (O'Mahony et al. 2012), although there is still relatively little known about Irish pine marten population densities in the westernmost part of their European range. The European pine marten has traditionally been considered a forest specialist. Zalewski and Jedrzejewski (2006) estimated that 2 km^2 is the minimum area of forested habitat necessary to support an adult pine marten in the temperate forest zone. Despite an extremely fragmented forest landscape (with <11 % forested area, Ireland represents the lowest forested land cover in their range), previous population studies in Ireland (Lynch et al. 2006; Mullins et al. 2010) have found pine marten density can be higher in Ireland than is typical throughout their British and European ranges (Birks 2002; Zalewski and Jedrzejewski

2006; Mergey et al. 2011; Caryl et al. 2012a). The Irish studies were conducted on the smaller population pockets in the south west (Killarney, Co. Kerry) and the south east (Portlaw, Co. Waterford). A population density estimate for the core population, and also the part of their range where they are believed to be much less common (i.e., the east of the country), has yet to be determined.

In Ireland and Scotland, it has been anecdotally suggested that the recovering pine marten population may be inhibiting the spread of the invasive North American grey squirrel (*Sciurus carolinensis*), and indeed that the grey squirrel population has retracted in the presence of pine martens in both countries (Carey et al. 2007; Paterson and Skipper 2008). Published information on red (*Sciurus vulgaris*) and grey squirrel distribution in Ireland (Carey et al. 2007) and more recent studies on squirrel distribution (unpublished data from Sheehy and Lawton) have found the grey squirrel to be rare in the midlands of Ireland, but their range potentially overlaps with that of the pine marten in the east. It has been suggested that the European pine marten preys preferentially on the larger, less arboreal of the two squirrel species; however, there has been no evidence to date of the European pine marten predating upon the North American grey squirrel anywhere in its invasive range, which only overlaps to a small extent with that of the pine marten (see maps in Carey et al. 2007 and O'Mahony et al. 2012).

Non-invasive genetic studies to identify species distribution and population size have become important tools to aid the study of wild, and particularly elusive, carnivore populations such as martens (Mowat and Paetkau 2002; Williams et al. 2009). Hair sampling is commonly used to non-invasively survey for the presence of mammal species (e.g., Scotts and Craig 1988; Lindenmayer et al. 1999). Lynch et al. (2006) found hair traps (fur-snagging devices) both quick and reliable in detecting pine marten presence in broadleaved woodlands. Subsequently, Mullins et al. (2010) optimised a panel of microsatellite loci to identify unique genotypes within the Irish pine marten population, thus enabling distribution and abundance to be established reliably through non-invasive field studies such as hair trapping. Faecal analysis of scats is used to determine species distribution (e.g., Palomares et al. 2002) and individual identity (e.g., Ruiz-González et al. 2013) of carnivores. The use of molecular techniques in the analysis of carnivore diet has also become popular in recent years (Deagle et al. 2005; Dunshea 2009; Shehzad et al. 2012), as prey DNA found in scats can be identified to taxon and species level and is not dependent on hard parts surviving digestion. Molecular techniques have recently been optimised to specifically detect the presence of mammalian prey in the diet of the Irish pine marten (O'Meara et al. 2013).

Using these recently developed non-invasive techniques, this study firstly aims to quantify pine marten abundance in the fragmented forest habitat in their core range in the midlands of Ireland and in the east of the country where they are considered to be less common (O'Mahony D, O'Reilly C, Turner P 2006 National pine marten survey of Ireland 2005; O'Mahony et al. 2012). Secondly, the study aims to quantify the frequency of occurrence of small mammals in the diet with emphasis on red and grey squirrels. In the process, we aim to quantify scat density in the midlands and eastern regions and to compare the findings of both molecular and macro dietary analysis techniques.

Materials and methods

Field methods

Study area

The primary study area consisted of counties Laois and Offaly, in the midlands of Ireland, and the secondary study area was county Wicklow, in the east of the country, where the pine marten population is considered to be less abundant (O'Mahony et al. 2012) (Fig. 1). Hair trapping sites (abundance study, $n=5$) and scat based survey sites (dietary analysis, $n=23$) are described below.

Abundance study

Five sites were selected, a broadleaved and a predominantly coniferous woodland from each study area (with two broadleaved woods examined sequentially in Co. Wicklow) (Fig. 1). Site 1, Charleville Forest, Co. Offaly, is a mature broadleaved wood (ca. 113 ha) in which oak (*Quercus robur*) is the dominant tree species. Site 2, Clonad (ca. 143 ha), Co. Offaly, is a mixed, mainly coniferous woodland situated 1.5 km from Charleville, where Norway (*Picea abies*) and sitka spruce (*Picea sitchensis*) are the dominant tree species. Site 3, Cloragh (ca. 160 ha) is located in Ashford, Co. Wicklow. The dominant tree species present are sitka spruce and Douglas fir (*Pseudotsuga* spp.). Site 4, Knocksink nature reserve (ca. 60 ha), is located in Enniskerry, Co. Wicklow. It consists of mature oak (*Quercus petraea*) and mixed woodland. Site 5, Tomnafinnoge (ca. 80 ha) in south Wicklow, is a broadleaved woodland consisting mainly of mature oak (*Quercus petraea*) (Fig. 1). Sites 2 and 4 are considered discrete woodlands as there is no forested habitat within 1 km of these sites. Site 1 is separated from adjacent forest habitat by a new primary road. Site 3 is separated from adjacent forestry by the Vartry river. Site 5 is not separated from adjacent forestry by either natural or man-made boundaries, and as such represents the only site which is indiscrete in this study (Fig. 1). Spring based hair traps as described by Messenger and Birks (2000) were installed in sites 1 ($n=5$), 2 ($n=7$), 3 ($n=8$), and 4 ($n=4$) in March 2011 for a period of

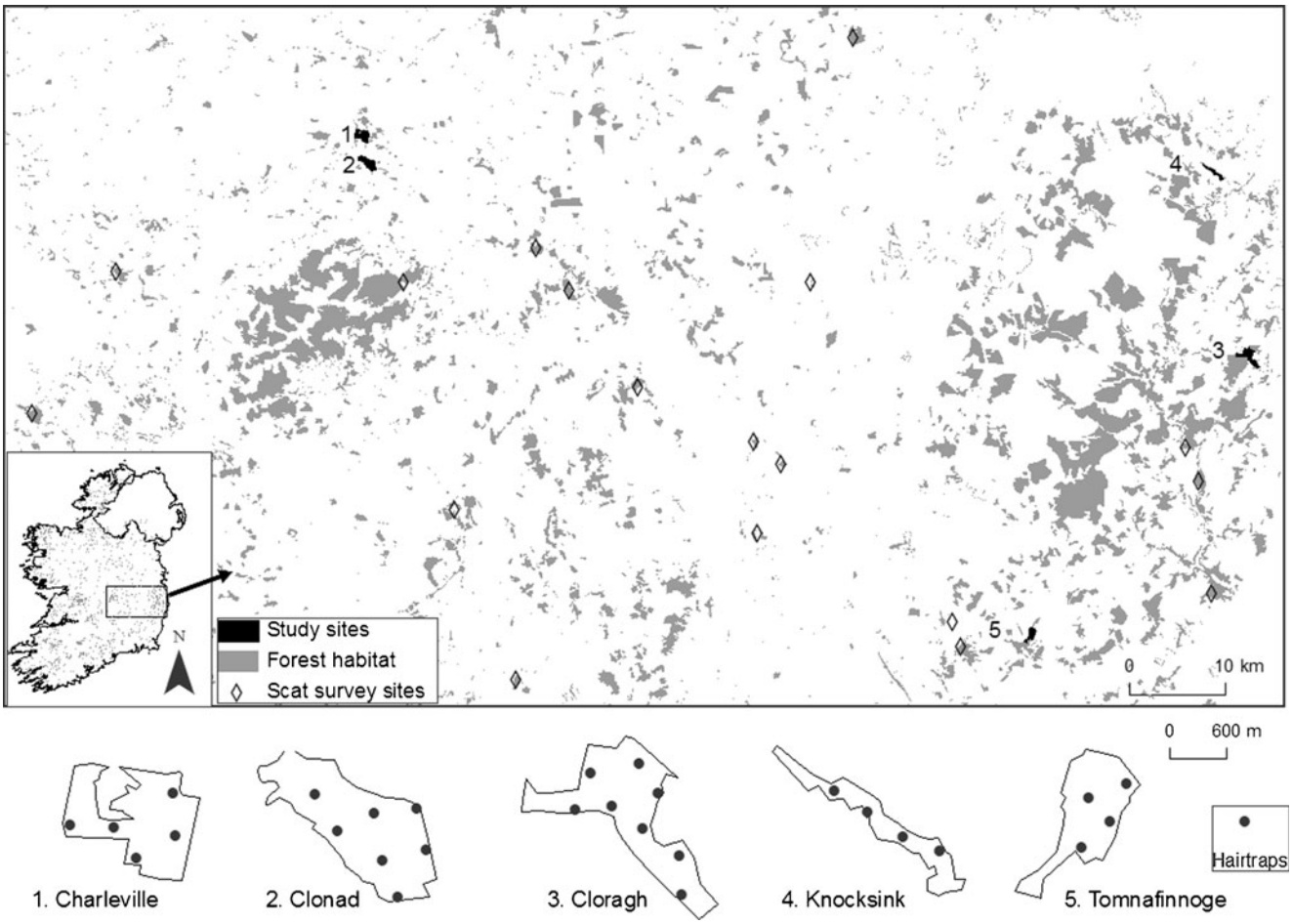

Fig. 1 Location of the study area in Ireland (*inset*) and the locations of the hair trap sites within the fragmented forested landscape. Non-forested land cover is *white*, forested habitat is *grey* and hair trap sites are represented in *black*. Scat survey sites are indicated by *open diamonds*. The position of each hair trap within the study sites are also shown

14 months, with the exception of site 4, where the hair traps were moved to site 5 in October 2011. Each trap was checked for samples and rebaited once per month with chicken and the tree trunk was smeared with marmalade. Traps were positioned 450 m apart at a density of one trap per 20 ha throughout the sites as previous home range sizes for the pine marten in Ireland have been reported as ≥0.2 km^2 (Birks 2002). Animals that were genetically identified in 3 or more months (including at least 1 month between November 2011 and April 2012) were assumed to be resident adults. Abundance values were then obtained by applying the number of residents adults identified at each site to the corresponding forested sampling area.

Dietary study

Scats were collected between March 2010 and August 2012 from 23 sites throughout the midlands and the east of Ireland (Fig. 1) and stored at −20 °C. In order to ensure reliability and validity of both scat density and dietary analysis, all scats collected as part of this study were subjected to DNA analysis in order to confirm they were of pine marten origin.

In order to determine whether a potential prey species was being preyed upon, it was necessary to establish first of all that it was available as a prey item. Of the 23 scat collection sites, 17 were classified as being either red squirrel or grey squirrel positive sites in accordance with the findings of concurrent squirrel distribution studies (Table 1) (unpublished data from Sheehy and Lawton). A sample of scats from the west of Ireland that were collected during the course of a red squirrel population study (Waters and Lawton 2011) were also included. Woodlands where both grey squirrels and pine marten were confirmed as being present together were identified as key sites and revisited where possible to increase the sample size of scats collected from these zones. In August 2012, a scent detection dog, specially trained to detect pine marten scat, carried out searches in three woodlands (including abundance study site 5, Tomnafinnoge and two further sites in the east). With the exception of the searches made by the scent detection dog, and a few scats that were collected opportunistically, the distance covered

Table 1 Scat surveys in sites where squirrel distribution studies had taken place (unpublished data from Sheehy and Lawton; Waters and Lawton 2011) resulted in a total of 361 scats for analysis of squirrel in the diet

Site	Location	RS	GS	RS site scats	GS site scats	RS prey	GS prey
Charleville	M	Y	N	162		4	
Clonad	M	Y	N	109		3	
Abbeyleix Demesne	M	Y	N	7		1	
Birr Castle	M	Y	N	1			
Ballykilcavan	M	Y	N	16			
Cappard	M	Y	N	6			
Emo Park	M	Y	N	9			
Ballyteige	M	Y	N	0			
Garryhinch	M	Y	N	11			
Derryclare	W	Y	N	6			
Croneybyrne	E	Y	N	1			
Clara	E	Y	N	1			
Tomnafinnoge[a]	E	N	Y		20		5
Dollardstown[a]	E	N	Y		8		
Ballygannon	E	N	Y		4		
Mullaghreelan[a]	E	N	Y		0		
Oakpark	E	N	Y		0		
Jenkinstown	E	N	Y		0		
Total				329	32	8	5
						2.4 % FO	15.6 % FO

RS red squirrel, *GS* grey squirrel, *M* midlands, *W* west, *E* east, *Y* squirrel species detected during field survey, *N* squirrel species not detected during field survey

[a] Site re-visited with scent detection dog

during the course of scat collection was used to estimate scat density, where scat density = (no. of scats collected)/(distance walked). All scats were collected by the lead author and transects were walked once per month in the abundance study sites, and in the rest of the sites either once or twice in total. A Fisher exact test was used to test for an overall difference in accuracy in scat identification in the field between the midlands and eastern sites, and to test for a significant difference in the frequency of occurrence of red and grey squirrels in the diet.

Laboratory methods

Abundance study

Molecular analysis

Genomic DNA was isolated from ($n = 158$) hair samples using The ZR Genomic DNA II Kit™ (ZYMO Research, California, USA) using the protocol for hair extraction (ZYMO RESEARCH Cat no. D3040). The DNA was eluted with 100 μl of deionised water. Real-time polymerase chain reaction (PCR) was used for species (targeting mitochondrial DNA) and sex determination (targeting ZFX and ZFY sequences on the X and Y chromosome) of the hair samples as described by Mullins et al. (2010). The C_t value for the ZFX gene was used to screen the samples for genotyping suitability. Samples with a C_t value of less than 36 were deemed to contain adequate quantities of nuclear DNA for genotyping.

Genotyping

Samples that were deemed suitable for genotyping were screened in duplicate at seven loci (Ma2-mini, Mel1, Gg7-mini, Mvi1341, Mvi1354, Mvis075, Ggu234) (Table 2). As the samples used for genotyping came from a non-invasive source (remotely plucked hairs), each sample was independently genotyped twice. Scores were only recorded if they were observed twice and exactly matched. Samples that were not replicated after the first two PCRs were repeated. Details of primers and multiplex setup are provided in Table 2. Fragment Analysis was conducted on an ABI PRISM 310® Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions with the standard run module. Alleles were scored with GS500 LIZ™ size standard using GeneMapper software v3.7 (Applied Biosystems). Two authors independently called alleles.

Data analysis

The two genotyping replicates were compared to assess the data for genotyping errors including the presence of allelic drop out and false alleles using GIMLET version 1.3.4 (Valière 2002). PCR success rates were also calculated using GIMLET version 1.3.4. The occurrence of repeated genotypes was identified using GENALEX version 6.4.1 (Peakall and Smouse 2006) and the number of replicates or individual recaptures was recorded. GENALEX was also used to estimate the probability of identity (PID). A final dataset was created with duplicated data removed and MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004) was used to further identify possible genotyping errors, including the presence of null alleles, large allele dropout, and scoring errors as a result of stutter peak (using default settings).

Gametic phase linkage disequilibria by Fisher's method (1,000 dememorizations and 5,000 iterations) and deviations from Hardy–Weinberg equilibrium were assessed (default settings, exact tests) using GENEPOP version 4.0.10 (Rousset 2009). Observed (H_O) and expected (H_E) heterozygosities and the number of alleles (a), were calculated using GENALEX version 6.4.1 (Peakall and Smouse 2006), and allelic richness (R_s) was estimated using FSTAT 2.94 (Goudet 1995).

Table 2 Microsatellite primers used in pine marten genotyping

Locus	Primer sequence 5′–3′	Size range	Reference
Ma2-mini	F: YAK-CCATGTACTTTTCCTATCTTTTAGGA R: ATCTTGCATCAACTAAAAAT	131–141	O'Reilly (This study) Davis and Strobeck (1998)
Mel1	F: FAM-CTGGGGAAAATGGCTAAACC R: GCTCTTATAAATCTGAAAATTAGGAATTC	106–116	Bijlsma et al. (2000) Mullins et al. (2010)
Gg7-mini	F: FAM-GTTTTCAATTTTAGCCGTTCTG R: GCTCTTCACTCTGTGTGGCATCTAC	132–140	Davis and Strobeck (1998) O'Reilly (This study)
Mvi1341*	F: PET-GTGGGAGACTGAGATAGGTCA R: GTTTCTTGGCAACTTGAATGGACTAAGA	164–178	Vincent et al. (2003)
Mvi1354*	F: FAM-CCAACTGGAGCAAGTAAAT R: GTTTCTTCATCTTTGGGAAAGTATGTTT	200–212	Vincent et al. (2003)
Mvis075*	F: FAM-GAAATTTGGGGAATGCACTC R: GTTTCTTGGCAGGATAGGATGTGAGCT	145–155	Fleming et al. (1999)
Ggu234	F: PET-TTACTTAGAGGATGATAACTTG R: GAACTCATAGGACTGATAGC	84–90	Duffy et al. (1998)

Reverse primers marked with an asterisk (*) were modified with a 5′ sequence of GTTTCTT to promote non-templated nucleotide addition (Brownstein et al. 1996). The Ma2-F and Gg7-R primers were redesigned to produce a smaller product. The primers were used in two multiplex mixes. Mix A contained Gg7-mini and Mvi1354 and Mix B contained all the other primers. Each primer pair was at a final concentration of 0.5 μM. Microsatellite amplifications were performed in a total volume of 10 μl with 4 μl DNA extract, 1 μl primer mix and 5 μl GoTaq® Hot Start Green Master Mix (Promega). The PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 90 s and 72 °C for 30 s, followed by 72 °C for 30 min

Dietary study

Molecular analysis

Approximately 0.2 g of scat was used for DNA extraction as described by O'Reilly et al. (2008), and using the ZR Genomic DNA II Kit™(ZYMO Research). Pine marten DNA was verified as described above. All samples with a C_t value lower than 32 were classified as pine marten and those with a greater C_t value were classified as non pine marten and excluded from further analysis. To test for prey DNA in the confirmed pine marten scats, species-specific Taqman assays designed to detect red and grey squirrel DNA were used. All PCR reactions and probes were as described by O'Meara et al. (2012). A sub-sample of 160 scats (80 each from sites 1 and 2) were also tested for small mammal prey DNA; woodmouse (*Apodemus sylvaticus*), bank vole (*Myodes glareolus*), pygmy shrew (*Sorex minutus*), and greater white toothed shrew (*Crocidura russula*) (O'Meara et al. 2013). Samples with C_t values of 36 or higher were discounted and positive results were replicated for verification. Percentage frequency of occurrence (%FO) in the diet for each prey species was calculated as the number of scats in which the species' DNA was amplified/total no. of scats tested × 100.

Macro analysis

A sub-sample of 110 scats was subjected to traditional hard part analysis to identify mammalian prey using keys to identify mammal bones (Yalden and Morris 1990) and hairs (Teerink 1991). The subsample of 110 scats comprised 40 scats from both the Charleville and Clonad subsamples, respectively (which had been tested for squirrel and other small mammalian prey DNA), and a further 30 scats from the grey squirrel positive sites (which had been tested for squirrel DNA only). The results from molecular and macro analysis were compared and then combined to determine an overall frequency of occurrence in the diet for each prey species. A chi square test was used to investigate significant differences in the frequency of occurrence of each species according to molecular, macro and combined results. Regression analysis was used to investigate whether a relationship exists between the frequency of occurrence of prey items using molecular and macro techniques. %FO for mammalian prey species was calculated as in molecular analysis and percentage relative biomass of prey ingested (%BPI) was calculated as: weight of dried remains for each species/total weight of dried remains. Previous studies investigating the contribution the main food groups make in terms of biomass to the diet have used pre-established correction factors in such estimations (Lynch and McCann 2007; Caryl et al. 2012b); these correction factors were derived from feeding trials in which the weight of the food item eaten was divided by the dry weight of undigested matter later identified in scats (Lockie 1961; Balharry 1993; Jedrzejewska and Jedrzejewski 1998; Lanszki et al. 2007). Individual correction factors for the mammalian prey species investigated in the current study were not available as they are usually simply grouped together in feeding trials as 'small mammals'.

Results

Density study

A total of 157 hair samples were collected out of 273 baited hair traps. Sites 1 and 2 in the midlands yielded the highest

success rates with 91 % and 78 %, respectively. In site 3, 37 % of potential trapping events yielded hair samples. In site 4, one hair sample was obtained out of a possible 24, and this was the only hair sample in the study to test as negative for pine marten DNA. Site 5 yielded nine hair samples, a success rate of 37.5 %. A further hair sample was collected from a roadkill animal in July 2011, ca. 3 km from site 2, bringing the total number of hair samples to 158.

Of the 158 hair samples, 157 were successfully genetically identified as pine marten and 139 were successfully sex-typed. 109 samples had a ZFX C_t value ≤36 and of these, 104 were successfully genotyped (at six or more loci) (i.e., 95 % of samples that passed the screening process, or 66 % of all hair samples collected). This success rate varied between individual sites (Fig. 2).

A total of 25 individual genotypes were obtained from the 104 samples; one from the roadkill animal near site 2, and 24 from the abundance study sites (site 1, $n=6$ pine marten detected with five hair traps; site 2, $n=10$ pine marten detected with four to seven hair traps; site 3, $n=6$ pine marten detected with eight hair traps; site 5, $n=2$ pine marten detected with four hair traps). Pine marten abundance values ranged from 0 to 4.42 per km^2 including adult residents only (Table 3). Mean abundance values for the midlands and east were 3.13 and 1.01, respectively, with an overall abundance value of 1.99 pine marten/km^2 (Table 3). The number of hair samples genotyped from each individual ranged from 1 to 14 with a mean value of 4.29 (±1.6, 95 % confidence interval [CI]) and the number of months each animal was captured ranged from 1 to 7, with a mean value of 3.29 (±0.96, 95 % CI) (Table 4). One individual (a male) was detected in both site 1 and site 2 (January 2012 and May 2011, respectively). These sites are located 1.5 km apart (Fig. 1) and together they comprise less than 3 km^2 of forested habitat. In total, eight adult residents were detected in the two sites and a further eight that are assumed to be either sub-adult or non-resident individuals. With the exception of the one animal there was no further crossover of individuals detected between these two woodlands, despite their close proximity to one another and the lack of surrounding forested habitat.

Assessing genotyping errors

The proportion of positive PCRs ranged from 90 % to 100 % across loci and from 86 % to 100 % across samples. Analysis of genotyping error revealed the presence of allelic dropout rates of 0.08 at locus Ma2-mini, 0.10 at locus Ggu234 and 0.46 at locus Mvis1354, with no false alleles detected. The overall allelic dropout error rate across all loci was 0.09, 0.32 across all samples, and 0.08 across all PCRs. We found that there was no systemic evidence of scoring errors and the data was not shown to be affected by the systemic presence of null alleles or large allelic dropout. The cumulative PID was $PI=1.9\times10^{-3}$, which is sufficient for the estimation of population size (0.01) (Mills et al. 2000).

Genetic variability

The number of alleles was low ranging from 2 at Ggu234 and Mvis075 to 4 at Gg7-mini (Table 5). Low levels of allelic richness per locus and per sample were also observed from 2 at Ggu234 and Mvis075 to 3.69 at Gg7-mini. Expected levels of heterozygosity averaged 0.386 and ranged from 0.106 at Mvis1354 to 0.570 at Ma2-mini. Observed levels of heterozygosity averaged 0.462 and ranged from 0.111 at Mvis1354 to 0.708 at Gg7-mini and Ma2-mini (Table 5). There were no significant deviations from Hardy–Weinberg expectations at any loci.

Dietary study

A total of 517 scats were collected between March 2010 and August 2011. Four hundred of these were collected in the midlands ($n=9$ sites) and 117 were collected in the eastern sites ($n=14$ sites) (Fig. 1). Overall, 86 % of scats collected in the midlands tested positive for pine marten DNA compared to 39 % in the east. As such, accuracy in the field was found to be significantly lower in the eastern region ($p<0.001$, Fisher exact). The majority of scats were included in scat density calculations as distance walked was known (333 out of 344 and 38 out of 46 in midlands and eastern sites, respectively). Thus scat density was estimated to be 1.745 scats/km in the midlands and 0.221 scats/km in the east. Although scat density was found to be higher in areas of higher pine marten occupancy, no statistically significant relationship was found between scat density and pine marten abundance. The scent detection dog succeeded in collecting seven pine marten scats from two of three woods visited over a 2-day period. When scats were categorised into red squirrel and grey squirrel sites (as per unpublished data from Sheehy and Lawton) a total of 329 were classified as coming from red squirrel positive sites, and 32 from grey squirrel positive sites (Table 1). Squirrels appeared in the diet as prey items at low frequencies during 9 months of the year, spring and early summer being most common (Table 6).

Molecular and macro analysis

Regression analysis found a linear relationship to exist between the %FO of mammalian prey items as detected by molecular and macro analyses ($y=-1.694+1.969x$, $R^2=0.895$, $p<0.05$) (Fig. 3). The woodmouse featured more frequently in the diet than any other mammal species in both the molecular ($\chi^2=41.17$) and the macro ($\chi^2=67.58$) analyses ($df=4$, $p<0.01$) (Fig. 4). Grey squirrels featured more frequently than red squirrels as prey items in both analyses,

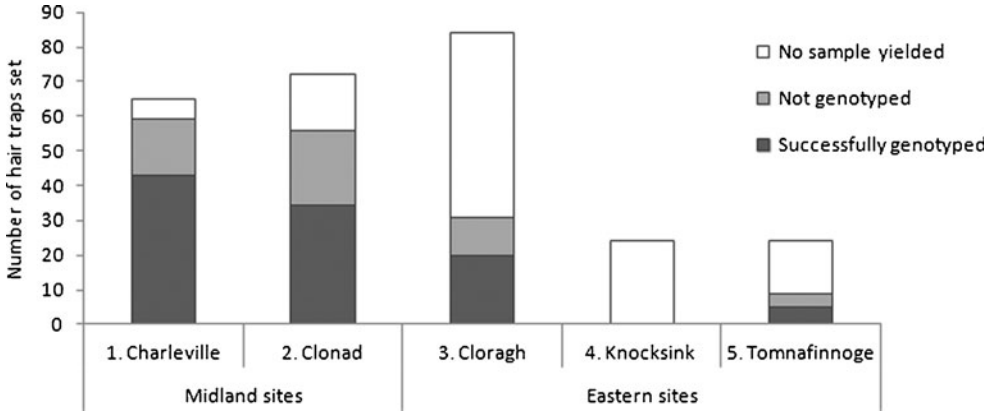

Fig. 2 The number of successful hair trapping events and the portion of hair samples successfully genotyped from each site

significantly so in the molecular analysis, despite the considerable difference in sample size ($p < 0.05$, Fisher exact).

When results from the reduced sample ($n = 110$) that was the subject of both molecular and macro analyses were combined, the %FO increased for all species; however, there was very little effect on the low frequency prey items (red squirrel, bank vole and pygmy shrew). The %FO for woodmouse and grey squirrel increased more considerably when results from both methods were combined, but not significantly so (Fig. 5). %BPI also found the woodmouse to be the most important prey species in the diet of the pine marten, followed by the grey squirrel where it was available, although ca. 30 % of mammalian remains could not be identified to species level (Fig. 6).

Discussion

Pine marten abundance

This study has provided an index of abundance for the pine marten population in both their core range and a considerably less populated part of their range in the east of Ireland. Abundance values are not directly comparable to European studies where density values were obtained from radio-tracking or snow tracking (e.g., Zalewski and Jedrzejewski 2006; Mergey et al. 2011), but are comparable to Irish studies that have used a combination of hair trapping and live-trapping to determine population density estimates. The abundance values obtained in this study suggest that the population in the midlands of Ireland (mean value of 3.13 adult residents/km^2) is currently living at a higher density than previously reported for the species in Europe (0.01–1.75 per km^2) (Zalewski and Jedrzejewski 2006) or Ireland (0.5–2 per km^2) (Lynch et al. 2006; Mullins et al. 2010) and thus quite possibly represents the highest density in their natural range. It is unclear why the European pine marten reaches these relatively high numbers in Ireland, particularly when their favoured habitat is so sparse and fragmented. In a review of available literature on European pine marten densities, Zalewski and Jedrzejewski (2006) found that between 41° and 68°N densities declined exponentially with decreasing winter temperature and increasing seasonality, and suggested that both winter severity and availability of rodents are

Table 3 The total number of pine marten identified by unique genotypes at each site, in total (All), in sites 1 and 2 combined (Midlands) and in sites 3, 4 and 5 combined (East)

	Site name	Site area	Total PM identified	Mean captures	No. adult residents	No. adult residents per km^2
Midlands	1. Charleville	1.13 km^2	6	4.67 (0.98)	5	4.42
	2. Clonad	1.43 km^2	10	2.8 (0.79)	3	2.10
East	3. Cloragh	1.58 km^2	6	2.83 (0.75)	2	1.27
	4. Knocksink	0.6 km^2	0	0.00	0	0.00
	5. Tomnafinnoge	0.8 km^2	2	2.5 (1.5)	1	1.25
Total	All	5.54 km^2	24	3.29 (0.46)	11	1.99
	Midlands	2.56 km^2	16	3.56 (0.63)	8	3.13
	East	2.98 km^2	8	2.75 (0.62)	3	1.01

Mean Captures = mean number of times each animal was captured per site (Std Er). No. adult residents = number of animals that were detected in ≥3 months including at least 1 month during November 2011 and April 2012

Table 4 The site each pine marten was recorded at and the sex assigned through DNA analysis

Animal	Site	Sex	GT	Months
CVF01	1. Charleville	Female	1	1
CVF02			3	3
CVF03			10	7
CVF04			6	4
CVM01		Male	9	7
CVM02			14	6
CDM05[a]			1	1
CDF01	2. Clonad	Female	4	4
CDF02			1	1
CDF03			3	3
CDF04			1	1
CDM01		Male	1	1
CDM02			3	2
CDM03			1	1
CDM04			12	8
CDM05[a]			1	1
CDM06			7	6
WWF01	3. Cloragh	Female	2	2
WWF02			3	3
WWM01		Male	7	5
WWM02			1	1
WWM03			6	5
WWM04			1	1
TFF01	5. Tomnafinnoge	Female	4	4
TFF02		Male	1	1
Roadkill			1	
Total			104	

GT the number of hair samples successfully genotyped for each animal, *Months* the number of months each animal was identified

[a] Animal was captured at two sites

Table 6 Squirrels as detected as prey items in molecular and macro analysis of pine marten scats, including the date and site at which the scat was collected

Species	Date	Site	Molecular	Macro
RS	2010-03-22	Abbeyleix	Y	
RS	2010-11-16	Clonad	Y	N
RS	2011-03-31	Charleville	Y	Y
RS	2011-04-01	Charleville	Y	
RS	2011-05-11	Charleville	Y	
RS	2011-06-07	Clonad	Y	Y
RS	2011-06-07	Clonad	N	Y
RS	2011-09-30	Charleville	Y	
GS	2011-10-01	Tomnafinnoge	Y	
GS	2012-02-03	Tomnafinnoge	N	Y
GS	2012-03-13	Tomnafinnoge	Y	Y
GS	2012-04-18	Tomnafinnoge	N	Y
GS	2012-05-18	Tomnafinnoge	Y	N

RS red squirrel, *GS* grey squirrel, *Y* positive, *N* negative (blank = not tested)

limiting factors on populations. Thus it is possible that Ireland's relative lack of seasonality and mild winters (the moderating influence of the Atlantic gulf stream results in mean minimum winter temperatures of 2–6 °C) (MetEireann 2012) contribute to the observed high pine marten abundance. Other contributory factors may include lack of competition and lack of predators, with the red fox (*Vulpes vulpes*) representing the pine marten's only real competitor or predator in Ireland.

Zalewski and Jedrzejewski (2006) estimated that 2 km^2 is the minimum area of forested habitat necessary to support an adult pine marten in the temperate forest zone. However, this does not appear to apply to the core Irish pine marten population and both midlands sites, Charleville (1.13 km^2) and Clonad (1.43 km^2), sustain relatively high pine marten numbers in comparison to other extensively forested parts of Europe (Zalewski and Jedrzejewski 2006). The European pine marten has traditionally been considered a forest specialist; however, recent studies have found the species to be less

Table 5 Descriptive statistics for microsatellite analysis of pine martens in four study sites in Ireland

	Ggu234	Mel1	Gg7-mini	Ma2-mini	Mvis075	Mvi1341	Mvi1354	Mean
N	24	24	24	24	24	24	18	23.14
a	2	3	4	3	2	3	3	2.86
R_s	2.0	2.99	3.69	3.0	2.0	2.75	3.0	2.78
a_s	87–93	108–116	132–142	131–137	151–153	170–180	200–208	
H_E	0.353	0.379	0.548	0.570	0.305	0.442	0.106	0.386
H_O	0.458	0.417	0.708	0.708	0.292	0.542	0.111	0.462
HW	0.145	0.861	0.775	0.889	0.834	0.632	0.996	

N denotes the number of individuals that successfully amplified at each locus, a is the number of alleles per locus, R_s is the allele size range, A_s is the allele size, H_E is the expected heterozygosity, H_O is the observed heterozygosity. There were no significant deviations from Hardy–Weinberg equilibrium

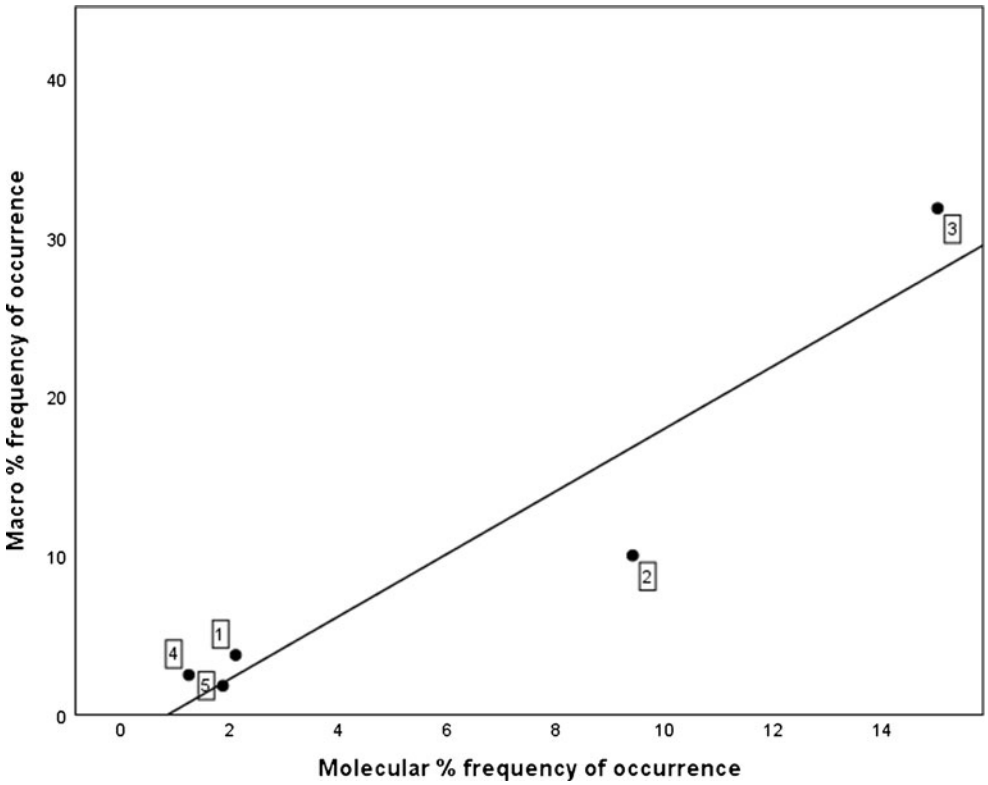

Fig. 3 A linear relationship was found to exist between the frequency of occurrence of mammalian prey items as determined using molecular and macro analyses ($y=-1.694+1.969x$, $R^2=0.895$, $p<0.05$)

restricted to large forests than previously believed and highlighted the importance of the surrounding landscape not only in providing habitat corridors but also in providing essential food resources and den sites throughout fragmented landscapes (Clevenger 1994; Pereboom et al. 2008; Mergey et al. 2011; Caryl et al. 2012a). In the current study, hair traps were only placed within the forested habitat and thus no data was obtained on the use of the surrounding landscape, or in the case of site 5, the adjacent forestry. Abundance values per km^2 are thus only applicable to forested area as use of surrounding landscape is not accounted for with this sampling technique. The fact that only one animal was detected both in sites 1 and 2 despite their close proximity to one another, supports the theory that these small woodlands can be

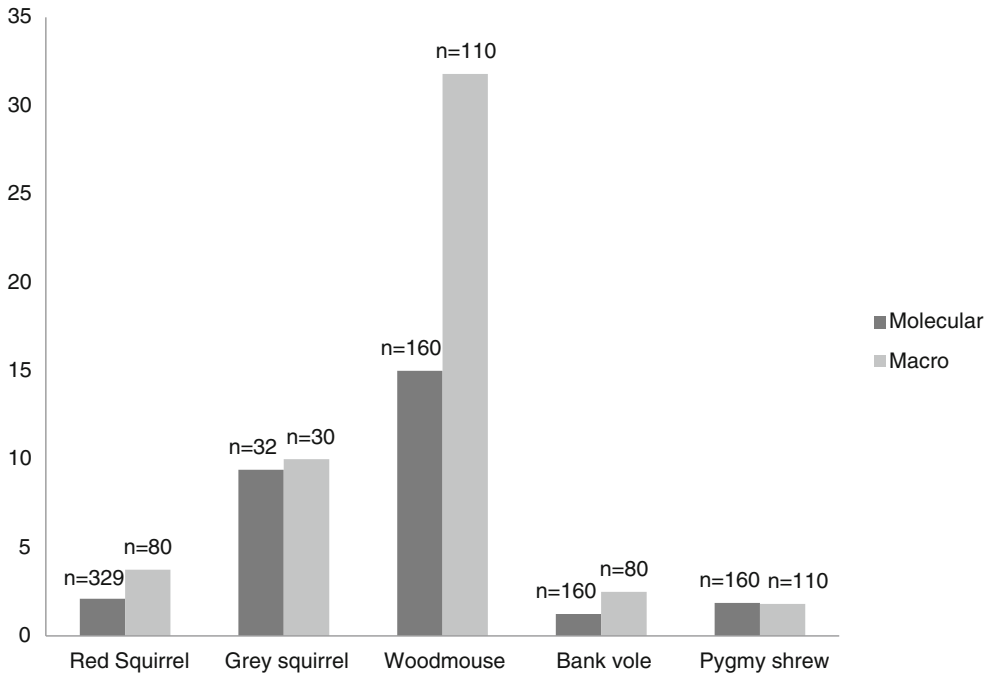

Fig. 4 Overall results for molecular and macro analysis of mammalian prey species in pine marten diet (n=number of pine marten scats tested). Those subject to macro analysis are a random sub-sample of the molecular samples except the sample from the grey squirrel positive sites. The woodmouse features significantly more frequently than any other prey species (molecular and macro analysis) and macro analysis detected significantly more woodmouse in the diet than molecular analysis ($p<0.01$, Fisher exact). Grey squirrels were more frequently detected than red squirrels as prey items (molecular analysis: $p<0.05$, Fisher exact)

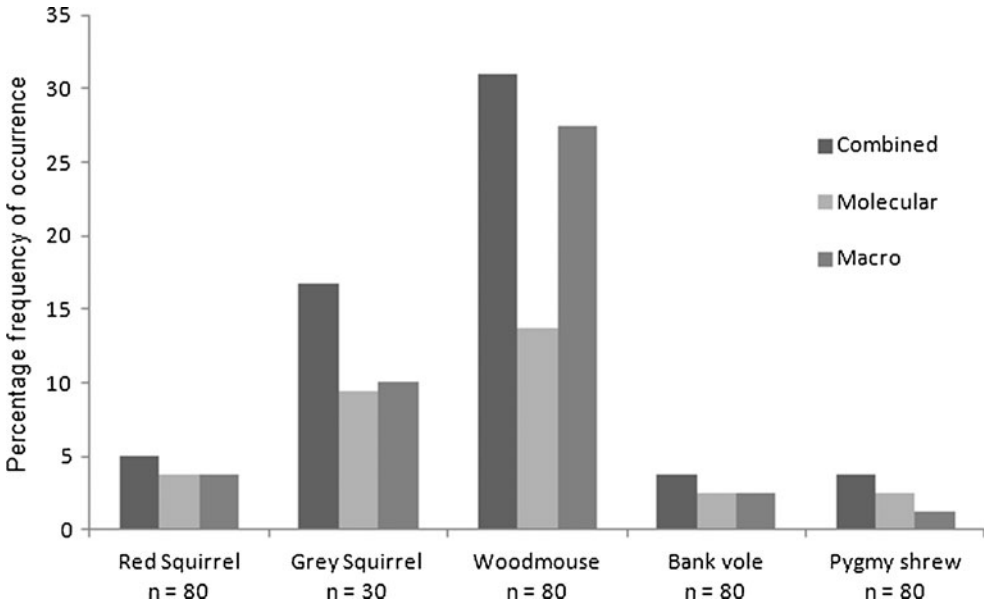

Fig. 5 Comparison of results from both molecular and macro analysis of pine marten scats, where all samples were subjected to both techniques (n = sample size). Frequency of occurrence was higher for all species detected when results were combined, and significantly so for the woodmouse (χ^2=6.04, df=1, p<0.05)

considered as relatively discrete in terms of pine marten occupancy; however, it is likely that surrounding non-forested landscape is also used to some extent.

Non-invasive techniques

When compared to other methods of determining pine marten density, such as snow-tracking (e.g., Zalewski 1999) (not feasible in Ireland), camera-trapping (e.g., Manzo et al. 2012) and radio-tracking, there are certain benefits and limitations to genetic tagging through hair samples. Genetic tagging does not provide specific biological information such as weight, reproductive activity and condition, nor does it provide detailed spatial information on home range size or territoriality. It is however the only non-invasive method that confidently differentiates between individual animals. The data collection process was non intrusive to the animal and time-efficient in terms of data collection. The hair traps themselves are inexpensive to construct and maintain. However it is clear from our data that hair trap density should be increased to optimise information gathered on the population. In the midlands sites there were more individuals detected per site over the study period than there were hair traps available per

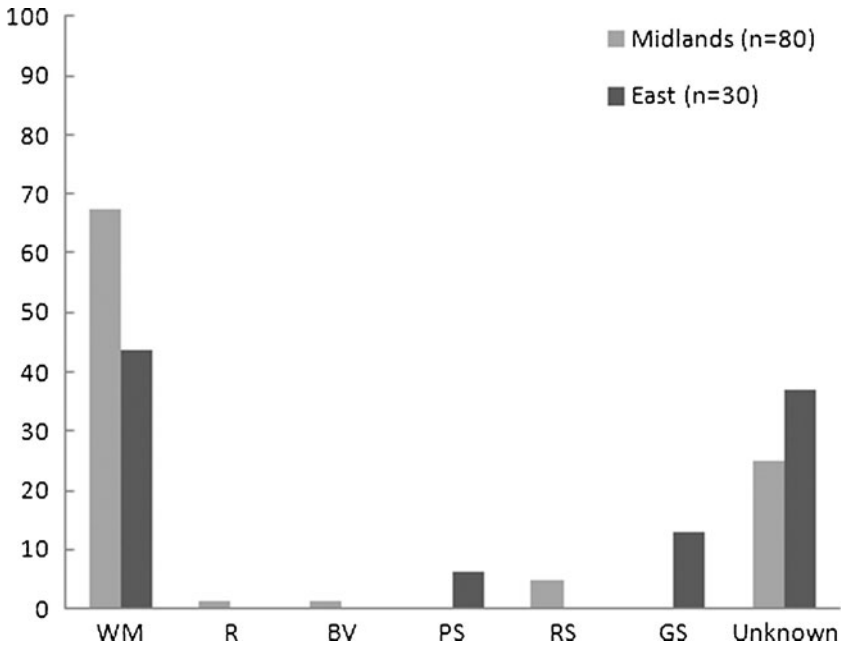

Fig. 6 The percentage relative biomass (%BPI) of mammalian prey items ingested as determined by macro analysis of pine marten scats. *WM* woodmouse, *R* rat, *BV* bank vole, *PS* pygmy shrew, *RS* red squirrel, *GS* grey squirrel

month; in site 1, six pine marten were detected using five hair traps and in site 2, ten pine marten were detected with as few as four hair traps (over the course of the sampling period, hair traps were persistently stolen from this site). It is possible that this resulted in an underestimation of the abundance value at this site as the number of hair traps available was not sufficient to give each individual present the opportunity to use a hair trap each month, in particular during the winter months when residency was being determined.

The proportion of hair samples collected that were successfully genotyped (66 %) could also be improved in future studies. A relationship has been found to exist between the number of hairs in a sample, and the probability it will be successfully genotyped (Mowat and Paetkau 2002). In the majority of hair trapping events in the current study more than ten hairs were captured, thus providing a relatively high amount of DNA. However, the samples were left in situ for a period of up to 1 month, which may have caused the DNA to degrade due to relatively high ambient temperatures and humidity. Lynch et al. (2006) suggested a survey period of 6 days is sufficient to detect pine marten presence (in lowland broadleaf woods). Screening the quantity of nuclear DNA in the samples using the sex typing assay prior to genotyping helped to increase the genotyping success (95 %) as the samples that were deemed to have insufficient high quality DNA did not proceed to the genotyping stage. This also helped reduce the overall cost and this technique combined with shorter sampling periods could substantially help improve the overall success rate in future studies.

The overall number of alleles and levels of heterozygosity in this study were very low. Mullins et al. (2010) also recorded low levels of genetic diversity (average H_E=0.35 and H_O= 0.34) in the Irish pine marten population, using a larger microsatellite panel than the current study. However, Mullins et al. (2010) used samples from a wider geographic range in Ireland than the current study. The low levels of genetic variability in both studies could be due to the low number of individuals that the current pine marten population have re-established themselves from. Furthermore, there has only been one mitochondrial DNA haplotype found in the contemporary Irish population (Jordan et al. 2012). The long-term effects of such low levels of genetic diversity in an expanding population are not known. However, the low diversity found in this study may also be partially due to the microsatellites used, as they were originally developed for use with other mustelids. This was also discussed as a reason for lower levels of genetic variability in the Iberian pine marten population by Ruiz-González et al. (2013).

An alternative form of quality screening to the method used in the current study involves preliminary analysis with a sub-group of microsatellites, as was undertaken by Ruiz-González et al. (2013). Samples that amplified well (>50 % positive PCRs) with the sub-group were then taken to the next stage of analysis. The approach taken in the current study may be more useful as data not used for genotyping at least provides further information on species and sex. The pre selection of DNA samples for genotyping removes samples that are unlikely to replicate or may cause a higher occurrence of genotyping error (Zhan et al. 2010) and thus may be more efficient.

Low genotyping error rates were reported in this study, and were at the lower end of the level of error when compared to other non invasive genetic studies using DNA extracted from hair (Broquet et al. 2007). Genotyping errors are easier to control and account for in small studies with fewer samples (Zhan et al. 2010). Mullins et al. (2010), also working with a relatively small dataset, similarly reported low genotyping errors. The high number of recaptures reported in the current study further supports the low occurrence of genotyping errors, and helps to validate the genotyping results. If an erroneous individual had been detected within the dataset, this individual would not affect the overall abundance estimates, as only animals identified within a site on at least three separate months were included in the abundance estimates.

Scat density

There are inherent problems with surveying for pine marten scats in areas of low population density (Birks et al. 2005); most notably misidentification of scats in the field, even by experienced surveyors. This problem is addressed in modern surveys by the use of genetic tests to confirm pine marten origin (O'Reilly et al. 2008; Balestrieri et al. 2011; Caryl et al. 2012b). The current study found that in the east of Ireland, where pine marten abundance is lower, a significantly lower portion of the scats collected were confirmed as being of pine marten origin than those collected in the midlands, where marten abundance is higher. A factor that may have contributed to this result is the likelihood that the surveyor was less discriminate about which scats were collected in the lower scat density sites. In areas where scats are more abundant, key features such as smell and shape are more easily taken into account, and the surveyor is likely to be more critical regarding the quality of the scat collected for the survey.

Furthermore, in areas of low pine marten population density, territorial scent marking behaviour may be greatly reduced (Macdonald et al. 1998). Lockie (1964) was the first to suggest that a relationship exists between the number of scats and pine marten abundance; however, in a review of nine previous scat surveys in the UK and Spain, Birks et al. (2005) found that the field relationship between scat abundance on transects and marten numbers was yet to be established. Indeed, whilst the current study found scat density to be higher in areas of higher pine marten abundance, regression analysis failed to define this possible relationship.

Scent detection dogs are increasingly being used in the study of elusive carnivores (Smith et al. 2003; Long et al.

2007; Reed et al. 2011), and have been found to have a superior detection rate to that of humans. In the current study, the scent detection dog was used over a 2-day period, and succeeded in collecting a total of 11 scats, seven of which were confirmed as pine marten through molecular analysis. Those that tested negative for pine marten DNA also tested negative for fox DNA (the species that pine marten scat is most likely to be misidentified as in Ireland), which suggests that the quality of the DNA in those samples was too degraded for genetic species identification. As such, it is not possible to determine whether the scats detected by the dog that tested negative for pine marten DNA were true or false negatives. The lead author only detected one pine marten scat during the 2-day survey without the aid of the dog, suggesting that the use of scent detection dogs in areas of low pine marten and low scat density can greatly improve sampling efficiency.

Dietary analysis techniques

In this study, both molecular and macro analyses detected prey species in similar proportions; therefore, molecular techniques can be accepted as a reliable method to detect mammals as prey items in pine marten diet. This is a useful tool in determining the small mammal composition of carnivore diet and also the spread (and possible decline) of both invasive and native mammal species in Ireland. However the macro analysis was significantly more sensitive in the detection of the woodmouse, which was the most frequently consumed mammal in the diet. It is recommended that any study aiming to determine exact frequencies of a species in the diet (as distinct from determining prey species presence or absence) be validated with traditional hard part (macro) analysis. In this study, a standard DNA extraction for both the species and dietary analysis was used (a cost effective strategy). However, to improve the molecular dietary detection of prey DNA, future molecular studies might increase the detection rate by sampling a larger amount of scat, extracting multiple samples from the same scat, or homogenising the scat prior to DNA extraction (see King et al. 2008). The woodmouse was found to occur in 31.8 % of scats tested, which is similar to the frequencies found in Northern Spain and Tuscany (De Marinis and Masetti 1995). Previous studies in Ireland have found the woodmouse to occur at around 13 % frequency (Lynch and McCann 2007) and 14.7 % (O'Meara et al. 2013) in pine marten scats.

Biomass or %BPI values could be better estimated for both macro and molecular analyses if feeding trials were conducted with captive pine marten to determine the appropriate correction factors for (a) the detection rates of the various mammalian prey species DNA after known amounts have been consumed and (b) the relationship between weight of dried remains and fresh weight ingested for red and grey squirrels as distinct from each other and from the 'small mammal' grouping.

Pine marten predation on squirrels

The absence of grey squirrel in the diet of the pine marten in the midlands most likely reflects their lack of availability as a prey item (Carey et al. 2007; unpublished data from Sheehy and Lawton). In Ireland and Scotland it has been speculated that the pine marten population has inhibited the grey squirrel population from spreading, and has even caused the grey squirrel population to crash in areas where they were once established (Carey et al. 2007; Caryl 2008; Paterson and Skipper 2008). No grey squirrel control measures had been carried out in any of the Irish midlands sites surveyed since the 1990s; therefore, human management of the alien squirrel population is not an explanatory factor in their rarity. Habitat is not a factor either, as red squirrel populations are found in the woodland, as until relatively recently were high numbers of grey squirrels. Whether predation was a factor in the retraction of the grey squirrel range historically is not possible to determine in retrospect, but evidence of predation on the alien squirrel species in the east confirms that the pine marten will indeed prey upon the grey squirrel, where it is available.

Molecular and macro analysis produced an overall frequency of occurrence for grey squirrel of 9.4 % and 10 %, respectively, in sites where grey squirrels are known to be present, that increased to 15.6 % when results from molecular and macro analysis were combined. The relative biomass of grey squirrels gave a similar estimate (13 % BPI). These figures are based upon a relatively small sample size however, and must be interpreted with caution as small sample sizes can cause a prey item to be either under or over represented in dietary analysis (Trites and Joy 2005). However, they do confirm that the North American grey squirrel forms part of the European pine marten diet when the two species' ranges overlap. Throughout the course of the current study, the grey squirrel was only confirmed as an available prey item in areas of low scat density, which made scat collection for dietary analysis in these areas very challenging. A larger sample size of scats will allow for a more robust dietary analysis in areas of low density. In contrast, the sample size of scats collected where red squirrels were confirmed as present was adequate to detect with confidence the frequency at which the red squirrel occurs in the diet. Red squirrels and pine marten have co-existed in Ireland and many other parts of Europe over many millennia, and the red squirrel has also appeared only as a very low frequency prey item in previous Irish pine marten dietary studies (Warner and O'Sullivan 1982; Lynch and McCann 2007; O'Meara et al. 2013). However, red squirrels have been recorded at higher frequencies in Russia and Sweden where other small mammal prey are less abundant (De Marinis and Masetti 1995). It is possible that the red squirrel's lower

frequency of occurrence in pine marten diet than that of the alien grey squirrel is a result of differences between red and grey squirrel ecology. Red squirrels live at lower densities (0.3–1.5 per ha) than grey squirrels (2–16 per ha) (Gurnell 1987) and would therefore be numerically less available as prey items. They are also lighter than the grey squirrel, capable of reaching the outermost branches of trees, and spend the vast majority of their foraging time in the canopy, whereas grey squirrels spend a larger proportion of their foraging time on the ground (Kenward and Tonkin 1986). This study introduces the possibility that there could be some form of density dependent effect on the grey squirrel population, where areas of high predator abundance might be discouraging the normally invasive grey squirrel from remaining in, or establishing in a woodland in the first place. The relationship between the native squirrel predator and the alien squirrel species is yet to be defined however, and what effect an increase in pine marten numbers in the east of Ireland will have on grey squirrel distribution and abundance merits further investigation.

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