Remotely plucked hair genotyping: a reliable and non-invasive method for censusing pine marten (, L. 1758) populations

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ORIGINAL PAPER

Remotely plucked hair genotyping: a reliable and non-invasive method for censusing pine marten (*Martes martes*, L. 1758) populations

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Abstract We investigated the feasibility of using genetic techniques to census pine marten (*Martes martes*) populations by genotyping non-invasively collected samples (plucked hair and scats), with particular reference to the genetically depauperate Irish population. Novel real-time polymerase chain reaction methods were developed for species and sex identification, targeting short DNA sequences. Background genetic variation at 17 microsatellite loci was very low in the Irish population, with an average of 2.29 alleles per locus and expected heterozygosity of 0.35. Despite such low polymorphism, a panel of eight loci with a sibling probability of identity of 0.011 reliably identified individual pine marten and their gender, as determined by reference to genotypes of live trapped individuals. With high nuclear DNA amplification success rates (93.8%) and low genotyping error rates (1.8%), plucked hairs may represent a more reliable and cost-effective DNA source than scats for monitoring populations of this elusive carnivore, and similar taxa such as the sympatric stone marten *Martes foina*.

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Introduction

The European pine marten (*Martes martes*, L. 1758) is a carnivore, a woodland dweller and a valuable furbearer. As such, this secretive mustelid has endured persecution in the form of habitat loss, overharvesting and the placement of poison to protect livestock, activities which contributed to significant range contraction on the islands of Britain and Ireland (Langley and Yalden 1977; O'Sullivan 1983). Recent range expansions from core refuge areas and reintroduction sites have been attributed to increased national afforestation, legal protection and the reduced use of strychnine for predator control (O'Mahony et al. 2006; Balharry et al. 2008). However, the status of many populations is unknown, and it is unclear whether faecal samples collected in peripheral areas are from resident or transient, non-breeding individuals (O'Mahony et al. 2006). Basic information such as population size and stability in core areas is fundamental for the assessment of conservation status, yet it can be difficult to obtain for carnivores, which are typically present at low densities with elusive behavioural patterns (Palomares et al. 2002).

The standard method for monitoring pine marten populations is scat transect surveys (Strachan et al. 1996; O'Mahony et al. 2006; Ruiz-González et al. 2008), but the relationship between scat density and local population abundance is unclear (Birks et al. 2004). Recent bait marking surveys have also indicated that few of the total number of scats in the environment are actually deposited on forest trails (Roche 2008), making associations between scat and population density even more difficult to interpret.

Hair-snagging devices have proven to be a viable alternative survey method, particularly in remote areas where the standardised collection of scats on trails is difficult (Statham 2005; Lynch et al. 2006).

These non-invasively obtained biological samples are often used to estimate population size and sex ratio (Sloane et al. 2000), through the amplification of hypervariable microsatellite sequences and sex-linked loci. There are currently no microsatellites developed specifically for *M. martes*, but cross-amplification of loci identified in related species is possible. Kyle et al. (2003) analysed the genetic structure of European pine marten populations using microsatellites developed for other mustelid species. In this study, the Irish population was found to have the lowest genetic diversity in Europe, comparable to that of the endangered *Martes americana* subspecies from the island of Newfoundland, and was genetically distinct from the populations in Britain and the continent. Many of the markers used by Kyle et al. (2003) were monomorphic or had very low heterozygosity (Statham 2005; Lynch 2006) and were therefore unsuitable for population size estimation in Ireland due to a lack of power to discriminate between individuals, the so-called 'shadow effect' (Mills et al. 2000). The aims of this study were to: (a) develop reliable and accurate methods for species, sex and individual identification and (b) evaluate the feasibility of applying these methods to census free-ranging pine marten populations by genotyping scats and plucked hair, with comparison of the results with direct estimates of abundance via live trapping procedures.

Materials and methods

Real-time PCR assays for species and sex identification

Two novel TaqMan™ MGB® probe real-time polymerase chain reaction (PCR) assays were designed to target short DNA sequences for species and sex identification from degraded DNA samples. TaqMan probes are dual-labelled oligonucleotides with a reporter and quencher dye at opposite ends (Livak et al. 1995). They are designed to hybridise to template DNA at a higher annealing temperature than the PCR primers. Annealing and extension of the primers displaces the probe through the 5′ to 3′ exonuclease activity of the *Taq* DNA polymerase, leading to an increase in fluorescent signal. This signal is detected by the real-time PCR instrument and measured against cycle number at an arbitrarily set threshold value, within the exponential phase of the PCR (Butler 2005). The number of cycles it takes to reach this threshold is called the C_T value which is correlated to the initial target DNA concentration in the PCR. In this way, different DNA samples can be compared in terms of their initial target DNA copy number (O'Reilly et al. 2008).

For species identification, an 85-bp fragment of the mitochondrial DNA control region was amplified using the primers PM3F (5′-CTTGCCCCATGCATATAAGCA-3′) and PM-REV2 (5′-GCCTGGTGATTAAGCTCGTGAT-3′), and pine marten samples were identified with the hybridization of a species-specific probe (PM3, 5′-6-FAM-CGTGCACCT CACTTAG-3′). Primers and probes were based on the pine marten *p* haplotype (AF336964), the only haplotype currently detected in the Irish population (Davison et al. 2001; Statham 2005; Lynch 2006). The PM3 probe sequence is also conserved in the other 17 European pine marten haplotypes described in Davison et al. (2001). Each 15 µl reaction contained 7.5 µl TaqMan™ Universal PCR mastermix (Applied Biosystems, Foster City, CA, USA) with 0.2 µM of each primer and probe and 1 µL of template DNA (isolated using the procedures described below).

Molecular sexing was based on amplification of zinc finger gene sequences on the X (ZFX) and Y (ZFY) chromosomes (Shaw et al. 2003). Homologous ZFX and ZFY introns were amplified and sequenced from male and female pine marten tissue using primers LGL331 (5′-CCAATCATGCAAGGA TAGAC-3′) and LGL335 (5′-AGACCTGATTCCAGA CAGTACCA-3′) as described previously (Statham et al. 2007). The ZFX product of 933 bp was sequenced directly on both strands from four females. The ZFY product of 1,023 bp was excised from an agarose gel and sequenced from two males. The ZFY sequence was used to design new primers, Y2F (5′-CTTGTCCAGAAACTTCATTCAATGTAA-3′) and Y3R (5′-TTCCGCAGTGCAATGTGCTATG-3′) which were used to specifically amplify a 754-bp fragment of the ZFY gene of four more male pine marten. No polymorphisms were found within the ZFX or ZFY sequenced regions, and there was 71.8% similarity between ZFX and ZFY. Sequences were deposited in GenBank (Accession numbers FN421124 and FN421125). A 5′ nuclease TaqMan assay was designed to distinguish the ZFX and ZFY genes, targeting 84 and 136 bp of the ZFX and ZFY, respectively. Sequence alignments and oligonucleotide-binding sites are shown in Fig. 1. The assay was validated using DNA from known gender tissue samples (21 males and 11 females). The multiplex PCR with TaqMan™ MGB® labelled probes was carried out with 7.5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.2 µM of each primer (MMXF, 5′-GGCAGAGCAACCCTGTCATAA-3′; MMXR, 5′-GGGCCTGAGGTTGGTACCACCA-3′; MMYF, 5′-GCATTGGGCTCCCTGCT-3′ and MMYR, 5′-AGATATCCAAATACATGTGGCTTTAAATG-3′), 0.2 µM of each probe (MMX, 5′-6FAM-TGTGTCTCTCTCTGTCAA-MGB-3′ and MMY, 5′-VIC-CCTGGTCTGAAAACT-MGB-3′) and 5 µl DNA extract in a total volume of 15 µl. Two PCR replicates were carried out for molecular sexing (Lynch and Brown 2006). Females were identified through the amplifi-

Fig. 1 Alignment of ZFX and ZFY introns showing the binding sites of the real-time polymerase chain reaction primers (underlined) and TaqMan MGB probes (*shaded*) for molecular sexing. A *dot* in the ZFY sequence indicates identity to ZFX

cation of ZFX only, while a signal from both ZFX and ZFY probes indicated male DNA was amplified. The ZFX allele therefore acted as an internal amplification control for the assay. All real-time PCR primers and probes were designed using Primer Express 2 software (Applied Biosystems). Amplifications were performed in an ABI 7300 real-time PCR system with Microamp® Optical 96-well reaction plates (Applied Biosystems). The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by cycling for 15 s at 95°C and 1 min at 60°C with 40 cycles for species typing and 50 cycles for sex typing to ensure any late ZFY amplification was detected. Applied Biosystems Sequence Detection Software version 1.2.3 was used for real-time PCR data analysis.

Microsatellite analysis

Twenty microsatellite loci identified in the genomic DNA of other mustelid species were tested for cross-species amplification and polymorphism in *M. martes*. These were from *Gulo gulo* (Gg7, Gg454 and Ggu234; Davis and Strobeck 1998; Duffy et al. 1998; Walker et al. 2001), *Lutra lutra* (Lut615, Lut604 and 04OT14; Dallas and Piertney 1998; Huang et al. 2005), *M. americana* (Ma1, Ma2, Ma5 and Ma8; Davis and Strobeck 1998), *Meles meles* (Mel1, Mel6 and Mel105; Bijlsma et al. 2000; Carpenter et al. 2003), *Mustela erminea* (Mer041; Fleming et al. 1999), *Mustela lutreola* (MLUT27; Cabria et al. 2007) and *Mustela vison* (Mvi1341, Mvi1354, Mvis072, Mvis075 and Mvis020; Fleming et al. 1999; Vincent et al. 2003). Microsatellite amplifications were performed in a total volume of 15 µl with 1 µl DNA extract, 1.5 mM $MgCl_2$, 0.2 µM each primer, 0.2 mM dNTP, 1X GeneAmp PCR Buffer (Applied Biosystems) and 1 U of AmpliTaq Gold™ polymerase (Applied Biosystems). The following PCR conditions were used for all amplifications: 95°C initial denaturation for 5 min, followed by 40 cycles of 94°C for 20 s and 58°C for 1 min, with a final extension time of 30 min at 72°C.

To facilitate genotyping of non-invasive samples, a new reverse primer was designed for the Mel1 locus (Mel1miniR: 5′-TGCTCTTATAAATCTGAAAATTAGGAATTC-3′) based on DNA sequences derived from two Irish pine marten tissue samples amplified using the original primer set (Bijlsma et al. 2000). Both primers were redesigned for MLUT27 (ML27miniF: 5-GTGATCACAGTTCAGCTAAATGTGT-3′ and ML27miniR: 5′-AAGCCAGTAATGAGAACCACAAT-3′). Primers were designed to bind as close to the repeat region as possible as smaller fragment sizes can reduce PCR failure and error rates in poor quality DNA samples (Frantzen et al. 1998; Butler et al. 2003). The reduction in size from the original primer set was approximately 160 bp (Mel1) and 80 bp (MLUT27). Forward primers were fluorescently labelled with different dyes (6-FAM, NED, PET, VIC or HEX) to enable multiplex electrophoresis of microsatellite products, and reverse primers were modified with a 5′ sequence of GTTTCTT to promote non-templated nucleotide addition (Brownstein et al. 1996). Fragment analysis was carried out on an ABI PRISM 310 genetic analyser under standard run conditions with 4% polyacrylamide. Alleles were scored against a GS500 LIZ™ size standard using GeneMapper software version 3.7 (Applied Biosystems).

Background genetic diversity was assessed using tissue DNA of 29 road-killed individuals collected across a wide geographic area in Ireland, using the mean number of alleles (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) per locus, calculated with the program MICROSATELLITE TOOLKIT (Park 2001). Pine marten tissue samples from France (n = 7) were also genotyped as

a continental population for comparison, along with one stone marten (*Martes foina*) tissue sample for amplification information in this sibling European marten species, for which there are also no specific loci available. Allelic richness was also calculated using Microsatellite Analyser (Dieringer and Schlötterer 2003) for the Irish samples as a measure of allelic diversity independent of sample size for comparisons with the smaller sample set from France. Tests for Hardy–Weinberg equilibrium and linkage disequilibrium were performed in GENEPOP version 4.0 (Raymond and Rousset 1995). Loci were arranged in order of decreasing per locus H_E values, and the cumulative probability of identity (PI) was calculated across polymorphic loci with the software GIMLET version 1.3 (Valière 2002). The PI for unrelated individuals (PI*ave*) and for full siblings (PI*sibs*) was calculated to give a lower and upper bound on the number of loci required for individual identification, with a target PI 0.01 or less (Waits et al. 2001).

Application to non-invasive samples

Sample collection

The study area in County Waterford, south-east Ireland is one of the regions outside the species stronghold in the west and mid-west which has maintained pine marten populations despite local extirpations in other areas (O'Sullivan 1983; O'Mahony et al. 2006). Hair and scat samples were collected from two commercial plantation forests in April and May 2008. The main study site was Curraghmore forest (52.25°N, 7.3°W; 2.5 km^2) which is part of a larger private estate of approximately 9 km^2. The second site at Kill (52.16°N, 7.3°W; 1 km^2) is situated 12 km south of Curraghmore in a highly fragmented forest landscape (Fig. 2). Lightweight polyvinyl chloride tubes (250 × 118 mm) fixed to trees were used for the collection of remotely plucked hair (Fig. 3). Hair-tubes were baited with chicken wing and marmalade, and commercial marten lure (Cumberland's Northwest Trappers Supply, PO Box 408, Owatonna, MN, USA, http://www.nwtrappers.com) was applied to a tree nearby. A patch of a strong glue-based mouse trap (stock code 3248; Solway Feeders, Kirkudbright, Scotland, http://www.solwayfeeders.com) removed hair for genetic analysis (Fig. 3). The study sites were divided into grid cells of 500 × 500 m, with one hair tube per grid cell in Curraghmore (n = 10) and two per cell in Kill (n = 8). The hair tubes were left in situ for the study duration and were spaced apart from those in adjacent cells as much as possible while still being placed at suitable sites. Hair traps were pre-baited every 3–8 days for 4 weeks. After this period, over 50% of the sites yielded hair samples. The tubes were then checked every day in Curraghmore and every 2–3 days in Kill for seven hair-capture sessions. Scats were collected from transects between the hair-tube sites during the same time period. The precise location of each sample was noted (Garmin eTrex). Both hair and scat samples were stored at −70°C until DNA extraction.

Molecular analyses

Genomic DNA was isolated from the root end of ten to 30 hairs per sample by digestion for at least 2 h at 56°C in a reaction mix containing 200 µL 5% (*w*/*v*) Chelex®-100 (Bio-Rad, Hercules, CA, USA), 35 mM DTT (Molekula, India) and 20 µg Proteinase K (Applied Biosystems). DNA extraction from scats followed the method of O'Reilly et al. (2008) using the ZR Genomic DNA II Kit™ (ZYMO Research, CA, USA). Species identification and molecular sexing were carried out as described above. For microsatellite analysis, each DNA extract was initially amplified in duplicate at all loci (Ma2, Mel1, Gg7, MLUT27, Mer041, Mvi1341, Mel105 and Mvi1354). The comparative multiple tubes approach was adopted for genotype assignment (Frantz et al. 2003), whereby consensus genotypes were constructed based on the observation of each allele at least twice for heterozygotes and three times for homozygotes. The first two PCR replicates were compared to the consensus genotype obtained from repeated amplification with the GIMLET software to detect genotyping errors. Samples with ambiguous results after a maximum of five amplifications per locus or with <50% successful amplifications across loci were removed from further analysis. Matching multilocus genotypes were grouped using GENALEX version 6.1 (Peakall and Smouse 2006), with the number of unique genotypes taken as the minimum population size estimate for each site.

Live capture

Live trapping was carried out for a radio-telemetry study in the Curraghmore forest in June 2008. The same area was covered as for the hair-tube survey, with the tubes (n = 10) left in situ but not baited for the interim 4-week period. A wire cage trap (23 × 23 × 74 cm, Tomahawk Live Trap, Tomahawk, WI, USA) was placed on the ground adjacent to each hair-tube (n = 10). Hair-tubes and traps (locked open) were pre-baited for two nights and set for capture on the third night. Glue patches were used to remove hair samples from the tail of each captured pine marten, which were genotyped at all polymorphic loci. The reference genotypes of the live-trapped individuals were compared to the unique genotypes identified from the non-invasive census to identify individuals captured by both survey methods and subsequently assess the reliability of the molecular techniques (Frantz et al. 2004).

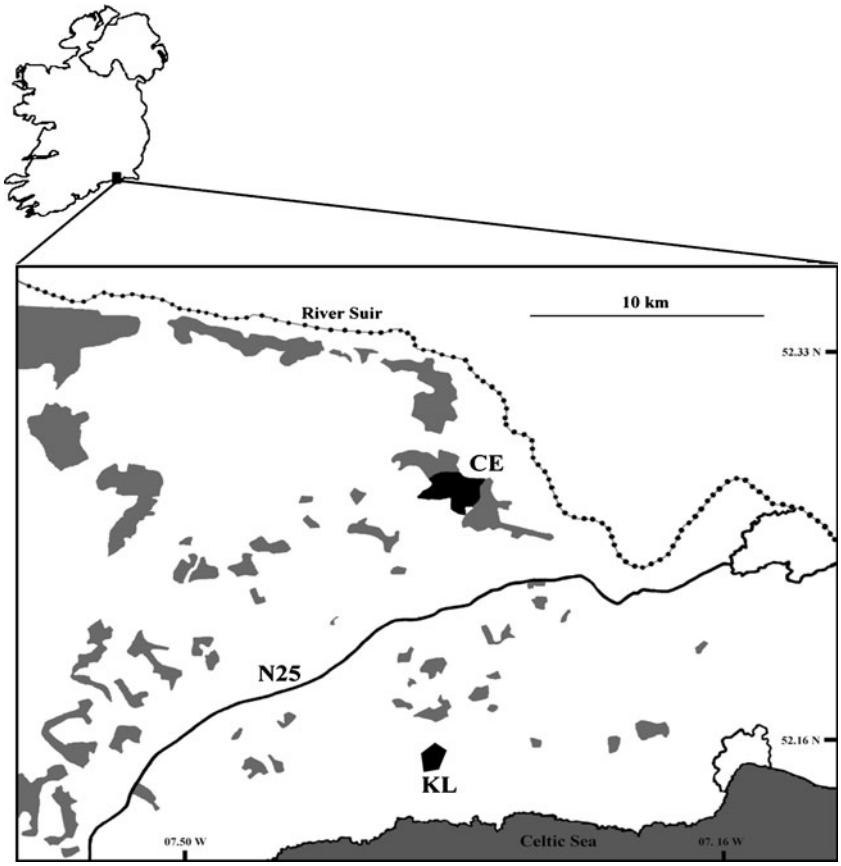

Fig. 2 Schematic map of the study area in the south-east of Ireland showing the location of the Curraghmore (CE) and Kill (KL) study sites along with other plantations (*grey polygons*), urban areas (*white polygons*), the River Suir and primary transportation routes (N25). Minor villages, rivers and roads were omitted for clarity

Results

Background genetic diversity

Seventeen out of the 20 microsatellite loci screened were polymorphic with a total of 55 alleles for the French pine marten samples, whereas only 39 alleles were found across the same loci for the Irish sample set (Table 1). Loci 04OT14, Mel6 and Ma5 were monomorphic across all samples tested; and Lut615, Lut604 and Ma1 were also monomorphic in the Irish samples. All 20 loci amplified and six were heterozygous for the stone marten tissue sample (Mel1, MLUT27, Mel105, Mvi1354, Lut615 and Ma1), indicating their potential use in population genetics of this species with more extensive genotyping. The average allelic richness and expected heterozygosity values were significantly lower (Wilcoxon test; $p < 0.001$) in the Irish samples (2.15 and 0.35) compared to the French samples (3.24 and 0.59). A significant heterozygote deficit was detected at locus Mvis020 ($p < 0.001$), presumably due to X-linkage as only females were heterozygous. Mvis075 was difficult to score due to excess stutter patterns and was dropped for the analysis of non-invasive samples. The first eight loci in Table 1 were finally chosen for genotyping non-invasive samples with an average of 2.88 alleles per locus, H_E of 0.51, a combined PI*ave* of 4.6×10^{-5} and a PI*sibs* of 1.1×10^{-2} for Irish pine marten.

Non-invasive population census

The hair-tubes successfully captured hair from free-ranging pine marten with 114 plucked hair samples obtained from 126 hair-tube trap nights (90.5%) across both sites. The last three of seven capture sessions were selected for genotyping (53 hair and 22 scat samples). The majority (97.3%) of the samples amplified mitochondrial DNA (all hair samples and 20 out of 22 scats), while amplification of nuclear DNA was more successful for hair samples, with lower C_T values, indicating the hair samples contained higher target DNA copy numbers (Table 2). The C_T values for sex typing were also higher than for species typing, which demonstrates the lower copy number of nuclear DNA compared to mitochondrial DNA, as expected. The remainder of the genotyping concentrated on the plucked hair samples for the population census due to the low microsatellite DNA amplification success rates for the scat samples in the first trial (Table 2). A high amplification success rate was achieved across eight loci for the 53 hair samples ($93.8 \pm 1.8\%$, $n = 848$ PCRs). Genotyping error rates were low with an overall error rate per reaction of 1.8%. Error

Fig. 3 A hair-tube in place during the population census. Remotely plucked hair (*top inset*) was obtained by placing two glue patches inside the tube (*bottom inset*), which the animal had to brush past to reach the bait. The entire device was attached to the tree with flexible aluminium wire, which was adjusted to suit the diameter of the tree trunk

rates per locus varied with three loci error-free in this analysis (Ma2, Mel1 and Mvi1341) and two loci (MLUT27 and Mer041) with error rates of 9.3% and 11.4% for allele dropout and false alleles, respectively. Full multilocus microsatellite genotypes were obtained for 49 plucked hair samples after multiple-tubes genotyping. Amplification success for the ZF gene was 93.8% (30 males and 16 females). Seven unique microsatellite genotypes were identified in total, giving minimum population sizes of five (three males and two females) in Curraghmore and two (one male and one female) in Kill. The number of times each individual was detected in the survey varied from one to 18 with some individuals detected at several sites within and across capture sessions, while others were detected only once in the survey.

Live trapping

Live trapping was successful with five pine marten (three males and two females) captured in one night in Curraghmore forest. The number and sex of captured pine marten was the same with both invasive (cage trap) and non-invasive (hair-tube) survey methods. Each of the five unique genotypes identified from the non-invasive census also matched the reference genotypes of the live trapped individuals at all microsatellite loci (Table 3). No incorrect gender assignments were identified by comparison of molecular sexing results (n = 46 PCRs) with the known sex of the live trapped pine marten, demonstrating the accuracy of the real-time PCR assay. The two males M325 and M279 were identified as a possible parent–offspring pair, as one allele was shared across all loci analysed, which was also the case for the two Kill individuals. However, more polymorphic microsatellites should be identified in future or isolated specifically from *M. martes* genomic DNA, as up to 20 loci may be required for investigations into relatedness structure given the level of heterozygosity in the population (Blouin 2003). Such analysis is technically feasible with the low genotyping error rates obtained in this study and should be more definitive in other populations such as in continental Europe where allelic diversity is higher (Kyle et al. 2003; Pertoldi et al. 2008; this study).

Discussion

Non-invasive genetic survey methods have been widely applied to studies of mustelid species (Mowat and Paetkau 2002; Frantz et al. 2004; Hung et al. 2004; Hedmark and Ellegren 2007), but have generally concentrated on species identification (Vercillo et al. 2004; Colli et al. 2005; Statham et al. 2005; Ruiz-González et al. 2008) and molecular sexing (Lynch and Brown 2006; Statham et al. 2007) for the European pine marten. Reliable amplification of microsatellite markers is a prerequisite for estimating the size and structure of populations within the landscape. Kyle et al. (2003) reported on the genetic variability and structure of pine marten populations in Europe, while Pertoldi et al. (2008) assessed genetic variation of Danish pine marten in both space and time. Of the 18 different microsatellite loci utilised in both studies, ten were also tested in this study, of which, only four (Ma2, Gg7, Mel1 and Mer041) had H_E values above 0.50 in Irish pine marten. This demonstrates the importance of carrying out a pilot study to select the most suitable set of markers for the population of interest based on their PI (Taberlet and Luikart 1999). The overall genetic diversity in Irish pine marten was very low and similar to mustelid species with documented population bottlenecks, such as Scandinavian wolverines (Walker et al. 2001; Hedmark and Ellegren 2007), black-footed ferrets *Mustela nigripes* (Wisely et al. 2008) and sea otters *Enhydra lutris* (Larson et al. 2002).

Table 1 Characteristics of polymorphic mustelid microsatellites for 36 *Martes martes* individuals from Ireland and France

Locus	Overall size range	Ireland (n=29)				France (n=7)		
		A	AR	H_O	H_E	A	H_O	H_E
Ma2	171–181	3	2.99	0.72	0.65	3	0.71	0.54
Mel1	106–116	3	2.96	0.59	0.58	3	0.57	0.52
Gg7	159–167	3	2.82	0.59	0.58	3	0.43	0.66
Mvi1341	164–178	3	2.57	0.55	0.54	3	0.57	0.61
Mer041	151–157	3	2.68	0.48	0.52	3	0.71	0.57
MLUT27	108–114	3	2.89	0.48	0.49	3	0.86	0.58
Mel105	123–135	3	2.42	0.48	0.42	4	0.57	0.68
Mvi1354	200–212	2	1.97	0.31	0.29	4	0.57	0.53
Mvis075	145–155	2	2.00	0.48	0.47	4	0.57	0.61
Mvis020[a]	175–187	3	2.80	0.21	0.45	3	0.29	0.57
Ma8	105–111	2	1.92	0.21	0.27	2	0.71	0.46
Ggu234	84–90	2	1.87	0.17	0.21	2	0.43	0.46
Mvis072	263–277	2	1.87	0.24	0.19	5	0.57	0.46
Ggu454	127–131	2	1.83	0.21	0.16	3	0.57	0.56
Lut615	230–234	1	1	0.00	0.00	3	0.14	0.36
Lut604	111–115	1	1	0.00	0.00	3	0.71	0.60
Ma1	197–203	1	1	0.00	0.00	3	0.14	0.44
Mean		2.29	2.15	0.34	0.35	3.24	0.54	0.59

Genetic variability was measured as the number of alleles (A), allelic richness (AR), observed heterozygosity (Ho) and expected heterozygosity (H_E)

[a] Significant deviation from Hardy–Weinberg equilibrium; $p < 0.001$

The origin of the pine marten in Ireland is presently unknown (Balharry et al. 2008). The mitochondrial DNA control region haplotype (hap *a*) present in Britain, Ireland's closest geographic neighbour, is among the most divergent from the single unique Irish haplotype (hap *p*), as are the two found in France (hap *c* and *u*) and the most common haplotype in the Netherlands (hap *l*). The closest haplotype to hap *p* is hap *i*, which was found in Iberia (Davison et al. 2001), but also more recently in Welsh museum specimens (C. O'Reilly, unpublished data), indicating a potential genetic missing link between Irish and continental European populations. Whether from Britain or the continent, it was possibly introduced by man as a furbearer or pet (Searle 2008) as the earliest archaeological evidence of its presence dates to the Bronze age, 2,780 ± 55 radiocarbon years before present (Woodman et al. 1997). This would explain the reduced genetic diversity, a result of historical founder effects compounded by population bottlenecks due to habitat loss and persecution extending into historic times. More extensive sampling is required to elucidate the colonisation source and current genetic structure of the pine marten in Ireland. Non-invasive genetic sampling can be used to increase the sample size and geographic coverage required for such analysis, as road-killed individuals and museum specimens are the primary sources of genetic information in the absence of a fur harvest.

In terms of estimating population size, nuclear DNA amplification was more reliable for plucked hair samples, with high DNA amplification success rates and low genotyping error rates. Different microsatellite genotyping protocols may be more successful for individual identification using scat samples, such as the multiplex pre-amplification approach which has been used successfully for wolverine and brown bear *Ursus arctos* faeces (Piggott et al. 2004; Hedmark and Ellegren 2005). Scats remain useful for assessing pine marten presence, distribution and diet in traditional surveys, which can be supplemented with genetic species identification. Intensive surveys in resident populations may be more cost effective by genotyping remotely plucked hair as a result of requiring fewer amplifications to obtain reliable genotypes. Preliminary trials indicate several loci can be easily amplified in multiplex reactions (Type-It™ Multiplex PCR Kit, Qiagen; unpublished data) which would reduce genotyping costs further, enabling genetic methods to be more easily integrated into small-scale monitoring programs. Hair-tubes can also be useful without genetic analysis, as visitation rates give valuable clues to the best sites for cage trap or camera trap placement, for a multi-evidence approach to population monitoring (Birks et al. 2004; Rosellini et al. 2008).

The population density estimate for this study of approximately 2 martens/km^2 was within the range obtained for other Irish populations (Lynch et al. 2006), though higher population densities have been documented (Birks 2002). Around 2 km^2 of woodland is regarded as the minimum area required to support adult pine martens

Table 2 Genotyping results for a single non-invasive sampling session

Site	Sample	Real-time PCR[a]				Microsatellite	
		Species and sex identification				Genotyping	
		PM3	ZFX	ZFY	Result[b]	Ma2	ID[c]
Curraghmore	Hair 1	22.00	29.93	31.00	PM, ♂	171/175	CE01
	Hair 2	22.18	29.86	31.12	PM, ♂	171/175	CE01
	Hair 3	23.06	31.55	32.12	PM, ♂	175/177	CE05
	Hair 4	24.57	31.41	33.05	PM, ♂	171/175	CE01
	Hair 5	23.09	31.57	31.67	PM, ♂	171/175	CE01
	Hair 6	24.80	33.00	33.02	PM, ♂	171/175	CE01
	Hair 7	24.82	29.39	U	PM, ♀	177/177	CE03
	Hair 8	23.49	30.74	U	PM, ♀	177/177	CE03
	Hair 9	22.89	30.63	U	PM, ♀	177/177	CE03
	Hair 10	22.60	29.99	30.35	PM, ♂	171/171	CE04
	Scat 1	31.40	U	U	PM	U	
	Scat 2	24.98	U	U	PM	U	
	Scat 3	24.80	36.56	38.05	PM, ♂	171/171	CE04
	Scat 4	22.26	37.40	U	PM, ♀	177/177	CE03
	Scat 5	24.03	35.54	38.71	PM, ♂	175/177	CE05
	Scat 6[d]	U			FOX		
Kill	Hair 1	23.51	31.64	32.47	PM, ♂	171/177	KL01
	Hair 2	22.39	30.47	31.78	PM, ♂	171/177	KL01
	Hair 3	22.09	29.99	30.58	PM, ♂	171/177	KL01
	Hair 4	23.80	29.39	31.72	PM, ♂	U	
	Hair 5	21.83	29.00	U	PM, ♀	171/171	KL02
	Hair 6	22.25	U	U	PM	171/177	KL01
	Hair 7	22.99	30.23	31.22	PM, ♂	171/177	KL01
	Hair 8	23.19	30.72	31.76	PM, ♂	171/177	KL01
	Scat 1	21.71	31.85	35.14	PM, ♂	U	
	Scat 2	29.36	30.07	U	PM, ♀	U	
	Scat 3	20.82	35.85	40.86	PM, ♂	U	

[a] C_T values, which are inversely, correlated to the initial target DNA copy number

[b] Pine marten (PM) samples were identified by C_T values for the species-specific PM3 TaqMan probe. Sex was determined by amplification of ZFX only (♀) or ZFX and ZFY (♂)

[c] Individual marten identified through genotyping of plucked hair. Note the individual CE02 (see Table 3) was not detected in this capture session

[d] Later identified as fox with a fox-specific TaqMan probe (method described in O'Reilly et al. 2008)

Table 3 Unique multilocus genotypes from remotely plucked hair (normal font) compared to reference genotypes of live trapped individuals (italic font) in Curraghmore forest

Ind	Sex	Ma2	Mel1	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354	*N*
M325	♂	*171/175*	*112/112*	*159/159*	*174/178*	*108/108*	*151/157*	*129/129*	*208/208*	
CE01	♂	171/175	112/112	159/159	174/178	108/108	151/157	129/129	208/208	11
F410	♀	*171/177*	*108/112*	*165/165*	*168/178*	*108/110*	*151/153*	*129/131*	*200/208*	
CE02	♀	171/177	108/112	165/165	168/178	108/110	151/153	129/131	200/208	5
F433	♀	*177/177*	*108/112*	*165/165*	*168/178*	*110/110*	*151/157*	*129/129*	*200/208*	
CE03	♀	177/177	108/112	165/165	168/178	110/110	151/157	129/129	200/208	6
M395	♂	*171/171*	*112/116*	*165/167*	*168/174*	*108/108*	*151/153*	*129/129*	*200/208*	
CE04	♂	171/171	112/116	165/167	168/174	108/108	151/153	129/129	200/208	3
M279	♂	*175/177*	*112/112*	*159/165*	*174/178*	*108/108*	*157/157*	*129/129*	*200/208*	
CE05	♂	175/177	112/112	159/165	174/178	108/108	157/157	129/129	200/208	1

N is the total number of hair-tubes the individual (Ind) was detected from, representing captures and recaptures during the survey

(Balharry 1993; Zalewski and Jedrzejewski 2006). Surprisingly, the male and female identified through hair-capture in Kill were also live-trapped in February 2009, and subsequently, collated telemetry data indicated the two animals were resident in the 1 km^2 site (P. Turner, unpublished data). Such small woodlands could easily be overlooked when conducting field surveys. The small size of available forests in fragmented landscapes (Fig. 2) may be offset by the use of non-forested habitat, such as scrub and limestone pavement in Ireland (O'Sullivan 1983), especially in countries with a mild climate and the absence of significant predators (Clevenger 1994; Pereboom et al. 2008). Recent range expansion into agricultural land in the north-west of Italy has been identified by the combined use of genetic analysis and road-kill data, which suggest dispersal movements may occur through the corridors of riparian woodland (Balestrieri et al. 2009), highlighting the importance of conserving such habitats for the maintenance of gene flow between populations. Pine marten hair and scat samples (Statham 2005; O'Mahony et al. 2006; this study) and road-kills (Smiddy and Berridge 2002) collected throughout the study area in Ireland also provide evidence for movement between forest fragments, which may be facilitated by the use of hedgerows (Pereboom et al. 2008) which are abundant in the Irish landscape. The ability to permanently tag individual pine marten without capture or observation through genotyping remotely plucked hairs has clear potential as a tool to characterise genetic variability, demographic parameters and habitat use in modified landscapes.

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